LIFE AND DEATH OF MESENCHYMAL STEM CELLS/ MULTIPOTENTIAL STROMAL CELLS (MSC): COUNTERVAILING REGULATION BY SURVIVAL AND APOPTOTIC SIGNALING

by

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Multipotential stromal cells/ mesenchymal stem cells (MSCs) can regenerate bone and adipose tissues, as these cells form osteoblasts and adipocytes. A major hurdle to using MSC however is cell-loss post-implantation. This cell death is partly due to inflammatory cytokines such as FasL generated in the implant site. In this study we found that FasL kills MSC by increasing reactive oxygen species in addition to activation of caspases. Thus we sought ways to protect MSC from FasL and other pro-death stimuli.

Our group previously reported that soluble epidermal growth factor (sEGF) promotes MSC expansion but does not support survival from FasL. Tethering EGF onto a two-dimensional surface (tEGF) altered MSC responses, causing sustained cell-surface activation of EGFR, protecting from FasL. However, for tEGF to be useful in bone regeneration, it needs to allow for MSC differentiation into osteoblasts, while also protecting emerging osteoblasts. Our lab has also shown that the matrikine Tenascin-C binds EGFR with a low affinity but a high avidity, and we proposed that Tenascin-C should also lead to cell-surface signaling of EGFR, causing survival.

We found that tEGF and Tenascin-C did not block induced differentiation of MSCs into osteoblasts, or adipocytes, a default MSC-differentiation pathway. tEGF protected differentiating osteoblasts from FasL mediated death. Differentiating adipocytes became resistant to FasL, with tEGF having no further survival effect. tEGF also protected MSC from combined insults of FasL, serum deprivation and hypoxia. Tenascin-C was found to protect MSC from FasL by activating sustained EGFR signaling from the cell surface.

We also found that MSC exist in a state of pre-autophagy, with cells filled with early autophagosomes that are not degraded. These autophagosomes are lost as MSC differentiate into osteoblasts, suggesting that MSC exist in a state of preparedness, to form new protein during nutrient challenge.

Our results suggest that MSCs and differentiating osteoblasts need protective signals to survive the inflammatory wound. tEGF or Tenascin-C can serve this function.

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1.0 INTRODUCTION

1.1 MESENCHYMAL STEM CELLS

1.1.1 Discovery

The first indication of the presence of a mesenchymal progenitor cell came from experiments in the late nineteenth century when E. Goujon transplanted marrow from the femur of a rabbit into the muscle of the same animal, and marrow from a chicken into pectoral muscles of other chickens and found “osseous productions”. Other labs were able to reproduce these observations at the time. (Goujon, 1869; Baikow, 1870). It was however not until the next century that elegantly designed diffusion chamber experiments with bone marrow being transplanted between two semipermeable membranes separated by a plastic ring showed that bone was formed not by the chemoattractant ability of the bone marrow, but by differentiation of the marrow itself (Danis 1960). The differentiation potential of the bone marrow sandwiched in the diffusion chambers was extended to forming not just bone, but also cartilage (Petrakova, Tolmacheva et al. 1963; Friedenstein, Piatetzky et al. 1966; Bruder, Gazit et al. 1990). Friendstein and colleagues then showed that it was the non-hematopoetic, tissue culture adherent cells of the bone marrow, which when singly isolated and cultured, form colonies, proliferate and form osteoblastic and chondrocytic cells. The colonies that formed were found to contain

fibroblastoid cells and the term colony forming unit-fibroblasts (CFU-f) was coined (Friedenstein, Chailakhjan et al. 1970; Friedenstein, Gorskaja et al. 1976). The term 'colony forming units' was adapted from hematologists describing hematopoetic stem cells forming colonies.

During this time, Till and McCulloch were describing hematopoetic stem cells and the differentiation potential of these cells (Till and McCulloch 1980). Owen was simultaneously describing a "stem cell" component of the bone marrow, which had the ability to give rise to the stromal lineage consisting of adipogenic, reticular, fibroblastic and osteogenic cells (Owen 1988). Subsequently, Arnold Caplan drew parallels between the migratory, space-filling, 'mesenchymatous' or 'middle' cells existing between the endoderm and ectoderm during embryonic development and the unique fibroblast-like stromal cells found in bone marrow of adult tissue that had the capacity to form repair blastemas, were capable of responding to local cues and differentiate to achieve regenerative repair. He was the first to call these cells 'mesenchymal stem cells'(Caplan 1987; Caplan 1991) and proposed that tissues should have repositories of these stem cells essential in the process of normal turnover and tissue homeostasis and predicted that 'we might be able to isolate such human mesenchymal stem cells, and place them in cell culture where we can mitotically expand their cell numbers. Eventually if we had enough of these cells, we could reintroduce them into the original donor in a manner that guaranteed that they would massively differentiate into a specified tissue, such as cartilage or bone, at a transplantation or repair site. Immunorejection would not be a problem because the donor and the host would be one and the same (Caplan 1991)

1.1.2 Characterization

Early isolation of human mesenchymal stem cells from the bone marrow showed specific differentiation into cells of the mesenchymal lineage in culture: osteoblasts, adipocytes and chondrocytes (Pereira, Halford et al. 1995; Pittenger, Mackay et al. 1999). It was shown that MSC were responsible for continuous renewal of cells in non-hematopoetic tissue including lung, calvaria, cartilage, long bone and skin (Pereira, O'Hara et al. 1998). Transdifferentiation of MSC into cells of the endoderm (Toma, Pittenger et al. 2002) and ectoderm (Kopen, Prockop et al. 1999) was also shown.

With increasing promise of MSC in regenerative therapy, investigators were reporting various modes of isolation, expansion and characterization (Wagner and Ho 2007). For purposes of standardization, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed the following minimal criteria for a cell to be defined as a mesenchymal stem cell: The cell has to be plastic adherent when grown under standard tissue culture conditions, the cell has to positively express CD105, CD90 and CD73, and not express CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Finally the cells have to have the tri-lineage differentiation potential of forming osteoblasts, adipocytes and chondroblasts in vitro (Dominici, Le Blanc et al. 2006).

1.1.3 Nomenclature

There exists the question of whether the tissue adherent fibroblast cells with differentiation ability can truly be termed ‘stem-like’. This is because firstly the characterization and naming of mesenchymal stem cells has been inferred by the clonal and non-clonal expansion

of in vitro expanded MSC and not by isolation of these cells directly from tissue source, due to the inability to currently do so (Nombela-Arrieta, Ritz et al. 2011). Secondly a common 'mesenchymal progenitor' for development of all mesenchymal tissues does not exist embryonically since tissues such as muscle and bone are derived from different progenitors. Thirdly, MSC from different tissue sources show differences in morphology, genetic profile and differentiation ability (Musina, Bekchanova et al. 2005). Finally MSC isolated from the same tissue from different donors show donor variation due to cellular heterogeneity during cell harvest (Phinney, Kopen et al. 1999). These reasons prompted the International Society for Cellular Therapy to refer to these plastic-adherent fibroblast-like cells with multilineage potential as 'multipotent mesenchymal stromal cells' abbreviated as MSC, regardless of the tissue from which these cells are isolated (Horwitz, Le Blanc et al. 2005).

1.1.4 Source

The intriguing question is how MSC reach the bone marrow. It was initially hypothesized that there could be two possibilities based on the mode of entry of MSC into the bone marrow. Firstly MSC could come along with invading vasculature along the abluminal surface of blood vessels or secondly these cells flow along the blood stream on the luminal side and home at the bone marrow. The second hypothesis would suggest that MSC are a circulating population of cells, which would make systemic delivery of MSC a viable option for cellular therapy (Dennis, Caplan et al. 2004). It is now widely accepted that MSC derive from the perivascular niche and predominantly evolve from pericytes of the microvasculature, which display conventional MSC markers: CD44, CD73, CD90 and CD105, can differentiate to form adipocytes, osteoblasts and chondrocytes and exhibit chemotaxis (Sarugaser, Lickorish et al. 2005; Crisan, Yap et al. 2008;

Zannettino, Paton et al. 2008; Crisan, Corselli et al. 2011). In addition to pericytes, another population of cells: CD146- CD34hi CD31- CD45- cells, from the tunica adventitia of arteries and veins have demonstrated the ability to give rise to MSC in long term culture (Corselli, Chen et al. 2011). Taken together there seem to currently be two sources of perivascular MSC: those derived from pericytes lining microvessels and those derived from adventitial cells surrounding larger blood vessels.

1.2 MSC IN BONE TISSUE ENGINEERING

1.2.1 Bone repair and regeneration

Bone regeneration is a tightly regulated process involving three major cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts produce and secrete structural proteins such as Collagen I, the major organic matrix of the bone, as well as the regulatory matrix proteins osteonectin, osteocalcin, and osteopontin, and growth factors including the BMPs. Osteocytes are fully differentiated osteoblasts that become encased in secreted matrix and embedded in the bone. Osteoclasts, derived from monocytes are involved in bone resorption. The interplay of these cells along with release of growth factors and deposition of matrix following injury in most cases leads to repair of fractures within 20 weeks (Littenberg, Weinstein et al. 1998). However there can be critical sized defects generated in long bones that lead to non-union of the bone and unhealed wounds. This can be the effect of shattered or splintered bone, open fractures, dissection of a tumor, inadequate blood supply, infection, or chronic disease. 4-10% of all fractures are considered non-union (Littenberg, Weinstein et al. 1998; Friedlaender, Perry et al.

2001) and there currently are 1.1 million fractures reported in the US every year. There exists a dire need for medical intervention in the treatment of nonunion bone wounds to prevent excessive pain and reduced productivity.

1.2.2 Conventional bone repair strategies

Bone grafts, both autogenous and allogeneic have been the traditional methods of treatment of critical sized defects, with autogenous grafts being the most reliable (Myeroff and Archdeacon 2011). Autogenous grafts most often obtained from the iliac crest provide sufficient transplant material, favor osteoinductive, osteoconductive and osteogenic substrates to fill bone voids but cause donor site morbidity. The problems include pain, infection, nerve injury and blood loss as well as time taken for surgery (Khan, Cammisa et al. 2005).

To circumvent these issues various biomaterials have been used for bone regeneration. Acrylate based bone cements have been used for dental reconstructs since the 1950s, have been approved by the FDA for hip prostheses in 1971 and have been used for different bone reconstructs since. Acrylates are preferred due to their high mechanical stability (Drosos, Babourda et al. 2012), however they do not exhibit osteogenic or osteoinductive properties. Bioinert ceramics such as alumina show low levels of wear and are commonly used for hip replacements (Sugano, Takao et al. 2011), but these ceramics such as acrylate are neither biodegradable nor osteoinductive. On the other hand ceramics comprising calcium phosphate and apatite integrate well into defects, have exceptional osteoconductive properties and are biodegradable. However their mechanical stability is much lower compared to acrylate based cements.

More recently demineralized bone matrix (DBM), a highly osteoinductive mixture, is being used either by itself or in combination with other biomaterials and has proven to be effective in regenerating bone (Kirk, Ritter et al. 2012). The downside of using DBM is donor variability. There have also been issues with DBM not being able to induce bone in non-bony sites (Kneser, Schaefer et al. 2006). There also have been osteoinductive growth factors such as the bone morphogenetic proteins (BMPs), specifically BMP2 and BMP7 that have been approved for clinical studies for treatment of non-union bone fractures and spinal fusions. BMPs however pose problems of heterotopic ossification, sub optimal release kinetics and high prices (Maniscalco, Gambera et al. 2002; Jones 2005) (Garrison, Shemilt et al. 2010). Since all these aforementioned treatments for non union bone wounds are still imperfect therapies, and MSC show the ability to regenerate bone, there have since been continued tests for using MSC for bone regeneration, especially in the treatment of non union wounds in long bones.

1.2.3 Bone tissue engineering

Langer and Vacanti described tissue engineering as 'an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function' (Langer and Vacanti 1993). In a classical sense 'tissue engineering' refers to the introduction of tissue specific cells (and not necessarily stem cells) along with a scaffold for regeneration of lost or injured tissue versus 'guided tissue regeneration' where implantation of a biocompatible scaffold by itself causes inherent cells at the transplant site to cause regeneration (Bianco and Robey 2001). On one end there are problems involved with conventional tissue replacement therapies, and on the other

there exists a solution where an autologous cell, the MSC, has the potential to form specific tissue like the bone, making MSCs highly sought after candidates for bone tissue regeneration.

1.2.4 MSC in reconstructing bone

Bone wounds generally involve structural defects involving bridging or filling, where healing is potentiated if MSC are delivered in a scaffold guided manner. The scaffolds tested for bone reconstruction using MSC are osteoconductive biomaterials such as hydroxyapatite and calcium phosphate. These scaffolds are loaded with MSC pre-cultured either in proliferation media or with bone differentiation inducers, and implanted at the wound site for reconstruction. However there are several caveats in the process of using MSC for bone reconstruction. Firstly there is a need for expansion of MSC both ex vivo and post implantation, to generate sufficient numbers of cells to reform bone (elaborated in Chapter 5). Secondly there is a need for improving integration to tissue and survival of MSC on implantation in vivo. This is because the overwhelming majority of MSC implanted in vivo are lost within 2 weeks of implantation due to factors described in Chapter 2 and Chapter 3. Finally MSC need to differentiate and form osteoblasts; differentiation into cells of other lineages including adipocytes is undesirable. The designed scaffolds must not only aid in these three vital stages of proliferation, survival and differentiation, they need to also be time-specific in not allowing MSC to differentiate before sufficient numbers of cells are obtained.

This thesis examines specifically death potentiated in MSC by non-specific inflammatory cytokines such as FasL. I look at death of MSC while undifferentiated as well as death of MSC as differentiation into osteoblasts proceeds (Chapter 6). I further describe two ways of enhancing MSC survival in the presence of death cytokines like FasL. Both these methods involve signaling

through the EGFR receptor, the first uses EGF based growth factor signaling (Chapter 6), the second Tenascin-C based matrikine signaling (Chapter 7).

2.0 ADULT STEM CELL SURVIVAL STRATEGIES

Melanie Rodrigues, Alan Wells, Linda Griffith

Chapter 18

Biomaterials and Stem Cells in Regenerative Medicine

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2.1 ABSTRACT

Stem cells have been proposed for the replacement of tissue lost to injury or disease because of the inherent ability of these cells to self-renew and differentiate into multiple lineages. An attractive source would be autologous cells from the patient's own bone marrow, fat or muscle. However initial attempts in both preclinical models and even early human trials show limited ability for such cells to directly generate the new tissue; rather their beneficial effects appear secondary to immunomodulation or paracrine signaling. Studies suggest that the failure to contribute to tissue replacement is due to the low rates of engraftment and survival of the stem cells in the harsh tissue microenvironment in which the cells are introduced. While adult stem cells by themselves are known to have low susceptibility to stress, in the presence of pro-death cytokines these cells not only become susceptible to stress but also undergo cell death leaving no cells to differentiate and reform tissue. This chapter on survival strategies of adult stem cells addresses the inherent survival signaling mechanisms of adult stem cells, responses to death signals and posits potential strategies to improve survival in order to develop better models for cellular therapy.

2.2 INTRODUCTION

2.2.1 Adult Stem Cells: Regenerative Potential

Stem cells are found in adult tissue at low frequencies and are capable of differentiating into specific tissues based on their differentiation potential, location, stimuli and relocation (Huh, Pan et al. 2006). Multipotent stromal cells or mesenchymal stem cells (MSC) present in the adult bone marrow are self-renewing clonal precursors of non-hematopoeitic stromal tissue and are capable of differentiating into mesenchymal lineages of bone, cartilage, fat, tendon, muscle, endothelial cells and marrow stroma (Pittenger, Mackay et al. 1999). These can be obtained by marrow aspiration. There are also stem and progenitor cells found in adipose tissue, called adipose derived stem cells (ASC) obtained at a higher frequency compared to bone marrow derived stem cells 0.5% compared to 0.01%. These cells which differ in expression of certain cell surface markers (Fraser, Wulur et al. 2006) possess similar multi-lineage differentiation potential though there is a predilection towards fat tissue (Brayfield, Marra et al. 2010). ASC are called several other names in literature including processed lipoaspirate cells (LPA) and adipose tissue derived stromal cells. Likewise the muscle also holds post natal stem cells called muscle derived stem cells (MDSC); these cells can differentiate spontaneously into cardiac and skeletal muscle and by modification ex vivo with growth factors can differentiate into the osteogenic and chondrogenic lineages (Usas and Huard 2007). There also exist stem cells with more restricted differentiation ability like neural stem cells (NSC) found in the adult hippocampus and lateral ventricle which can differentiate into neurons and glia (astrocytes and oligodendrocytes) (Massirer, Carromeu et al. 2011). There are other less well clinically characterized epithelial stem cells in tissues with high homeostasis, turnover rates and regeneration such as the skin

which form hair follicles (Zhang, Cheong et al. 2009), sebaceous glands (Jensen, Collins et al. 2009) and epidermal cells (reviewed (Barker, Bartfeld et al. 2010)). Other stem cells are found in the mammary and prostate glands (Shackleton, Vaillant et al. 2006; Stingl, Eirew et al. 2006).

Gastrointestinal stem cells, which help renew cells of the stomach, small intestine and colon, are readily identifiable in the crypts of the GI tract (reviewed (Barker, Bartfeld et al. 2010; Mills and Shivdasani 2011)). Colonic stem cells hold special status, as they are necessary for normal organ homeostasis during adult life (Shaker and Rubin 2010). This evidence of ongoing physiological functioning is shared only with hematopoietic stem cells.

The need for organ stem cells does not appear to be universal. For instance, skin can be regenerated from basal keratinocytes and dermal fibroblasts (Werner, Krieg et al. 2007). Still in these situations, the adnexal structures of the skin (hair follicles, sebaceous glands) are not formed in the absence of the epidermal stem cells from the hair follicle bulge (Yang and Peng 2010). A second organ in which the parenchymal cells may reconstitute the organ is the liver. Hepatocytes have been shown to have the capacity to repopulate injured livers via serial transplantation (Fausto 1997). In fact, the existence of liver stem cells in adults has not been conclusively demonstrated. A comprehensive list of adult stem cells, with their origin and differentiation potential is listed in Table 1.

Thus, while the role of adult stem cells is not certain in most instances, their presence allows for novel interventions.

Table 1. Adult Stem Cells: Types and Differentiation Potential

Stem Cell Type	Tissue of Residence	Differentiation Potential
Mesenchymal Stem Cells/ Multipotent Stromal Cells	Bone marrow	Bone, cartilage, muscle, marrow, adipose tissue, tendon, connective tissue, vasculature (Pittenger, Mackay et al. 1999)
Adipose derived Stem Cells/ Lipoaspirate cells	Adipose Tissue	Adipose tissue (Zuk, Zhu et al. 2002; Choi, Cha et al. 2006) bone(Haimi, Suuriniemi et al. 2009; McCullen, Zhu et al. 2009), muscle (Kim, Choi et al. 2006; Marra, Brayfield et al. 2011), cartilage (Xu, Balooch et al. 2007), cardiovascular cells (Planat-Benard, Menard et al. 2004)
Muscle derived Stem Cells	Muscle Tissue	Muscle, cartilage, bone
Neural Stem Cells	Subgranular zone of the Hippocampus and subventricular zone of the Lateral ventricle (Alvarez-Buylla and Lim 2004; Suh, Consiglio et al. 2007)	Neurons and glia (Duan, Kang et al. 2008)
Colonic Stem Cells	Base of Crypts of Lieberkuhn (Bjerknes and Cheng 1999; Bjerknes and Cheng 2002)	Intestinal Epithelial Cells and Paneth Cells
Stomach Stem Cells	Pyloric and Corpus Region(Nomura, Esumi et al. 1998)	Epithelial Cells of the Stomach (McDonald, Greaves et al. 2008)
Mammary Stem Cells	Terminal End Buds (Kenney, Smith et al. 2001)	Mammary Epithelial cells
Prostrate Stem Cells	Basal layer of prostratic epithelium (reviewed(Miki 2010))	Prostatic epithelial cells

2.2.2 Adult Stem Cells: Clinical Need

There exists a strong clinical need for replacement of conventional transplantation procedures due to problems of donor availability, cost and rejection. Adult stem cells are great candidates for such replacement therapy due to their potential for expansion and differentiation into the multiple cell types that constitute an organ. A single cell type will not be sufficient to regenerate the lost tissue as the stromal and vascular (endothelial and smooth muscle cells) supports are critical to functioning. Due to the multipotential differentiation of adult stem cells, one can readily imagine organotypic replacement proceeding from essentially a singular population of stem cells.

The three organs most extensively studied are fat, bone and heart. For fat, there exists the approach of direct transplantation of small fat organoids extracted from other, autologous locations (Kantanen, Closmann et al. 2010; Stallworth and Wang 2010). However, the clinical outcome of using autologous fat is highly unpredictable as lack of revascularization leads to graft resorption (reviewed in (Rubin and Marra 2011)). Adipocyte stem cells on the other hand are a propitious alternative as they promote angiogenesis and vasculogenesis (Cao, Baig et al. 2005) in addition to being pericyte-like with respect to their interactions with endothelial cells (Traktuev, Merfeld-Clauss et al. 2008).

Bone regeneration constitutes a greater challenge. After trauma, tumor resection or surgical procedures, critical-size bone defects do not heal on their own, and require indwelling prostheses. The use of autologous bone grafts that are adopted as treatment for such wounds is limited due to availability, donor site morbidity, geometry and cost. Research in rats (Song, Rao et al. 2011), rabbits (Zhao, Zhou et al. 2011), dogs (Liao, Chen et al. 2011) and sheep (Boos, Loew et al. 2010) suggest that bone graft substitutes such as tricalcium phosphate or degradable

polymeric scaffolds combined with cells of regenerative potential such as MSC increase both the osteoconductivity and the efficacy of bone formation.

Likewise heart transplants are limited by donor supply, immunosuppression and high cost. Use of stem cells as an alternative heart regeneration therapy is feasible in terms of differentiation into cardiomyocytes and safe also in the lung where these cells might migrate (Oguz, Ayik et al. 2011; Wang, Jiang et al. 2011). However repeated studies with stem cells to regenerate heart tissue have failed due to death of the transplanted cells in the excessively harsh ischemic microenvironment; this death situation will subsequently be addressed in greater detail.

2.2.3 Adult Stem Cells: Current Use and Gaps in Therapy

One of the first issues with the use of stem cell therapy is obtaining sufficient number of cells for tissue differentiation since stem cells are present in scarce numbers in the body. Obtaining high cell numbers involves expansion of the stem cells ex vivo with defined media such as growth factors that need to remain neutral and not lead to differentiation of the stem cells (Tamama, Fan et al. 2006; Tamama, Kawasaki et al. 2010). However, even if the hurdle of cell numbers is overcome, despite the innate differentiation potential of stem cells, most of the therapeutic effects currently attributed to the cells are based on the ability to home to sites of injury, release trophic factors and suppress inflammation (Prockop, Kota et al. 2010) instead of direct tissue generation. Current literature shows that MSC (Park, Kim et al. 2010) and ASC (Sadat, Gehmert et al. 2007; Ikegame, Yamashita et al. 2011) release growth factors including VEGF, IGF-1, HGF and TGF-β which protect injured tissue from hypoxia and serum deprivation apoptosis by activating and upregulating anti-apoptotic molecules and supporting new vessel formation (Park, Kim et al. 2010). MSCs also inhibit MMP signaling, prevent breakage of cell-

matrix interactions and impede anoikis and apoptosis in surrounding cells (Scuteri, Ravasi et al. 2011). In addition these cells express inflammatory cytokines including INFY, TNF, ILF1a and ILF1b that suppress T-cell and other immune cell activity (Ren, Zhang et al. 2008; Singer and Caplan 2011) aiding in immunosuppression.

All the trophic effects just described are thought to occur immediately following homing of the stem cells in vivo, probably in the first days to week of implantation since these cells release higher amounts of growth factors under stresses like hypoxia. However these stem cells show markedly reduced cell numbers from the time of implantation until 7 days after implantation with no surviving cells after 2 weeks likely caused due to the same stressors at the graft site (Hoffmann, Glassford et al. 2010; Rodrigues, Griffith et al. 2010).

2.3 ADULT STEM CELL SURVIVAL AT GRAFT SITE

2.3.1 Death of Stem Cells on Implantation

Although stem cells display several beneficial effects with regards to release of trophic factors and immunosuppression when tested on small animals, pre-clinical and clinical trials, few of these cells can be tracked beyond a few days of implantation. In one study involving MSC for treatment of inflammatory bowel disease, there were clinical improvements in all mice injected with MSC, however only 40% of mice showed persistence (possibly engraftment) of MSC, with as little as 0.13% of MSC traceable in the intestine after 3 days (Semont, Mouiseddine et al. 2010). In another study where high numbers of ASC were injected for treatment of colitis and intestinal sepsis, there was a transient attachment of cells seen in the intestine at Day 2, with very few detectable MSC at Day 6 (Gonzalez-Rey, Anderson et al. 2009). Similarly decreased numbers of MSC have been observed after delivery to the ischemic kidney (Mias, Trouche et al. 2008).

Both ASC and MSC rapidly die after injection/ implantation into infarcted hearts, with less than 5% of the cells engrafting and fewer cells being identifiable over the first 14 days (van der Bogt, Schrepfer et al. 2009; Noort, Feye et al. 2010). So also MSC transplanted as bone graft substitutes show decreased cell numbers after implantation. One study showed cell numbers decrease from 31±2% on day 1, to 9±1% on day 3 to 0.3±0.1% on day 7, and no identifiable MSC after fourteen days. The decreased cell numbers correlated with increased cellular disintegration (Zimmermann, Gierloff et al. 2011).

The reduced stem cell numbers on implantation in the body has been consistent in studies across tissues suggesting that there is reduced attachment and increased loss of these cells that

could be occurring due to a combination of effects such as nutrient deprivation and hypoxia, increased cytokine production in the wound milieu, ischemia, enhanced reactive oxygen species and oxidative stress in the tissue microenvironment (Menasche 2008). These pathways are summarized in Figure 1.

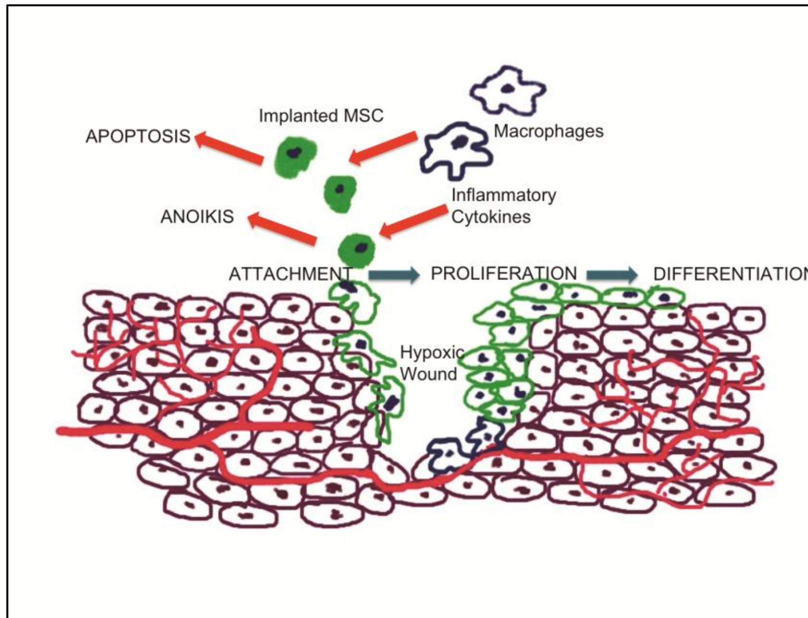

Figure 1 Stem Cell Expansion and Survival at Graft Site. Stem cells, in therapeutic application, are generally implanted in ischemic wound regions or inflammatory tissue for tissue regeneration. However the implanted cells need to attach to the microenvironment, proliferate to generate substantial cell numbers for differentiation into tissue and differentiate. This progression is hampered by the harsh microenvironment, devoid of oxygen or nutrients, which increases oxidative stress and prevents attachment of the stem cells, leading to death by anoikis. Non-specific immune responses brought about by invading immune cells such as macrophages, increase pro-inflammatory cytokines causing death of the stem cells by apoptosis. Most studies done with stem cells observe a massive reduction of the implanted cell numbers within 14 days, most likely occurring due to these mechanisms.

2.3.2 Effects of Hypoxia and Serum Deprivation on Stem Cell Survival

MSC reside in the bone milieu under conditions of 4-7% oxygen (Kofoed, Sjontoft et al. 1985). Studies suggest that when oxygen is dropped below 4% indicative of hypoxia in addition to subjecting MSC to serum deprivation, cells display caspase-3 mediated programmed cell death with loss in membrane potential, cytochrome c release and accumulation of Bax in a p53 dependent manner, all mediators of the mitochondrial death pathway involving caspase 9, with very little death occurring via the death receptor pathway mediated by caspase 8 (Zhu, Chen et al. 2006). Additionally, MSC in the presence of hypoxia generate increased levels of ROS due to increased expression of NAD(P)H oxidase and reduced catalase, display lower levels of the pro-survival protein survivin and increased Bcl-2/Bax ratios which culminates in cell death (Peterson, Aly et al. 2011). However serum deprivation proves to be the stronger stimuli compared to hypoxia (Zhu, Chen et al. 2006) since glucose depletion in the presence of hypoxia leads to massive cell death in MSC which is not seen in the presence of hypoxia alone (Deschepper, Oudina et al. 2010). Additionally, recent studies show that MSC are able to tolerate hypoxia for up to 72 hours following which they undergo caspase mediated death and this death is exacerbated on addition of the glycolysis inhibitor 2-deoxyglucose, suggesting that glycolysis maybe a major pathway supporting survival in ischemic conditions (McGinley, McMahon et al. 2011).

ASC are also susceptible to hypoxic death (Follmar, Decroos et al. 2006) and survive to a much lesser degree even than bone marrow derived MSC or cord blood derived stem cells, either in vitro in the presence of hypoxia or in vivo in infarcted mouse hearts (Gaebel, Furlani et al. 2011).

2.3.3 Effects of Cytokines

The wound environments in which adult stem cells are generally implanted are sites of high inflammatory cytokine activity. Similarly, implantation of any space-occupying substance into the body, such as polymeric scaffolds containing stem cells, generates a non-specific immune response that the stem cells need to overcome and survive. MSC are susceptible to FasL and TRAIL induced cell death, with TRAIL preferably killing fetal MSC and FasL preferably killing adult MSC (Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011). There have also been contrary reports wherein activation of neither of these pathways stimulates death in MSC (Mazar, Thomas et al. 2009; Szegezdi, O'Reilly et al. 2009). To clarify these reports studies in our lab show that cytokines such as FasL promote cell death in MSC via caspase3 mediated apoptosis and also induce the production of ROS in MSC that contributes to apoptotic death in these cells (Rodrigues, Turner et al. 2012)

2.3.4 Effects of Reactive Oxygen Species and Oxidative Stress

Stem cells are thought to possess high amounts of anti-oxidants glutathione and superoxide dismutase and concurrently low inherent levels of ROS (Urish, Vella et al. 2009). MDSC for example display lower levels of oxidative stress related death compared to their more differentiated phenotype, myoblasts (Oshima, Payne et al. 2005). However this reduced baseline ROS does not prevent these cells from undergoing cell death in the presence of oxidative stress. Pretreatment of MDSC with an ROS scavenger N-acetylcysteine (NAC) prior to implantation of cells in the heart increases cell survival which is dependent on signaling by the MAP kinase Erk, while on the contrary addition of a pro-oxidant di-ethyl maleate decreases survival (Drowley,

Okada et al. 2010). Similarly, application of hydrogen peroxide, one of the major contributors of oxidative damage to MSC in vitro causes apoptosis by activation of p38MAPK in the early stages and JNK in the late stages; this activates caspase3 via the mitochondrial pathway by causing Bax translocation to the mitochondrial membrane, releases cytochrome-c and simultaneously causes activation of caspase-12 by ER-stress induction (Wei, Li et al. 2010). Another effect seen due to paracrine ROS signaling on adult stem cells is anoikis. Treatment of MSC with hydrogen peroxide causes reduced attachment and cell spread by reducing focal adhesion related molecules including p-FAK and p-Src as well as integrin related molecules (Song, Cha et al. 2010). Contrarily, supplementing MSC with NAC increases attachment of MSC to the heart infarct region, provides a survival advantage and revives infarcted hearts better. The various death-signaling pathways have been illustrated in Figure 2.

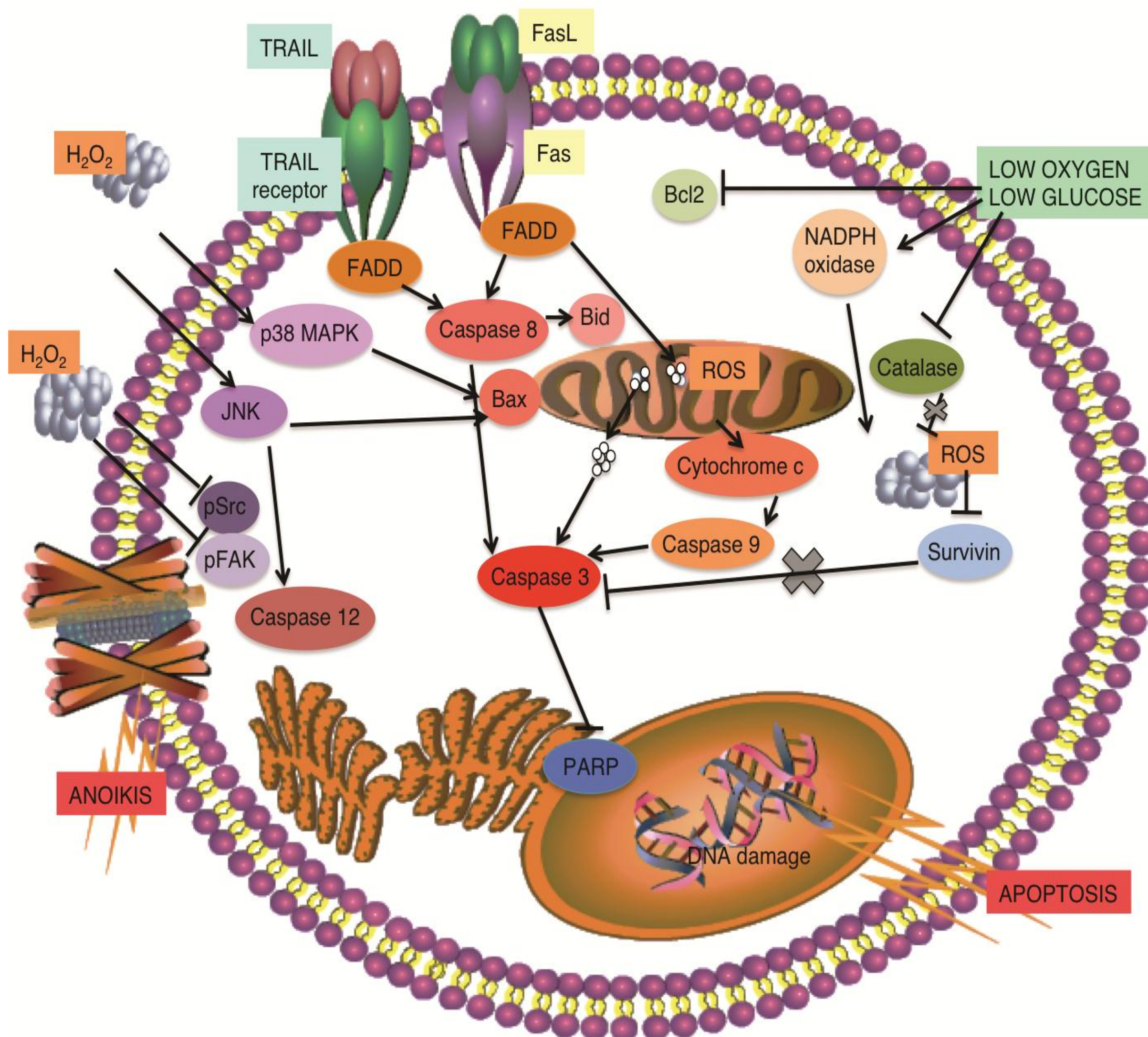

Figure 2 Adult Stem Cell Death Signaling. Hydrogen Peroxide inhibits integrin signaling by inhibiting phosphorylation of FAK and Src, causing cells to detach and die by anoikis. H_2O_2 also activates the JNK and p38MAPK stress pathways, causing Bax to translocate to the mitochondria and initiate the mitochondrial death pathway involving loss of mitochondrial membrane potential, release of cytochrome-c, activation of caspase9 and activation of the effector caspase3. p38MAPK is involved in the early stages, while JNK is activated in the later stages of apoptosis. In addition JNK activates the ER stress related caspase12 death pathway. Pro-death cytokines such as TRAIL and FasL also activate cell death via caspase8 and mitochondrial pathways leading to caspase3 activation, in addition to generating reactive oxygen species (ROS) in the mitochondria, which also cause activation of caspase3. Although both low glucose and hypoxia are used to mimic ischemic conditions in vitro, low glucose is the stronger deaths stimulus. In combination these two conditions activate NADPH oxidase and inhibit catalase enzymes, which enhance ROS production in the cells, blocking the pro-survival protein survivin and Bcl2, causing caspase3 activation and cell

death. DNA repair enzyme PARP is just one among several substrates of caspase3, which the apoptotic effector cleaves and inactivates.

2.4 IMPROVING STEM CELL SURVIVAL AT GRAFT SITE

2.4.1 Gene Therapy for Increased Survival

Introducing genes to enhance survival signaling in stem cells prior to implantation has been tested for prolonging the lifespan of stem cells in vivo. This mode of inducing survival is specific to the stem cells carrying the gene alone and does not affect cells of the surrounding tissue directly. In one study adult rat MSC were genetically modified ex vivo to over-express the anti-apoptotic protein Bcl-2. These cells were injected into the rat myocardium bordering the infarct region. While there still was a reduction in cell numbers over 6 weeks, MSC over-expressing Bcl-2 showed a 2.2 fold increases in cell survival on day 4, 1.9 fold increase in survival after 6 weeks and 1.2 fold increase over vector control cells after 6 weeks (Li, Ma et al. 2007). While these are miniscule improvements, the experiment did show some protection from death.

There have been other studies where MSC over-expressing GSK-3β when injected into infracted mice hearts showed increased survival due to increased production of VEGF-A (Cho, Zhai et al. 2011). Another showed introduction of the human insulin growth factor 1 (IGF-1) gene in MSC increased IGF-1 production to nearly 6 times that of native cells, enhanced Erk signaling and greater MSC survival. However these modified MSC could differentiate only into the endodermal and mesodermal lineages and not into neuronal cells (Hu, Wu et al. 2008).

Lentiviral vector-mediated over-expression of pro-survival genes HSP27, HSP 70, SOD1 and SOD3 in MSC have shown increased survival in the presence of hypoxia, glucose and nutrient deprivation, with HSP70 overexpression most significantly increasing survival and decreasing caspase 3-mediated death (McGinley, McMahon et al. 2011). Similarly in another

study where rat MSC over-expressing Hsp20 were introduced in a rat left anterior descending ligation model of infarct via intracardial injection, there was a two-fold increase in survival in vivo on day 4, compared to MSC alone, owing to enhanced signaling of the pro-survival AKT pathway and increased production of the growth factors FGF-2, VEGF and IGF-1(Wang, Zhao et al. 2009).

Stable overexpression of the pro-survival protein AKT1 (Protein kinase B) in swine bone marrow MSC shows stable AKT activation for 2 weeks and increased attachment of the MSC to swine myocardial walls after autograft into the infracted myocardium (Mangi, Noiseux et al. 2003; Rodrigues, Griffith et al. 2010; Yu, Shen et al. 2010). All these studies, while showing only minor improvements, did demonstrate proof-of principle to indicate that pro-survival gene therapy can be an advantageous option to increase stem cell viability in vivo. However the issues of gene therapy remain, and lie in the fact that the effects last for several generations, including post-differentiation of the stem cells and the integration itself may cause deleterious mutations.

2.4.2 Protein Factors for Increased Stem Cell Survival

Presentation of proteins avoids the issues of integration with second site mutagenesis and possible neoplastic transformation that is inherent in gene therapeutic approaches. While the issues of timed released and concentration distributions remain, these are potentially solvable via cell engineering manipulations. A number of possible proteins have been forwarded to accomplish this task.

2.4.2.1 Heat shock proteins

Heat shock proteins are molecular chaperones, which act as checkpoints and either direct cells into survival or death under conditions of molecular stress. Treatment with recombinant human heat shock protein 90α (rhHSP90α) increases rat MSC survival in the presence of hypoxia and serum deprivation in a dose dependent manner by increasing pro-survival proteins Bcl-2 and BclxL and decreasing the pro-apoptotic protein Bax. The change in expression of these proteins is thought to occur via interactions with Toll like receptor-4 (TLR-4) and the receptor tyrosine kinase ErBb-2. rhHSP90α binds ErBb-2 on its extracellular domain activating PI3K and Erk; similarly it binds TLR-4 causing NFκB activation and production of nitric oxide. There is also PI3K-Akt activation via MyD88, reducing apoptosis in all cases (Gao, Hu et al. 2010).

2.4.2.2 Growth factors/ Angiopoetins/ G-Protein coupled receptor signaling

Various growth factors have been queried for the increase in survival of stem cells based on their ability to activate pro-survival pathways including Erk and Akt. There is a long-appreciated role for these factors based on their trophic effects such as inducing blood vessel growth in the immediate surroundings, reducing hypoxia and nutrient starvation. A number of studies with VEGF show ASC and MSC numbers increasing on pre-treatment with VEGF or introduction with a VEGF peptide at ischemic sites, due to increased Akt and induction of angiogenesis (Pons, Huang et al. 2008; Behr, Tang et al. 2010).

Treatment with neurotrophic factor and nerve growth factor show increased MSC homing and survival after introduction in a traumatic brain injury model (Mahmood, Lu et al. 2002). Liver growth factor improves viability of neural stem cells grafted in rat brains in a study involving treatment of Parkinson’s by raising levels of the anti-apoptotic protein Bcl-2 four

times, without changing expression of Bax (Reimers, Osuna et al. 2010). EGFR signaling is also known to play a major role in survival of neural stem cells, by activation of downstream JAK-STAT and PI3K AKT pathways (Grimm, Messemer et al. 2009; Ayuso-Sacido, Moliterno et al. 2010; Tham, Ramasamy et al. 2010).

In bone marrow MSC, use of a bioengineered EGF ligand where restriction of the ligand to the cell culture substratum causes restriction of EGFR receptors to the cell membrane and sustained EGFR signaling and sustained downstream Erk signaling protects cells from FasL mediated cell death. Use of EGF ligand in the soluble, free state however does not lead to the same survival effect due to internalization of the receptor and transient EGFR and Erk signaling (Fan, Tamama et al. 2007). The advantage of using EGFR signaling for increasing MSC survival lies in the fact that EGFR signaling does not affect MSC differentiation while increasing proliferation and expansion, making it the ideal growth factor option for a lot of model systems (Tamama, Fan et al. 2006).

Another highly studied growth factor with respect to stem cells is fibroblast growth factor-2 (FGF-2/b-FGF). Overexpression of FGF-2 in neural progenitor cells shows increased engraftment to the ischemic cortex and higher rates of survival (Jenny, Kanemitsu et al. 2009). Cord blood derived MSC loaded onto a fibrin gel with FGF2 and transplanted intramuscularly into the ischemic hindlimbs of mice display significantly lower apoptosis of MSC post implantation. The same study showed MSC with FGF2 enhanced expression of host derived PDGFβ and NG2, which induces recruitment and homing of endothelial cells and pericytes, protecting muscles from ischemic degeneration (Bhang, Lee et al. 2011).

Angiopoetins are protein growth factors that stimulate angiogenesis and vasculogenesis by binding to their protein kinase Tie receptors and are known to have pro-survival effects on

several cell types including neurons, endothelial cells and cardiomyocytes. Angiopoetin 1 (Ang-1) was found to induce Tie phosphorylation in MSC, activated PI3K-AKT, increased Bcl-2/Bax ratios and prevented caspase9 and caspase3 activation by hypoxia and serum deprivation (Liu, Jiang et al. 2008).

Lisophosphatidic acid is a bioactive lipid that induces apoptosis in some cell types, but also protects a number of cell types from hypoxia, serum deprivation and drug induced apoptosis, including MSC (Chen, Baydoun et al. 2008). LPA binds the G=protein coupled receptor LPA-1 which is the only LPA receptor expressed on MSC, activates Gi proteins, which activates downstream PI3K-AKT and Ras-Erk pathways, shuts Bax translocation to the mitochondria and prevents death via hypoxia and serum induced mitochondrial death pathway. In vivo studies in ischemic rat hearts with rat MSC pre-treated with LPA survive much better than MSC without any prior conditioning although there are reduced cell numbers in both cases. The number of LPA treated MSC found in the ischemic myocardium after 1 hour of implantation was 51%±12% compared to 24%±2% of untreated MSC; the numbers after 1 day were 35±12% of LPA treated MSC compared to 15%±1% of untreated MSC and only 1% of LPA treated MSC compared to 0.7% after 1 week. So also the number of apoptotic cells in the peri-implant region after 1 day of implantation was 6%+2% with MSC treated with LPA and 16%+3.61% with untreated MSC indicating that LPA has a major role to play in retaining stem cell numbers at the ischemic site (Liu, Hou et al. 2009).

Growth factors such as EGF therefore have the advantage of increasing stem cell numbers ex vivo and possibly in vivo, which makes for a defined media for the culture of the cells while maintaining their undifferentiated state and increases survival in vivo. The challenge with the use of growth factors is delivery and current bioengineering studies in regenerative

medicine probe deeply into various biocompatible scaffolds and polymeric materials that can be used which will carry the growth factor in its bioactive form, restrict its activity to the stem cell compartment of regenerating tissue and degrade as soon as its function is complete minimizing any aberrations in the tissue.

2.4.2.3 Chemokines

Chemokines are a group of small sized proteins that were initially discovered as chemoattractants but have since been increasingly studied for their role in pro and anti-inflammatory signaling and cellular migration. Human MSC express chemokine receptors in each of the CC, CXC, C and CX(3) C categories: CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5 and CXCR6, while murine MSC only express CCR6, CCR9, CXCR3 and CXCR6, receptors well studied for recruitment of cells into regions of inflammation (Ponte, Marais et al. 2007; Chamberlain, Wright et al. 2008). Pancreatic islet cells for example secrete CX3CL1 and CXCL12, which recruit MSC (Sordi, Malosio et al. 2005). In vitro, MSC display chemotaxis in response to CXCL 10, CXCL12 and CX3CL1 (Croitoru-Lamoury, Lamoury et al. 2007) and the expression of these chemokine receptors in MSC is regulated by death cytokines like TNFα and INFγ indicating that on inflammation during injury, there is upregulation of chemokine receptors, which cause MSC to respond to chemokines and migrate towards the wound site(Croitoru-Lamoury, Lamoury et al. 2007; Ponte, Marais et al. 2007). However culture expanded MSCs lose chemokine receptor levels and display decreased chemotaxis in response to chemokines (Honczarenko, Le et al. 2006).

Pre-treatment of MSC with stromal derived factor-1 (SDF-1) which binds the chemokine receptor CXCR4 decreases cell death and increases homing and survival of MSC in infarct and peri-infarct areas. The increase in survival is due to activation of PI3K AKT survival pathway by CXCR4 signaling (Pasha, Wang et al. 2008). Also MSC over-expressing the SDF-1 gene show better engraftment and angiogenesis in a rat myocardial infarction model due to enhanced production of the pro-angiogenic growth factor VEGF and subsequent signaling by AKT and eNOS (Tang, Wang et al. 2009). CXCL9, the ligand for CXCR3 is known to significantly increase MSC attachment, spread and crawling (Chamberlain, Smith et al. 2011). These effects are primarily due to downstream activation of Erk1/2 and STATs (Honczarenko, Le et al. 2006).

2.4.3 Hormones for Increased Survival

The pineal hormone melatonin has been used to increase survival of rat bone marrow derived MSC delivered to the ischemic kidney. Melatonin binds the melatonin receptor (both Melatonin recpetor1 and melatonin receptor II are expressed in MSC) and activates the enzymes catalase and superoxide dismutase responsible for reducing reactive oxygen species mediated cell death. In addition melatonin increases production of growth factors such as FBF-2 and HGF and increases surrounding vascularity, improving conditions of survival for the MSC in the ischemic environment (Mias, Trouche et al. 2008).

2.4.4 Anti-Ischemic Drugs for Increased Survival

Since the largest threat to stem cells once implanted at the site of injury is ischemia and loss of nutrients, one of the best ways to study survival would be to use methods to directly

counteract ischemia. Two such studies with anti-ischemic drugs have been conducted. Trimetazidine (1-[2,3,4-trime- thoxybenzyl]piperazine; TMZ; also known as Vastarel) is a drug used to reduce metabolic damage induced due to ischemia by reducing the rate of fatty acid oxidation and increasing rates of anaerobic glycolysis (Lopaschuk, Barr et al. 2003), a means of metabolism found by several studies to be favorable to stem cells. MSC pre-conditioned with TMZ show increases in HIF-1α, pAKT, and the pro-survival proteins Bcl-2 and survivin and show reduced cell death in the presence of oxidative damage caused by H_2O_2 (Wisel, Khan et al. 2009).

The prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) is the other example of an anti-ischemic drug tested to increase MSC. The enzyme prolyl hydroxylase under conditions of normoxia allows HIF1α interaction with VHL, targeting the complex towards degradation. DMOG, by inhibiting prolyl hydroxylase, prevents the formation of HIF1α- VHL complex, and allows HIF1α to shuttle to the nucleus, bind to p300-CBP and transcribe genes involved in angiogenesis, energy metabolism and survival. In addition DMOG prevents translocation of Apoptosis inducing factor (AIF) from the mitochondria to the nucleus, preventing a caspase 3 independent apoptosis pathway, blocks release of cytochrome-c from the mitochondria and activates PI3K mediated survival. DMOG however does not affect the Erk pathway (Liu, Wang et al. 2009).

2.4.5 Increasing Engraftment of MSC: Coating of MSC with Antibodies

Targeting stem cells to sites of inflammation, attachment to sites of inflammation and survival at these sights pose major challenges. There have been studies where MSC have been coated with antibodies to target the cells to sites of inflammation. MSC cell membranes coated with addressin antibodies target MSC to TNF-α secreting endothelial cells which are rich at sites of inflammation (Ko, Kean et al. 2009). This model has been studied in vivo in mice with inflammatory bowel disease. MSC coated with addressin antibodies displayed increased delivery and engraftment to the inflamed intestinal mucosa and exerted immunosuppressive effects to bring about survival of the mice (Ko, Kim et al. 2010). This study is a one of its kind focusing on increasing delivery and engraftment of MSC to inflammatory sites and can be used effectively along with methods to increase survival of MSC to bring about better effects.

2.4.6 Anti-oxidants/ Reduced Temperatures/ Mitochondrial Death Blockers

As indicated earlier, reactive oxygen species rich at sites of injury pose a major threat to stem cells survival by initiating stress pathways and promoting cell de-attachment. Also as mentioned earlier, pro-inflammatory cytokines are able to induce ROS increases in stem cells making the cells responsible in promoting their own death. Studies with addition of the anti-oxidant NAC to muscle derived stem cells have indicated a surge in survival, which would be the case with a majority of other stem cell types (Drowley et al. 2010).

Immunosuppressant such as Cyclosporine A (CsA) have also been tested for enhancement of stem cells survival. CsA regulates membrane permeability in MSC as in several other cell types, prevents drop of membrane potential, release of cytochrome c and prevents

activation of the mitochondrial apoptotic pathway. In addition CsA deactivates the pro-apoptotic moiety BAD via calcineurin and protects MSC from hypoxia/reoxygenation induced death (Chen, Wang et al. 2008).

The various survival pathways that have been studied in stem cells are described in Figure 3.

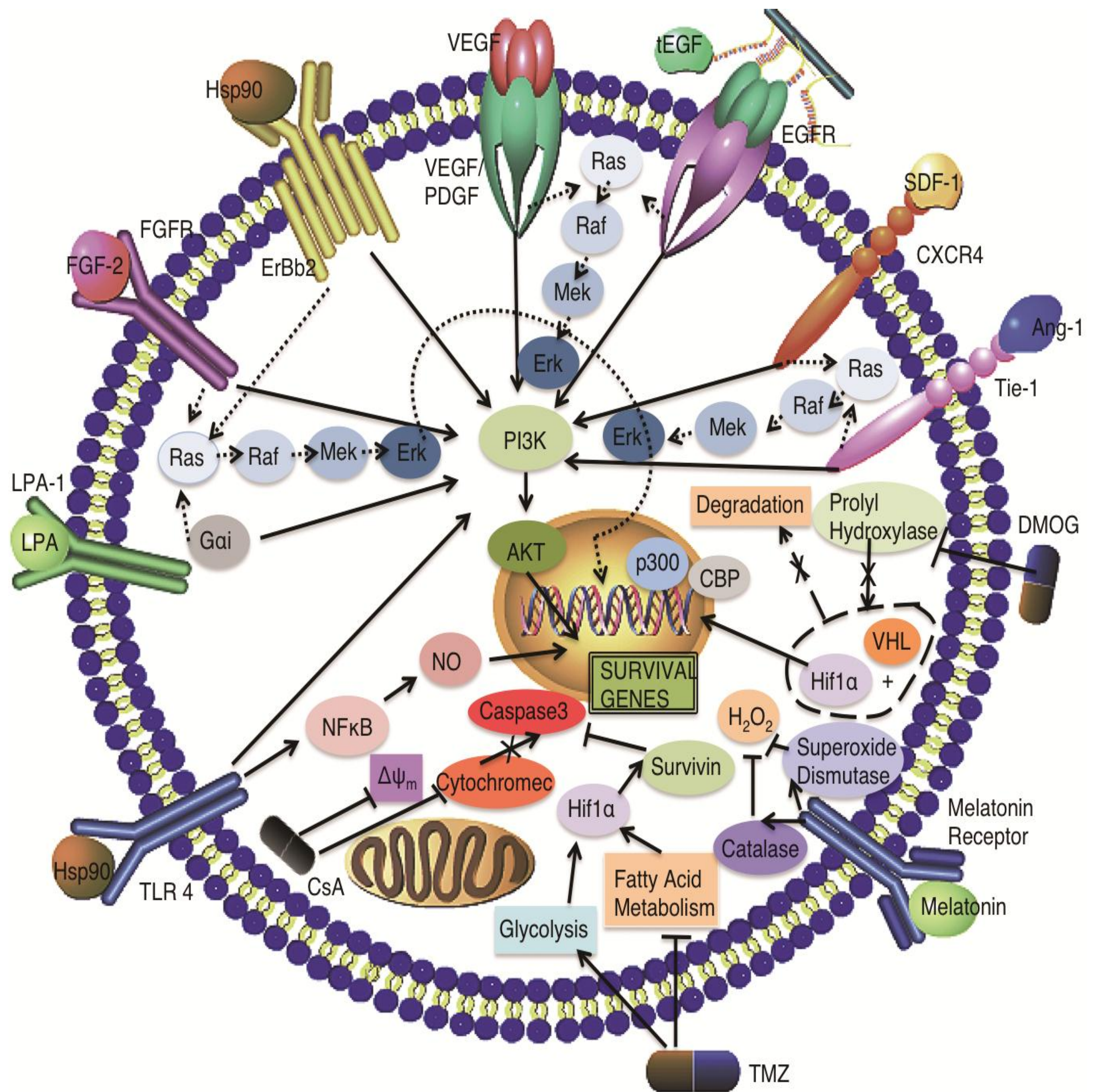

Figure 3. Adult Stem Cell Survival Signaling. Heat shock Protein 90 (Hsp90) binds the orphan growth factor receptor ErBb-2 on its extracellular domain activating PI3K-AKT and Erk signaling pathways. It also binds TLR-4 causing both NFκB activation and nitric oxide (NO) synthesis and activation of PI3K-AKT. Growth factors, such as Fibroblast Growth Factor-2 (FGF-2) binding its receptor FGFR; Vascular endothelial growth factor (VEGF) binding its receptor VEGFR, Platelet derived growth factor (PDGF) bindings its receptor PDGFR, and

Angiopoetin-1 (Ang-1) binding its receptor Tie-1, activate the PI3K-AKT and MAPK-Erk pathways, which stimulate transcription of survival genes. Bioengineered models of growth factors tethered to a substratum in contact with the cell membrane such as a tethered epidermal growth factor (tEGF) restrict the activated growth factor receptor (in this case EGFR) to the cell surface and cause sustained signaling of the downstream Erk pathway prolonging survival signaling. Similarly bioactive lipids including Lisophosphatidic acid (LPA) binding its G-protein coupled receptor LPA-1 and chemokines such as Stromal derived factor-1 (SDF-1) binding its receptor CXCR4, signal survival via the MAPK-Erk and AKT pathways. Hormones such as Melatonin binding its receptor increase reactive oxygen species reducing enzymes Catalase and Superoxide dismutase, thus lowering hydrogen peroxide in cells and protecting cells from oxidative damage. Anti-ischemic drugs such as TMZ increase glycolysis in the cells and decrease fatty acid metabolism, causing increases in Hif1α and pro-survival proteins survivin and Bcl-2, which inhibit caspase3 in addition to activating Akt. The prolyl hydroxylase inhibitor DMOG by inhibiting prolyly hydroxylase, inhibits the complex formed by Hif1α which targets it to degradation, allowing Hif1α to shuttle to the nucleus, bind CBP and p300, allowing the transcription of pro-survival genes. Also shown in another drug, Cyclosporin-A that acts in the mitochondria, preventing the drop in membrane potential and release of cytochrome-c, thereby inhibiting the mitochondrial death pathway.

2.5 DISCUSSION

Reduction of stem cell numbers within the first two weeks of implantation remains a major hurdle in the application of stem cell therapy for regeneration of tissue. The increasing number of pre-clinical and clinical trials using stem cells that have been successful are due primarily to the ability of the stem cells to release trophic factors and cause immunosuppression, increasing vasculogenesis, increasing survival of surrounding cells and preventing graft rejection. However the prime potential of these cells, of differentiating into specific cell lineages, is lost as the cells die in the ischemic, oxidative and inflammatory wound environment. The cells are lost in the presence of low oxygen, low nutrient concentrations, high levels of reactive oxygen species and pro-death cytokines. Thus, the field needs to develop ways to improve survival during the initial challenge period.

This chapter has brought together current literature on increasing attachment and survival of stem cells in the harsh graft environment. There have been successful gene transfer studies both in vitro and in vivo, under conditions of ischemia and oxidative stress where introduction of pro-survival genes have shown substantial increases in survival. However gene therapy poses a threat of causing aberrations during gene integration, especially in a cell type whose main feature involves self-renewal. Use of protein factors on the other hand, overcomes the problem of neoplastic transformation via gene integrations. Growth factors, chemokines and heat shock proteins have been tested and have shown significant improvements in retaining cell numbers at sites of inflammation. However these proteins have to be chosen in a manner that will improve both stem cell proliferation to obtain sufficient numbers of these scarcely occurring cells, and survival, without affecting differentiation of the stem cells into an undesirable tissue lineage. Also, a limiting factor with the use of proteins currently is their mode of presentation.

Bioengineering studies currently underway will be a major factor that can bridge this gap. Development of biocompatible polymeric scaffolds, that can function as a carrier of both stem cells and proteins, maintaining proteins in their active conformation and restricting the signaling of proteins to the stem and progenitor cell compartment alone for a desired duration of time until tissue repair has been established will take cell therapy to newer heights. Use of growth factors with anti-oxidants to alleviate oxidative stress or drugs that counter ischemia might also be a useful way of improving stem cell survival in vivo. While these studies require further improvements prior to even preclinical testing of relevant defects, the recent findings with supportive and tethered factors hold promise that these challenges will be overcome.

3.0 PRODUCTION OF REACTIVE OXYGEN SPECIES BY MULTIPOTENT STROMAL CELLS/MESENCHYMAL STEM CELLS ON EXPOSURE TO FASL

Melanie Rodrigues, Omari Turner, Donna Stolz, Linda Griffith and Alan Wells

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3.1 ABSTRACT

Multipotent stromal cells (MSC) can be differentiated into osteoblasts and chondrocytes, making these cells candidates to regenerate cranio-facial injuries and lesions in long bones. A major problem with cell replacement therapy however is the loss of transplanted MSCs at the site of graft. Reactive oxygen species (ROS) and non-specific inflammation generated at the ischemic site have been hypothesized to lead to MSCs loss; studies *in vitro* show MSCs dying both in the presence of ROS or cytokines such as FasL. We questioned whether MSCs themselves may be the source of these death inducers, specifically whether MSCs produce ROS under cytokine challenge. On treating MSCs with FasL we observed increased ROS production within two hours, leading to apoptotic death after 6 hours of exposure to the cytokine. N-acetyl cysteine, an antioxidant, is able to protect MSCs from FasL-induced ROS production and subsequent ROS-dependent apoptosis, though the MSCs eventually succumb to ROS-independent death signaling. Epidermal growth factor (EGF), a cell survival factor, is able to protect cells from FasL induced ROS production initially; however the protective effect wanes with continued FasL exposure. In parallel, FasL induces upregulation of the uncoupling protein UCP2, the main uncoupling protein in MSCs, which is not abrogated by EGF; however, the production of ROS is followed by a delayed apoptotic cell death despite moderation by UCP2. FasL-induced ROS activates the stress-induced MAPK pathways JNK and p38MAPK as well as ERK, along with the activation of Bad, a pro-apoptotic protein, and suppression of Survivin, an anti-apoptotic protein; the latter two key modulators of the mitochondrial death pathway. FasL by itself also activates its canonical extrinsic death pathway noted by a time-dependent degradation of c-FLIP and activation of caspase8. These data suggest that MSCs participate in

their own demise due to non-specific inflammation, holding implications for replacement therapies.

3.2 INTRODUCTION

Multipotential stromal cells, also called mesenchymal stem cells, (MSCs) have the ability to differentiate into a wide range of cell types *in vitro* including cells of the bone, cartilage, and fat ((Pereira, Halford et al. 1995; Pittenger, Mackay et al. 1999; Bianco, Riminucci et al. 2001; Kolf, Cho et al. 2007)). However, there lies a major void between studies done with these cells *in vitro* and with their use in a wound microenvironment *in vivo* since MSC once implanted rarely survive. While there have been reparative effects seen after MSC implantation into sites of injury, the regeneration is attributed to the paracrine signaling by the MSC, or immunosuppressive aspects, rather than MSC differentiation into the desired tissue ((La Manna, Bianchi et al. 2010; Tate, Fonck et al. 2010; Angoulvant, Ivanes et al. 2011)). Tests conducted *in vivo* in small animals show that MSCs implanted both in the ischemic as well as the normal heart start dying within 7-10 days and completely disappear by the fourth week (38). Similar engraftment studies display less than 3% of the administered MSCs after two weeks, a number which is hardly sufficient to reform lost tissue ((Liu, Ding et al. 2010),(Pittenger and Martin)). MSCs used for treatment of inflammatory bowel disease display clinical improvements in all mice injected with MSCs, however only 40% of mice showed engraftment of cells, with as little as 0.13% of MSCs traceable in the intestine after 3 days ((Semont, Mouiseddine et al. 2010)). Similarly decreased numbers of MSC are observed after delivery to the ischemic kidney ((Mias, Trouche et al. 2008)). The reduced MSC numbers are consistent in studies across tissues.

Several factors have been hypothesized and reported for making the environment in which MSCs are injected poorly conducive to cell survival. Lack of trophic factors is one. But also these cells must survive a surge of non-specific inflammation involving the release of pro-death cytokines ((Fan, Tamama et al. 2007; Rodrigues, Griffith et al. 2010)). Other non-

exclusive causes may include reduced blood flow at the wound site causing hypoxia ((Zhu, Chen et al. 2006)), ROS generation ((Wei, Li et al. 2010)) and nutrient starvation ((Kim, Meliton et al. 2010; Mantel, Messina-Graham et al. 2010)), leading to cellular stress. All these factors would culminate in loss of the transplanted MSC.

The microenvironment in which MSC are implanted are generally wound regions with high pro-death cytokine activity. Based on the assumption that MSC may be susceptible to pro-death cytokines, Fan et al tested for survival of MSC in the presence of several pro-death factors and found that FasL was the most efficient in triggering MSC cell death ((Fan, Tamama et al. 2007)). This cell death is enhanced when MSC are stressed with nutrient deprivation/protein limitation; in vitro this is mimicked with the protein synthesis inhibitor cycloheximide ((Fan, Tamama et al. 2007)). MSC have been shown to be killed by CD8+ T lymphocytes and activated NK cells ((Crop, Korevaar et al. 2011; Gotherstrom, Lundqvist et al. 2011)), the two cell types which abundantly and perhaps exclusively produce FasL ((Strasser, Jost et al. 2009)) with several groups showing IL-2 activated NK cells to be detrimental to MSC ((Rasmusson, Ringden et al. 2003; Poggi, Prevosto et al. 2005; Spaggiari, Capobianco et al. 2006)). Le Blanc's group also notes that both fetal and adult MSC are susceptible to death by activated NK cells, dependent on FasL, an innate immune response triggered upon implantation of foreign materials into the body ((Gotherstrom, Lundqvist et al. 2011)). However there is one contrary report where Mazar et al show that activation of Fas, the receptor for FasL by anti-Fas antibody does not lead to MSC death ((Mazar, Thomas et al. 2009)). These differences in outcomes may be explained by the mode of activation of Fas and culture surfaces, the study by Fan et al being done using Fas ligand on bio-engineered polymer surfaces and the studies by Mazar et al using anti-Fas antibody on tissue culture plastic ((Fan, Tamama et al. 2007; Mazar, Thomas et al. 2009)).

The target of MSC therapy is either a wound/injury bed and/or implantation; in both these cases there is active inflammation, which involves the generation of ROS. ROS evoke many intracellular events in cells including cellular proliferation, migration and cell cycle arrest, but mainly cell death ((Groemping, Lapouge et al. 2003; Lee, Kang et al. 2004; Chess, O'Reilly et al. 2005)). Increased ROS in cells brings about damage not only by oxidizing DNA, proteins and lipids, but also by activation of cellular stress-activating pathways. Recently there have been studies indicating that paracrine signaling of ROS on MSC can be detrimental to these cells. According to one report hydrogen peroxide, a major contributor to oxidative damage when added to MSC in culture, activated the caspase cascade and induced apoptosis in MSC (37). In another study ROS was seen to inhibit adhesion of MSC to the ischemic myocardium ((Song, Cha et al. 2010)). Additionally, senescent human umbilical cord MSC have shown to be killed in the face of increased ROS ((Liu, Ding et al. 2010)). These reports suggest that MSC will undergo cell death in an ischemic environment where there is excessive ROS production.

Based on the independent studies that reported FasL as a first phase immune response cytokine detrimental to MSC ((Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011)), the primary goal of our study was to investigate whether cytokines such as FasL induce ROS production in MSC and contribute to death by causing oxidative damage. Our hypothesis is based on reports that FasL generates ROS in several cell types, which causes further death receptor clustering, activation of caspases and apoptosis ((Zhang, Yi et al. 2006; Zhang, Yi et al. 2007; Circu and Aw 2010)). We also probed for Uncoupling Proteins (UCP), protein transporters present in the inner mitochondrial membrane that are upregulated to counter increases in ROS in several cell types ((Le Minh, Berger et al. 2010; Pi and Collins 2010; Affourtit, Jastroch et al. 2011; Basu Ball, Kar et al. 2011; Bugger, Guzman et al. 2011)). As such, UCP-deficient animals

show higher ROS production and greater oxidative damage ((Brand, Affourtit et al. 2004)). We found that FasL increased ROS within a few hours of treatment, causing high levels of intracellular ROS levels and subsequently caused activation of caspase3 leading to MSC death. MSC with UCP2 reduced by genetic means, showed faster onset of apoptosis upon treatment with FasL compared to MSC without UCP2 knockdown suggesting that UCP2 does negate intracellular ROS produced by FasL. To our knowledge this is the first time FasL has been linked to changes in uncoupling protein expression in terms of moderating oxidative stress in any cell type. Our data suggest that MSC will not only be susceptible to paracrine signaling of ROS when implanted in ischemic wound regions, but will also produce damaging ROS in response to implantation owing to non-specific inflammation.

3.3 MATERIALS AND METHODS

3.3.1 Reagents

Human recombinant epidermal growth factor [354052] was procured from BD BioSciences (Franklin Lakes, NJ) and human soluble recombinant SuperFasL [ALX-522-020-3005] was obtained from Enzo Lifesciences (Plymouth Meeting, PA). Mitosox Red [M36008] and CM-H2DCFDA [C6827] were from Molecular Probes, Invitrogen (Carlsbad, CA). RNeasy Minikit and QuantiTect Reverse Transcription kit were procured from Qiagen (Valencia, CA). Brilliant SYBR Green qPCR Master Mix was from Stratagene (Santa Clara, CA). Ac DEVD AFC [556574] and Ac IETD AFC [556552] were from BD Pharmigen (San Diego, CA). UCP2 [SAB 2501087] antibody was from Sigma (Saint Louis, MO), c-FLIP antibody [ab8421] was from AbCam (Cambridge, MA), phospho-JNK antibody [17-466] was from (Billerica, MA), phospho-ERK1/2 antibody [4377], phospho-Bad antibody [9291] and Survivin antibody [2808] were from Cell Signaling Technology (Boston, MA). Caspase inhibitor Z-VAD-FMK [G7231] was from Promega (Madison, WI). UCP1 and UCP2 forward and reverse primers were from Integrated DNA Technologies (Coralville, IA). siRNA for UCP2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

3.3.2 Cell Culture

Immortalized human bone marrow stromal cells (imhMSC) ((Okamoto, Aoyama et al. 2002)), a kind gift from Dr. Junya Toguchida's lab (Kyoto University, Japan), were cultured in DMEM low glucose

From Cellgro (Mediatech, Washington, DC) with 10% fetal bovine serum, 1mM Sodium pyruvate, 1 μM non-essential amino acids and 100units/ml penicillin-streptomycin. The tenth passage of these cells was used for experiments. Primary human marrow stromal cells (prhMSC) ((Sekiya, Larson et al. 2002)) provided by Dr. Darwin Prockop (Texas A&M) were cultured in α-MEM without ribonucleotides or deoxyribonucleotides from Gibco (Carlsbad, CA) with 16.5% FBS from Atlanta Biologicals (Norcross, GA), and 2mM L-glutamine. We have previously characterized both these cell lines in terms of differentiation potential, proliferation in response to growth factors and death in the presence of cytokines (21,65). The use of the human cells has been approved the IRB committees of the University of Pittsburgh and Pittsburgh VAMC.

3.3.3 Concentration of Cytokines/ Reagents

MSCs were treated with 100nM EGF, 100ng/ml FasL, 20μM cycloheximide (CHX), 50μg/ml n-acetyl cysteine (NAC), 0.03% H_2O_2 and 50μg/ml zVAD-FMK.

3.3.4 Mitosox Red Assay

Mitosox red is a live cell permeant probe that gets oxidized rapidly by superoxide in the mitochondria and fluoresces red on superoxide generation. imhMSC and prhMSC were grown in 6 well plates until 60% confluent. Cells were washed in PBS, 20μM Mitosox red reagent in PBS and Hoescht 33342 were added and cells incubated at 37°C for 10 min to allow for cells to intake the probe. Cells were rinsed in PBS to wash out all extracellular mitosox red and MSC treated with EGF/FasL/CHX/NAC. Images were taken at regular time periods up to 8 hours and

quantified by Image J analysis. Since cell numbers were less with onset of death, increases in ROS were represented as the ratio of Mitosox intensity to total cell numbers.

3.3.5 CM-H2DCFDA Assay

CM-H2DCFDA is a cell permeable indicator that fluoresces only upon oxidation and after loss of its acetate group and is a marker for hydroxyl radical. imhMSC and prhMSC were grown in 96 well plates. On the day of assay, media was aspirated, cells washed with PBS and 10μM CM-H2DCFDA reagent in PBS added to cells. Cells were treated with EGF/FasL/CHX/NAC alone and in combinations, incubated at 37°C and fluorescence at an excitation of 485nm and emission of 535nm measured at regular intervals up to 8 hours.

3.3.6 Immunoblots

After treatment of cells with cytokines at defined time points, both floating cells in the media and cells left on the culture plate were lysed in SDS lysis buffer containing 0.1 M Tris-HCl, 4% SDS, 0.2% Bromophenol Blue and 5% β-mercaptoethanol. Cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were probed for UCP2 and GAPDH primary antibodies, followed by HRP conjugated secondary antibodies and developed with an ECL kit.

3.3.7 FLICA (Fluorochrome Inhibitor of Caspase Assay)

MSC were grown on Lab-tek 8 chamber slides and once the cells were 70% confluent, cells were changed into media with 0.5% dialyzed FBS. Cells were treated with cytokines such as FasL alone, EGF + FasL etc for 6 hours, following which FLICA reagent and Hoescht 33342 dye was added to cells in media and incubated for 30 minutes at 37°C. Media were aspirated, cells washed and live cells imaged for fluorescence. FLICA intensity was measured by Image J analysis software. To account for cell loss, intensity of FLICA was normalized to the number of remaining cells.

3.3.8 Real Time Quantitative PCR Analysis

Total RNA from MSC was isolated using RNeasy Minikit. 1µg of RNA was reverse transcribed into first strand c-DNA using QuantiTect Reverse Transcription kit. Real time PCR was performed on a MX3000P instrument from Stratagene using 1µl of c-DNA and Brilliant SYBR Green qPCR Master Mix and the following primers: UCPI Forward Primer: 5'-CTGCCACTCCTCCAGTCGTT-3'; UCP1 Reverse Primer: 5'-CCG CCTCTCTCAGGATCGGCCT-3'; UCP2 Forward Primer: 5'-GACCTATGACCTCATCAAGG-3'; UCP2 Reverse Primer: 5'-ATAGGTGACGAACATCACCACG-3'; GAPDH Forward Primer: 5'-GAGTCAACGGATTTGGTCGT-3'; GAPDH Reverse Primer: 5'-TTCATTTTGGAGGGATCTCG-3'; the GAPDH primers were the normalization controls. All reactions were performed in triplicates and the fold change in transcript levels was calculated based on ΔCt method with GAPDH as reference.

3.3.9 Caspase 3/ Caspase 8 Activity Assay

To 15μg of protein sample, obtained by lysis of imhMSC with RIPA buffer and measurement of protein by BCA assay, 2μl of 2mM Ac DEVD AFC or 2μl of 2mM Ac IETD AFC was added to 200μl of buffer to detect Caspase3 or Caspase8 activity respectively. The buffer comprised of 20mM PIPES, 100mM NaCl, 1mM EDTA, 0.1% CHAPS, 10% sucrose in water, with Ph of 7.2. The samples were incubated with the reporter substrate for 8 hours and fluorescence measured at an excitation of 400nm and an emission of 505 nm.

3.3.10 UCP2 siRNA Knock-down

0.5nM siRNA was transfected into imhMSC using Lipofectamine 2000 for 6 hours (in OptiMem), followed by a complete media change. Cells were left in media for 24 hours and assayed for Mitosox and FLICA after treatment with FasL and CHX and FasL. A fluorescein-conjugated scrambled RNA sequence also obtained from Santa Cruz Biotechnology (Santa Cruz, CA) was used as control siRNA.

3.3.11 Statistical Analysis

ROS levels and cell numbers were analyzed using paired t tests. Significance was set at $p<0.05$ or a more stringent $p<0.01$ as noted in the figure legends. The graphs of ROS increases with different treatment conditions were compared to each other using 2-way ANOVA and TUKEY's tests on SPSS software. Significance for these tests was set as $p<0.05$.

3.4 RESULTS

3.4.1 FasL Induces Superoxide Anion Production in MSC

Pro-death cytokines such as FasL and TNF-α cause increased ROS generation in several cell types causing intensive damage to cells and leading to cell death ((Gulbins, Brenner et al. 1996; Chen, Chen et al. 2003; Sato, Machida et al. 2004; Jin, Ray et al. 2008)). In the present study we used Mitosox red staining to determine increases in levels of the potent ROS superoxide anion after treatment of MSC with FasL. MSC are known to undergo cell death in the presence of FasL and this death is further enhanced when protein synthesis is inhibited using cycloheximide (CHX) ((Fan, Tamama et al. 2007)). CHX was used at sub-optimal concentrations, enough to induce cell stress, but not strong enough to kill MSC. As seen in Figure 4, imhMSC treated with FasL showed Mitosox fluorescence within 2 hours and the fluorescence intensity kept increasing through 8 hours after treatment. While addition of CHX to FasL increased Mitosox red staining to a much greater extent, addition of the antioxidant NAC to FasL treated cells prevented the increase of superoxide anion generation. The positive control comprising MSC treated with H_2O_2 showed pronounced increase in Mitosox fluorescence. We were thus able to conclude that FasL increases superoxide anion synthesis in MSC.

The intensity of Mitosox fluorescence was normalized to cell numbers and is displayed in Figure 2A. Treatment of imhMSC with FasL alone showed a two and a half fold steady increase in Mitosox fluorescence over the eight hour time frame compared to untreated cells, while treatment with CHX and FasL showed a 3 fold increase of Mitosox fluorescence after eight hours compared to untreated imhMSC. Subjecting MSC to H_2O_2 showed a trend similar to CHX + FasL treatment of cells with the fluorescence after eight hours being three times more than

untreated control cells. NAC protected cells from FasL for six hours, after which the effects of NAC appeared to wear out, as re-addition of NAC after the first four hours was able to protect imhMSC from FasL induced ROS even at eight hours (data not shown). Addition of EGF to both FasL alone and to CHX and FasL was able to delay onset of superoxide generation by two hours and alleviated the increased superoxide seen with both FasL alone and with CHX + FasL. EGF alone or CHX alone did not bring about any change in superoxide anion levels over the eight hours compared to untreated imhMSC. The comparison of the various treatments over time was analyzed by ANOVA and TUKEYs test and is represented in Figure 6.

To determine whether the increase in ROS was specific to immortalized MSC, we also tested primary human MSC with the death cytokine. prhMSC showed a similar trend of Mitosox intensity normalized to cell numbers with the various treatments as imhMSC. The primary MSC were more sensitive to both FasL and CHX + FasL treatments, with Mitosox fluorescence increasing to three and a half times that of untreated cells after eight hours on addition of FasL alone and five times that of untreated prhMSC with CHX + FasL treatment after eight hours (Figure 2B). EGF added to both MSC treated with FasL alone and with CHX + FasL, offered protection against increase of ROS. EGF alone or CHX alone did not bring about any changes in ROS, however H_2O_2 showed an increase in mitosox staining with time, as expected. The statistical comparison of the curves over time is represented in Figure 7.

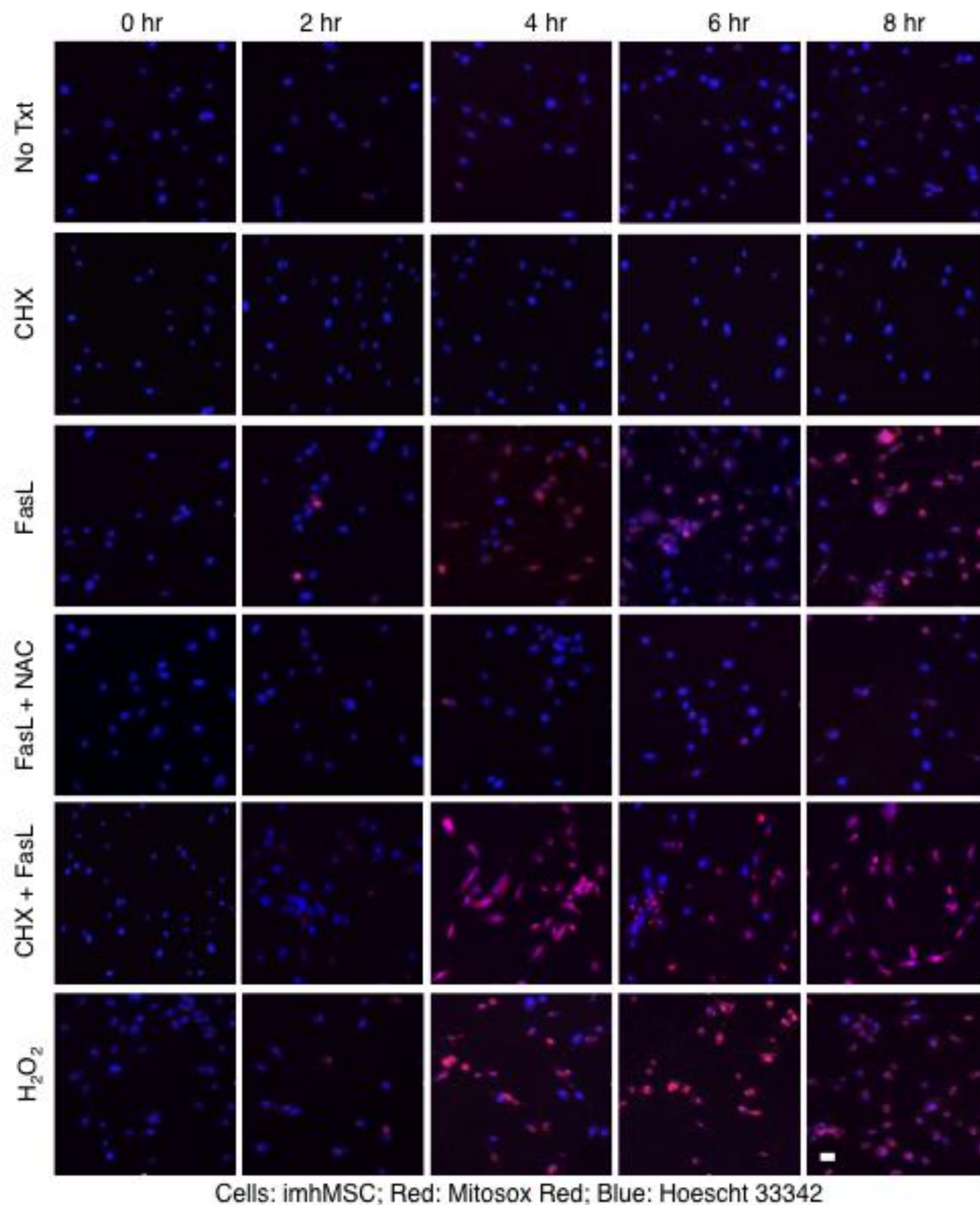


Figure 4 FasL stimulates production of mitochondrial superoxide anion in MSC. imhMSC were treated with various factors and then tested for Mitosox red flourescence at the indicated time points. Mitosox red fluorescence (red) is an indicator for mitochondrial superoxide generation. Shown are representative

photomicrographs of cells, of three independent experiments. Magnification 20X, scale bar 10μm. Not shown are similar experiments with primary human MSC.

3.4.2 FasL Induces Severe Oxidative Stress in MSC

As high levels of superoxide anion were generated with FasL in MSC, we hypothesized that there should also be high levels of other damaging ROS such as hydroxyl radicals. This would be the case since superoxide anion gets dismutated spontaneously or by superoxide dismutases to generate hydrogen peroxide and molecular oxygen, and hydrogen peroxide further breaks down by the Fenton reaction to produce hydroxyl radical ((Fridovich 1978)). To look for hydrogen peroxide and hydroxyl radicals, we used CM-H_2DCFDA. CM-H_2DCFDA fluorescence after treatment of imhMSC with FasL showed a linear increase with time and ended six times higher than untreated cells after eight hours, while CHX + FasL rapidly increased CM-H_2DCFDA fluorescence to over ten times that of untreated imhMSC within the first two hours and maintained this value to over five times that of untreated cells even after eight hours of treatment (Figure 2C). Addition of NAC to FasL treated cells entirely suppressed hydroxyl radical generation during the eight hour time frame, and addition of EGF to FasL alone and CHX + FasL reduced hydroxyl radical generation in both cases. CHX alone and EGF alone did not bring about any change in hydrogen peroxide or hydroxyl radical generation compared to untreated cells over the entire treatment time-frame as seen with the mitosox results. H_2O_2 was used as the positive control and showed rapid increases in CM-H_2DCFDA fluorescence. The statistical comparison of the different treatments over time by ANOVA and TUKEYs test is displayed in Figure 8.

Demonstrating that this hydroxyl generation is not unique to the immortalized MSC, prhMSC showed a thirty fold increase in generation of hydroxyl radicals with CHX + FasL compared to untreated cells after eight hours and a ten fold increase with FasL compared to untreated cells in the same time frame (Figure 2D). NAC again was able to completely block this

increase of hydroxyl radicals, while EGF protected both prhMSC treated with FasL alone and with CHX + FasL significantly, although these cells still display CM-H_2DCFDA fluorescence. CHX alone and EGF alone did not produce any hydrogen peroxide or hydroxyl radicals compared to control cells during the entire treatment. From the results obtained with both imhMSC and prhMSC, we were able to conclude that FasL produces severe oxidative stress in MSC and this stress is amplified when protein synthesis is curtailed. The comparison of the effects on oxidative stress in prhMSC with the different treatments is represented in Figure 9.

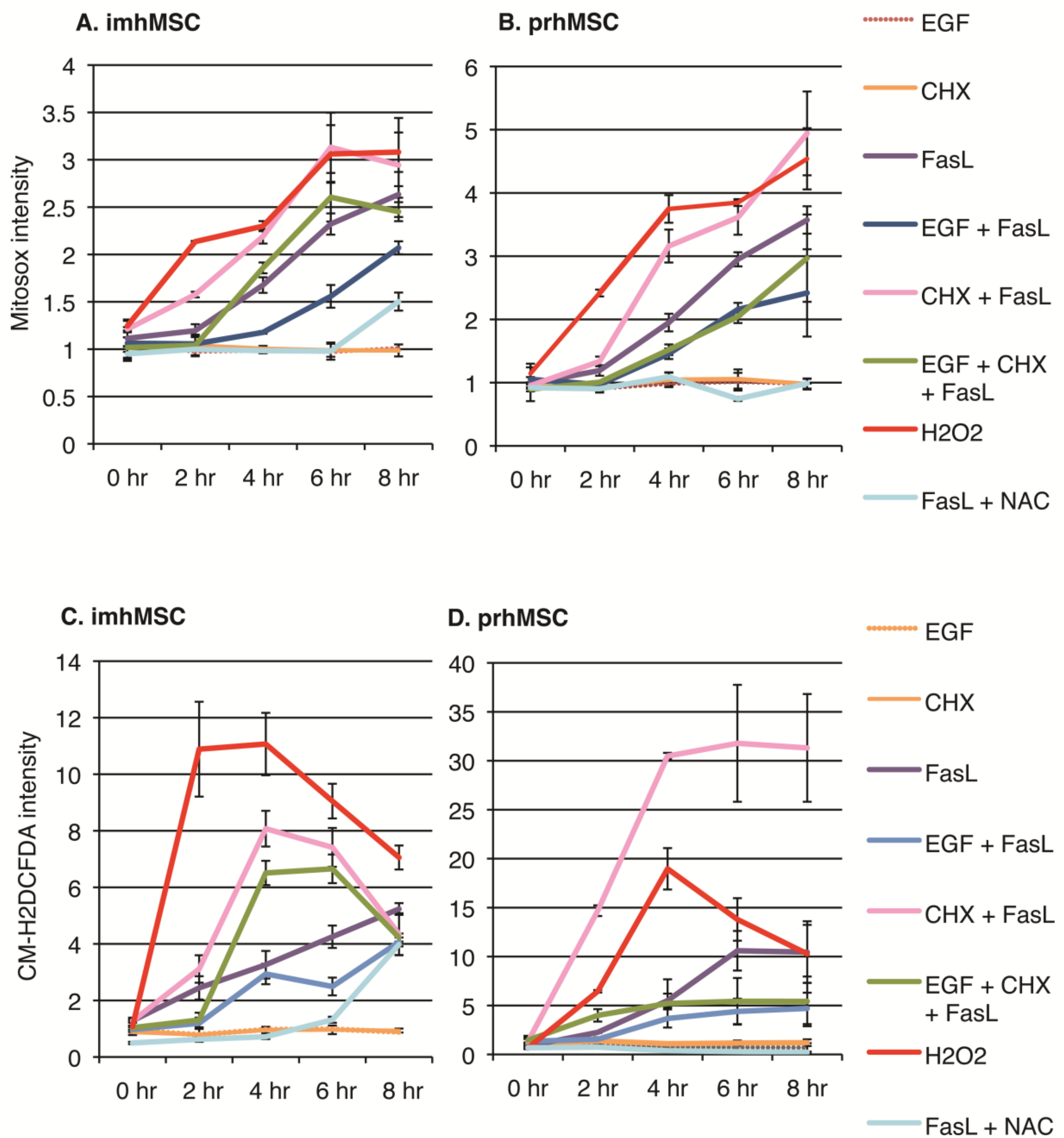


Figure 5 FasL causes severe oxidative stress in MSC. Both imhMSC (A, C) and prhMSC (B, D) were treated and analyzed of superoxide generation as in Figure 1 (A, B) or hydroxyl radical generation (C, D). Superoxide anion generation was detected by Mitosox red fluorescence (A, B) and hydrogen peroxide and hydroxyl radical synthesis by CM-H_2DCFDA fluorescence (C, D). Shown are graphs of mean ± s.e.m. of three independent experiments, each normalized to cell number and no treatment.

Mitosox: hTERT MSC	No txt	EGF	CHX	FasL	EGF FasL	CHX FasL	EGF CHX FasL	H_2O_2	FasL NAC
No txt	NA	NS	NS	+++	++	++++	+++	+++++	+
EGF	NS	NA	NS	+++	++	++++	+++	+++++	+
CHX	NS	NS	NA	+++	++	++++	+++	+++++	+
FasL	- - -	- - -	- - -	NA	-	++	+	++++	- -
EGF FasL	- -	- -	- -	+	NA	++	++	+++++	-
CHX FasL	- - - -	- - - -	- - - -	- -	- -	NA	-	+++	- - -
EGF CHX FasL	- - -	- - -	- - -	-	- -	+	NA	++++	- -
H_2O_2	- - - - -	- - - - -	- - - - -	- - - -	- - - - -	- - -	- - - -	NA	- - - - -
FasL NAC	-	-	-	++	+	+++	++	+++++	NA

Significant increase/ decrease in Mitosox staining with $p<0.05$ between
0-5,000 denoted by +/ -
5,000-10,000 by ++/ - -
10,0000-15,000 by +++/ - - -
15,000-20,000 by ++++/ - - - -
Above 20,000 by +++++/ - - - - -

Figure 6 Change in Mitosox Fluorescence in imhMSC with various treatments over time analyzed by two-way ANOVA and TUKEYs tests.

Super oxide: prh MSC	No txt	EGF	CHX	FasL	EGF FasL	CHX FasL	EGF CHX FasL	H_2O_2	FasL NAC
No txt	NA	NS	NS	+++	++	+++++	++	+++	NS
EGF	NS	NA	NS	+++	++	+++++	++	+++	NS
CHX	NS	NS	NA	+++	++	+++++	++	+++	NS
FasL	- - -	- - -	- - -	NA	-	++	-	+++	- - -
EGF FasL	- -	- -	- -	+	NA	++	NS	+++++	- -
CHX FasL	- - - - -	- - - - -	- - - - -	- -	- -	NA	++	+++	- - -
EGF CHX FasL	- -	- -	- -	+	NS	- -	NA	+++++	- -
H_2O_2	- - - - -	- - - - -	- - - - -	- - - -	- - - - -	- - -	- - - - -	NA	- - - - -
FasL NAC	NS	NS	NS	+++	++	+++	++	+++++	NA

Significant increase/ decrease in Mitosox staining with $p<0.05$ between
0-5,000 denoted by +/ -
5,000-10,000 by ++/ - -
10,0000-15,000 by +++/ - - -
15,000-20,000 by ++++/ - - - -
Above 20,000 by +++++/ - - - - -

Figure 7 Change in Mitosox FLuorescence in prhMSC with various treatment over time analyzed by two-way ANOVA and TUKEYs tests.

CM-H2DCFDA: hTERT MSC	No txt	EGF	CHX	FasL	EGF FasL	CHX FasL	EGF CHX FasL	H_2O_2	FasL NAC
No txt	NA	NS	NS	+++	++	++++	+++	+++++	+
EGF	NS	NA	NS	+++	++	++++	+++	+++++	+
CHX	NS	NS	NA	+++	++	++++	+++	+++++	+
FasL	- - -	- - -	- - -	NA	-	+	NS	++++	- -
EGF FasL	- -	- -	- -	+	NA	++	++	+++++	-
CHX FasL	- - - -	- - - -	- - - -	-	- -	NA	+	+++	- - -
EGF CHX FasL	- - -	- - -	- - -	NS	- -	+	NA	++++	++
H_2O_2	- - - - -	- - - - -	- - - - -	- - - -	- - - - -	- - -	- - - -	NA	- - - - -
FasL NAC	-	-	-	++	+	+++	- -	+++++	NA

Significant increase/ decrease in Mitosox staining with $p<0.05$ between
0-5,000 denoted by +/ -
5,000-10,000 by ++/ - -
10,0000-15,000 by +++/ - - -
15,000-20,000 by ++++/ - - - -
Above 20,000 by +++++/ - - - - -

Figure 8 Change in CM-H2DCFDA Fluorescence in imhMSC with various treatments over time analyzed by two-way ANOVA and TUKEYs tests.

CM-H2DCFDA: prh MSC	No txt	EGF	CHX	FasL	EGF FasL	CHX FasL	EGF CHX FasL	H_2O_2	FasL NAC
No txt	NA	NS	NS	++	+	+++++	+	++++	NS
EGF	NS	NA	NS	++	+	+++++	+	++++	NS
CHX	NS	NS	NA	++	+	+++++	+	++++	NS
FasL	- -	- -	- -	NA	-	+++	-	++	- -
EGF FasL	-	-	-	+	NA	++++	NS	+++	- -
CHX FasL	- - - - -	- - - - -	- - - - -	- - -	- - - -	NA	- - - -	- -	- - -
EGF CHX FasL	-	-	-	+	NS	++++	NA	+++	-
H_2O_2	- - - -	- - - -	- - - -	- -	- - -	+ +	- - -	NA	- - - -
FasL NAC	NS	NS	NS	++	+	+++	+	++++	NA

Significant increase/ decrease in Mitosox staining with $p<0.05$ between
0-5,000 denoted by +/ -
5,000-10,000 by ++/ - -
10,0000-15,000 by +++/ - - -
15,000-20,000 by ++++/ - - - -
Above 20,000 by +++++/ - - - - -

Figure 9 Change in CM-H2DCFDA Fluorescence in prhMSC with various treatments over time analyzed by two-way ANOVA and TUKEYs tests.

3.4.3 Heightened ROS levels on FasL Treatment leads to the Onset of Apoptosis and Death in MSC

As FasL enhanced ROS and earlier reports show that FasL causes death in MSC, we hypothesized that the increased oxidative stress on FasL treatment would cause activation of caspases and apoptosis. To investigate this, we examined caspase3 activity in prhMSC using FLICA. After 6 hours of treatment with FasL or CHX + FasL, prhMSC showed active caspase3 indicated by the red fluorescence in Figure 10A. EGF added to both these treatments was protective at 6 hours (though longer challenges do lead to enhanced cell death ((Fan, Tamama et al. 2007))). Addition of NAC to FasL suppressed caspase3 activation. H_2O_2 alone also induced caspase3 activation. When zVAD-FMK the pan-caspase inhibitor was added to prhMSC treated with CHX + FasL, caspase3 activity was completely suppressed. EGF alone and CHX alone did not bring about any caspase3 activation. The intensity of FLICA fluorescence was analyzed using ImageJ and is represented in Figure 10B. These results indicate that FasL induced ROS activates apoptosis in MSC.

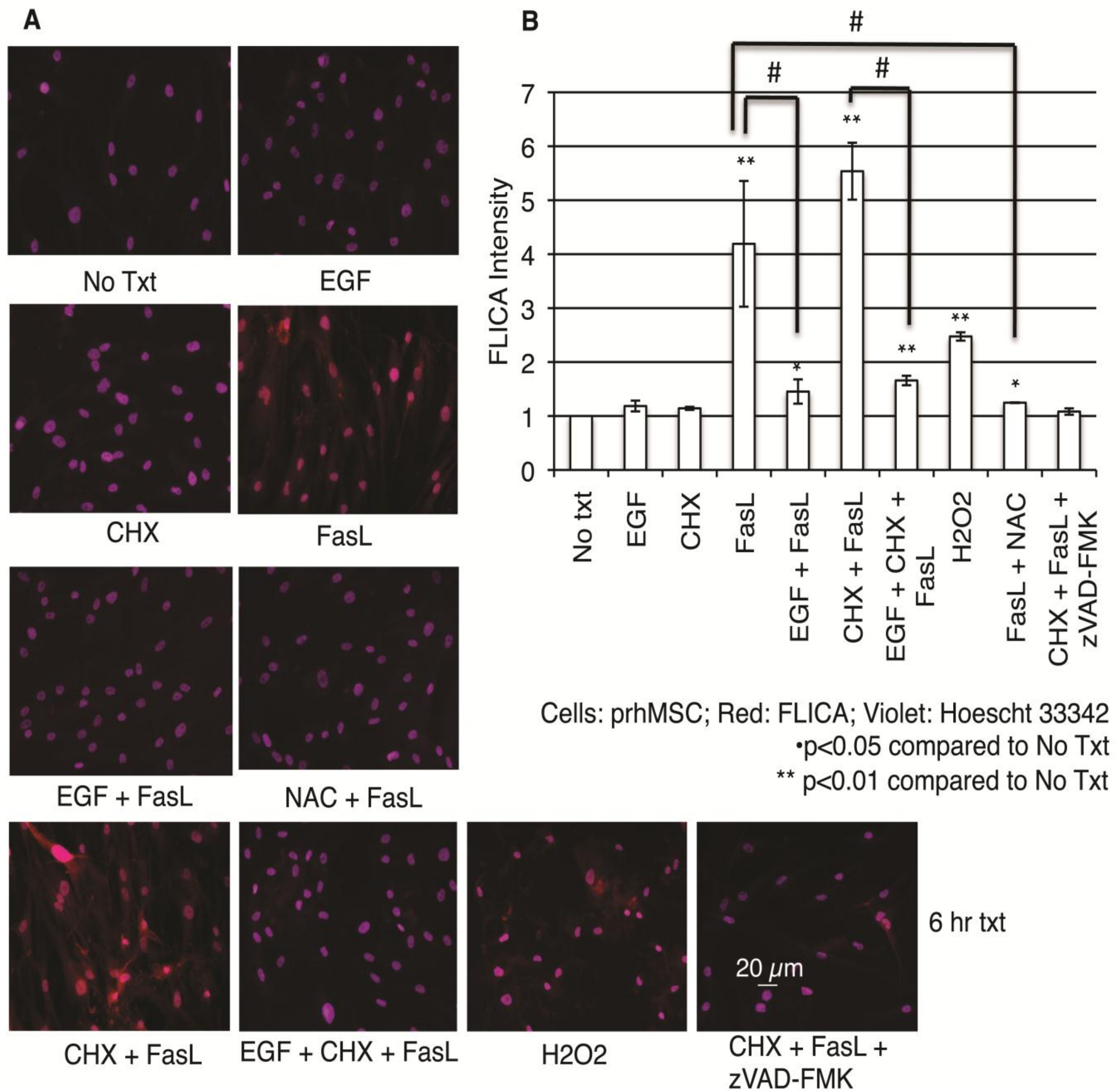


Figure 10 FasL induced ROS leads to apoptosis in MSC. (A) prhMSC were treated with the indicated agents for 6 hours and tested for caspase3 activation by FLICA. The red fluorescence represents FLICA, the violet fluorescence is Hoescht 33342 stain for nuclei. Shown are representative fields. The fluorescence is quantified in (B) with *$p<0.05$ and represents mean ± s.e.m. of three independent assays each viewing multiple fields.

3.4.4 FasL also kills MSC via the Extrinsic Death Pathway Independent of ROS

MSC were next subject to more prolonged treatment with FasL or CHX + FasL with or without the protective factors EGF or NAC. 8 Hours after treatment, MSC subjected to CHX + FasL displayed very few cells, as most cells had died by this time-point and had detached from the surface. The few cells left behind on tissue culture plastic, at the last stage of apoptosis showed diminished FLICA fluorescence. Addition of either EGF or NAC to CHX + FasL treated cells was unable to protect from caspase3 activation. Addition of zVAK-FMK however curbed caspase3 activation (Figure 11A). We were able to conclude from these results that the ROS produced in response to FasL generates enough oxidative stress to cause caspase3 activation and death of MSC; EGF is able to delay this death. NAC is able to suppress ROS generation until 6 hours; however MSC die after 8 hours of exposure to FasL even in the presence of NAC, most likely through direct signaling of FasL via the caspase8 pathway. To test further if EGF does not protect MSC from death on longer exposures with the death factor FasL, we treated MSC with the different factors for 8 hours and the lysates collected at this time-point were immunoblotted for PARP, the DNA repair enzyme, which is a caspase3 substrate. We observed FasL-induced degradation of PARP after 8 hours and this degradation increased with CHX + FasL treatment (Figure 11B). Addition of EGF was unable to protect against CHX + FasL, observed by a distinct cleaved PARP band. Addition of the pan-caspase inhibitor zVAD-FMK to CHX + FasL treated MSC prevented PARP cleavage.

To confirm that FasL was activating the caspase8 pathway, we examined expression of cellular caspase8 FLICE like inhibitory protein (c-FLIP) which modulates the activity of caspase8 by binding the Fas signaling complex and preventing the binding and activation of pre-casapse8 to this complex. Upon treatment with FasL alone or CHX and FasL, FLIP levels were

reduced in a time dependent manner (Figure 11C), consistent with reports in other cell types ((Jung, Park et al. 2009)). Directly probing for caspase8 by Ac IETD AFC demonstrated significant activation of caspase8 after 8 hours of treatment with FasL or CHX + FasL. EGF was not able to protect from caspase8 activation (Figure 11D). Similar increases with FasL alone/ CHX + FasL/ EGF + CHX + FasL was seen in caspase3 activity. Although H_2O_2 displayed increased caspase3 activity, the activated caspase8 levels were not as high as either FasL alone or CHX + FasL confirming that H_2O_2 and ROS has no role to play in activating the extrinsic death pathway.

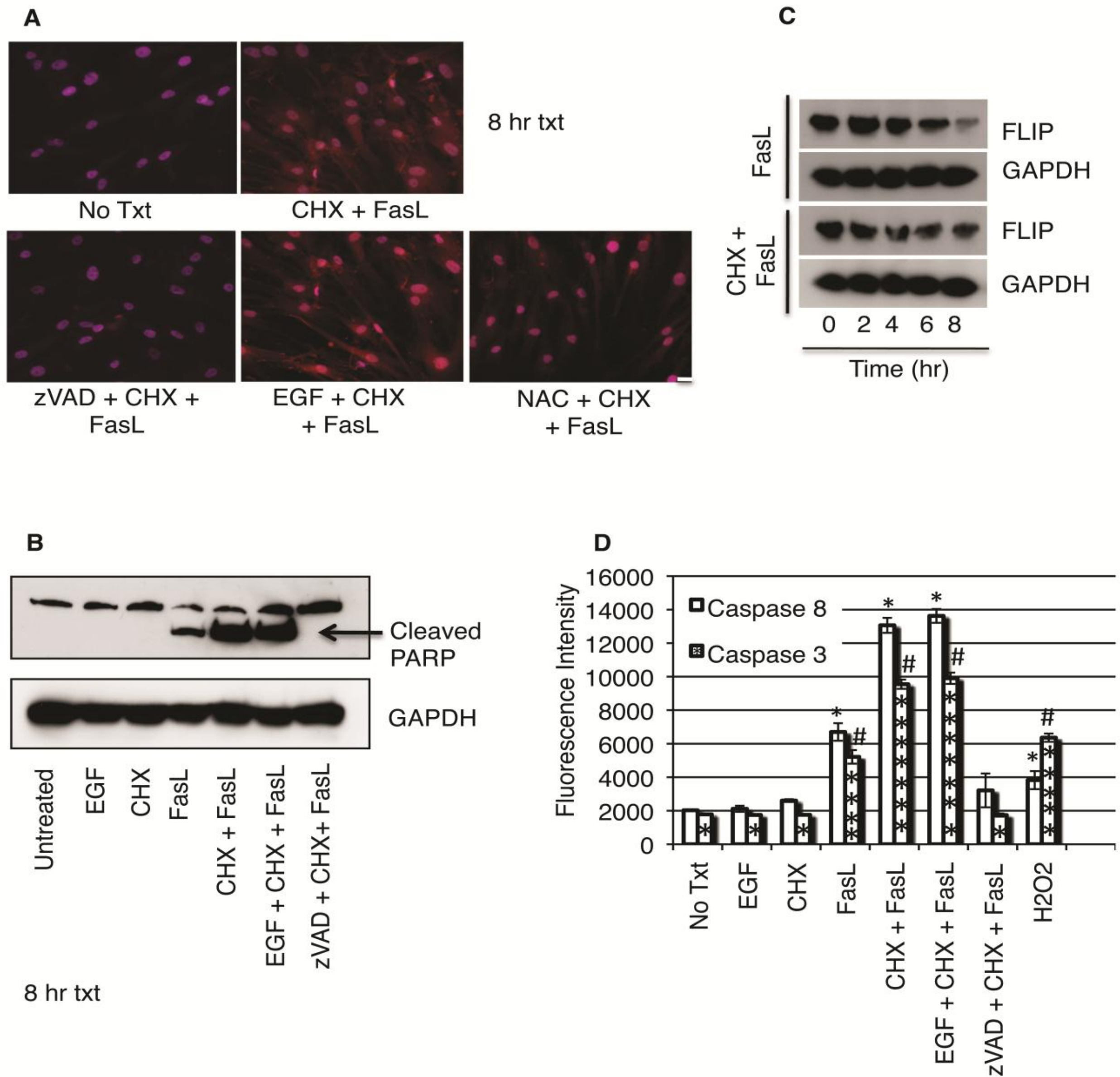


Figure 11 FasL kills MSC via the extrinsic pathway independent of ROS. (A) prhMSC were treated with the indicated agents for 8 hours and tested for caspase3 activation by FLICA. Levels of Magnification 20X, scale bar 20μm. Shown are representative fields. (B) imhMSC were treated with the indicated agents for 8 hours and tested for apoptosis by full length and cleaved PARP Immunoblot. The immunoblots are representative of at least three experiments. (C) Levels of c-FLIP in hTERT MSC assessed by Immunoblot after treatment with indicated agents. The immunoblots are representative of at least two experiments. (D) Caspase8 and caspase3 activity in imhMSC by Ac IETD AFC and Ac DEVD AFC detection, after 8 hour of treatment with indicated agents. The y-axis represents emission at 505nm and is mean ± s.e.m. of two independent assays

3.4.5 Uncoupling Protein 2 Expression Increases in Response to FasL Induced Oxidative Stress

As cells upregulate mitochondrial uncoupling proteins (UCP) as a feedback loop to attenuate increases in ROS ((Negre-Salvayre, Hirtz et al. 1997; Echtay, Roussel et al. 2002; Echtay and Brand 2007; Toime and Brand 2010)), we first looked at basal expression levels of UCPs by qPCR and found that UCP2 was by and large the main form in these cells (Figure 12A). This prompted us to examine the changes in UCP2 levels after treatment with FasL alone and CHX + FasL, the two conditions with which we saw distinct increase in ROS over the time course of eight hours. We found increases in UCP2 expression within two hours of treatment in both these cases (Figure 12B). In addition we asked whether protective factors EGF or NAC when added to FasL or CHX + FasL changed UCP2 since these factors reduced ROS. Inclusion of NAC with FasL prevented the increased UCP2 expression; this correlated with the suppression of ROS. EGF also was able to suppress CHX + FasL induced UCP2 expression for the first four hours; again correlating with lessened ROS generation. As H_2O_2, the positive control, increased UCP2 expression as expected (Figure 12B), we take this to suggest that the UCP2 upregulation is compensatory.

Since we earlier observed that high levels of ROS were established on FasL treatment after four hours, we examined the differences in UCP2 expression across treatments at this time point. UCP2 expression was increased upon exposure to FasL alone and CHX + FasL. Addition of NAC to CHX + FasL treated cells resulted in levels similar to untreated cells and much lower than cells treated with FasL alone as expected. The changes in protein were mirrored by changes in UCP2 transcript levels (Figure 12D).

The trend of UCP2 increase was similar to the trend of ROS increase after four hours (Figure 12E). EGF added to FasL or to CHX + FasL at four hours showed no significant decrease in UCP2 production compared to FasL alone or CHX + FasL. These results suggest that as ROS levels increase in MSC, levels of UCP2 also increase as a compensatory mechanism to bring about attenuation of ROS and limit oxidative stress.

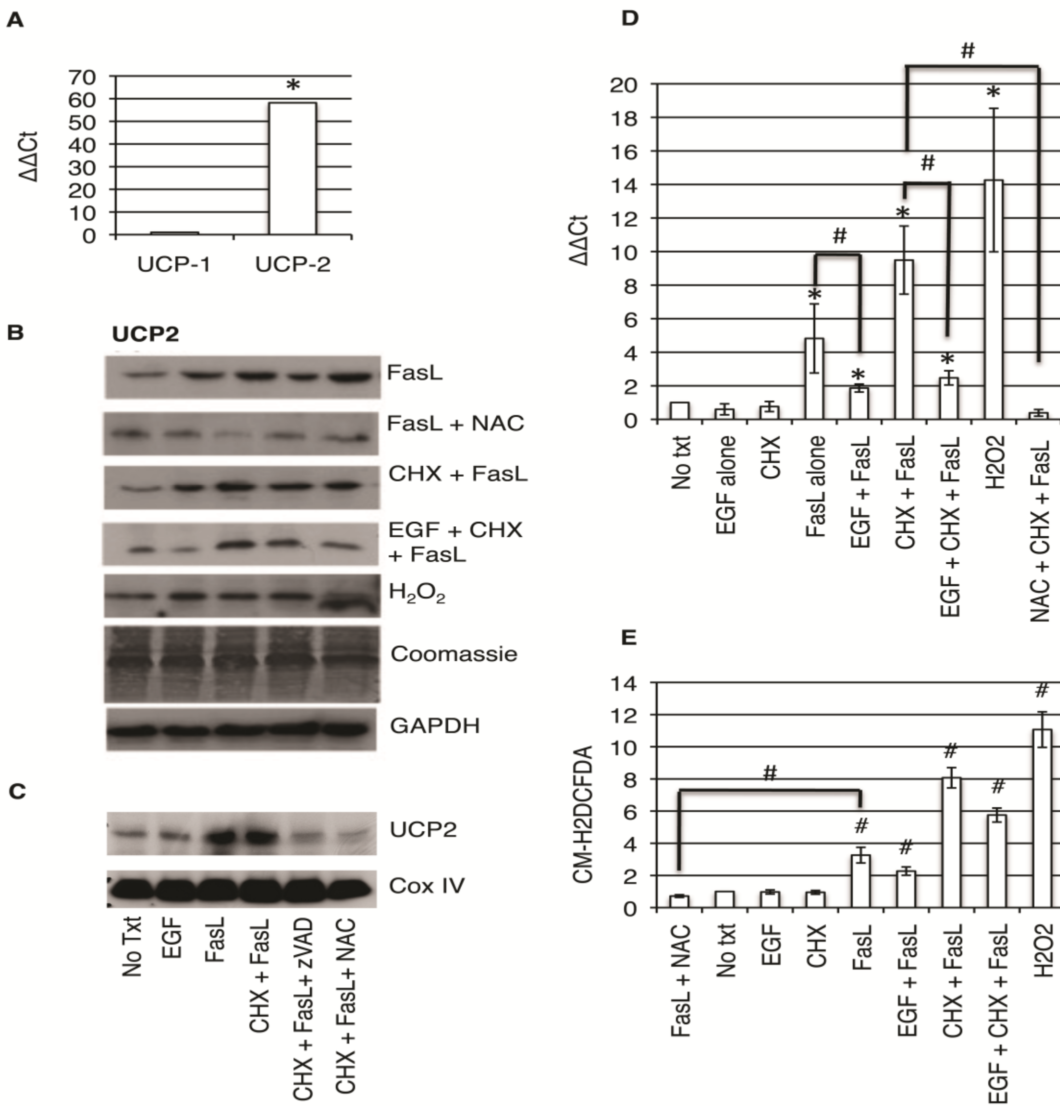


Figure 12 FasL exposure leads to an increase in UCP2 expression. (A) Levels of UCP mRNA in imhMSC were assessed by quantitative RT-PCR. (B) Cells were treated with the indicated agents and then UCP2 protein level assessed at indicated times. (C) Immunoblotting after 4 hours of treatment to show direct comparison of UCP2 levels. (D) qPCR analysis of UCP2 transcripts after 4 hours of treatment. Cycle times are compared to GAPDH and further normalized to no treatment condition. *$p<0.05$ compared to No Treatment. #$p<0.05$. (E) Changes in CM- H_2DCFDA fluorescence in imhMSC after 4 hours of treatment. *$p<0.05$ compared to No Treatment. #$p<0.05$. The immunoblots are representative of at least two experiments, and the graphs are mean ± s.d. (A) or s.e.m. (D, E) of three independent experiments.

3.4.6 Abrogation of UCP2 leads to higher levels of Apoptosis in the Presence of FasL

Since FasL increased UCP2 expression and UCP2 is a negative regulator of ROS, we wanted to see if UCP2 either prevents ROS or delays ROS increase in MSC. UCP2 levels were downregulated in imhMSC using siRNA and reduced levels were confirmed by an immunoblot for UCP2 (Figure 13A); cells transfected with a control scrambled siRNA showed no downregulation of UCP2. Non-transfected imhMSC, imhMSC with downregulated UCP2 and imhMSC transfected with scrambled RNA were treated with media alone, FasL and CHX with FasL and ROS generation was evaluated. Cells in which UCP2 was downregulated showed higher amounts of ROS compared to both non-transfected cells and cells with scrambled RNA, even in the absence of challenge. Cells with downregulated UCP2 showed greater amounts of ROS when further confronted with FasL alone, or CHX + FasL (Figure 13B). This suggested that UCP2 upregulation brought about by FasL reduces ROS levels in MSC.

When we looked at the onset of apoptosis after four hours of treatment with FasL we saw imhMSC downregulating UCP2 showing much higher levels of apoptosis and on treatment with CHX and FasL, MSC with UCP2 siRNA with fragmented nuclei due to enhanced cell death (Figure 13C). This led us to conclude that increases in UCP2 levels in the presence of FasL likely reduce ROS levels in MSC and may delay the onset of apoptosis in MSC, but that these compensatory changes are insufficient to alter ultimate outcome.

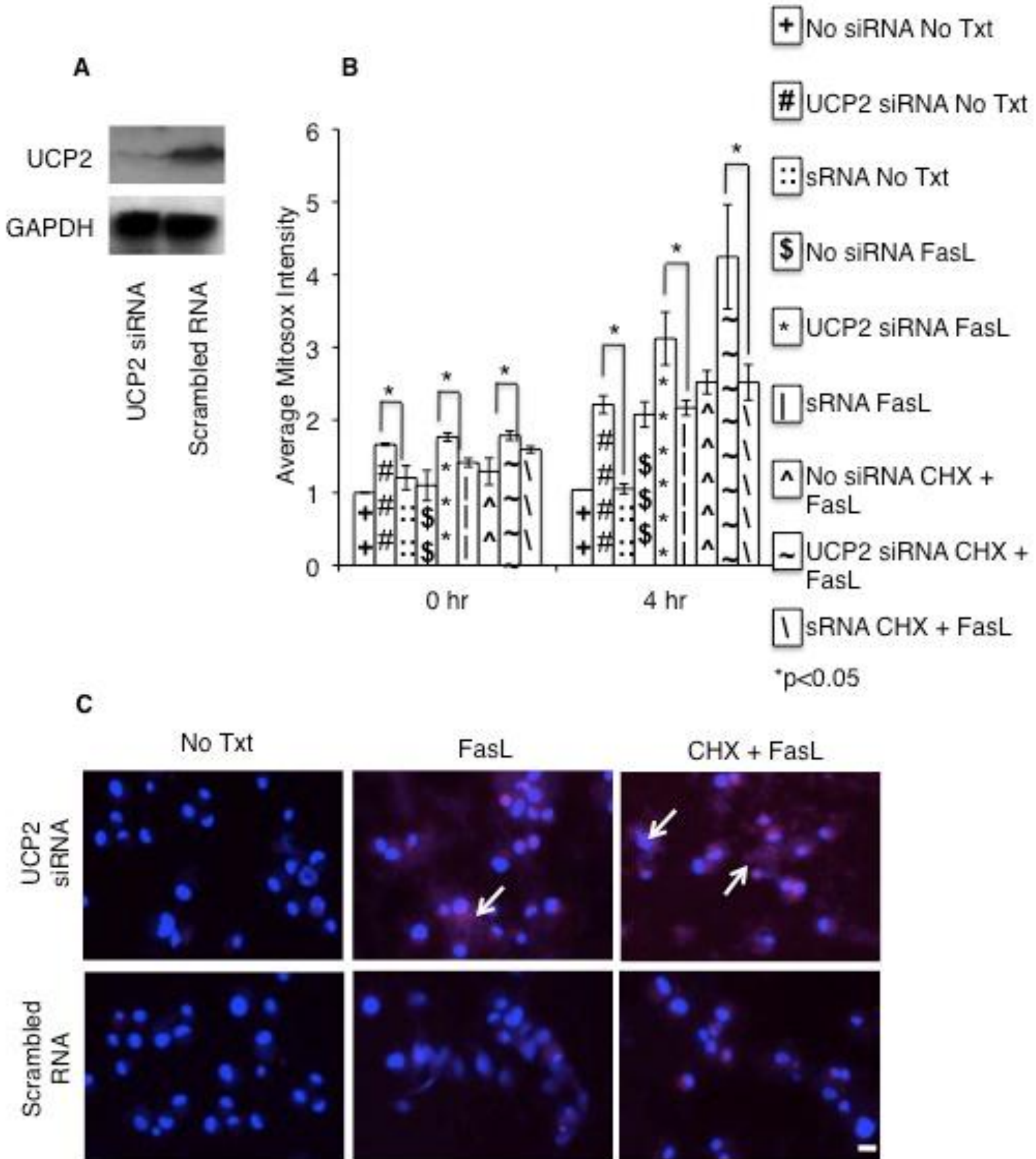


Figure 13 UCP2 moderates ROS increase in MSC upon exposure to FasL. (A) UCP2 levels were downregulated upon transfection of UCP2 siRNA compared with scrambled RNA. (B) Levels of ROS as assessed by average Mitosox intensity in untreated MSC, MSC with downregulated UCP2 or MSC with scrambled RNA (sRNA) with and without FasL or CHX and FasL. (C) Onset of apoptosis in MSC with downregulated UCP2 4 hours after treatment with FasL. The immunoblot (A) graph (B) and micrographs (C) are one of two replicates (*P < 0.05). Magnification 20X, scale bar 10μm. White arrows indicate dying cells.

3.4.7 FasL Induced ROS Activates Mitogen-Related Protein Kinases and Alters Apoptotic Pathway Signaling

Studies suggest that oxidative stress and ROS activate the MAPK pathways including ERK, JNK, and p38 Kinase ((Kefaloyianni, Gaitanaki et al. 2006),(McCubrey, Lahair et al. 2006)). Moreover, studies in MSC suggest that H_2O_2 induces phosphorylation of both JNK and p38 MAPK pathways leading to caspase3 activation and cell death ((Lee, Kang et al. 2004)). We investigated if FasL-induced ROS was responsible for the activation of these MAPK pathways (Figure 14). MSC were treated with H_2O_2 alone as positive control or FasL alone or CHX and FasL over a time-course of 8 hours. In the presence of oxidative stressors (H_2O_2 or FasL), the MAPK pathways were activated, though the time courses varied, with some peaking prior to the full 8 hours of observation. The addition of NAC to CHX and FasL treated cells prevented the increases in phosphorylation of these pathways, suggesting that it is the oxidative stress aspects that trigger these signalings.

We next probed for specific pro- and anti-apoptotic proteins of the mitochondrial death pathway which might display changes in expression on FasL treatment and production of mitochondrial ROS. Survivin belongs to the inhibitor of apoptosis protein (IAP) family, and plays a role in inhibiting mitochondrial apoptosis, inhibiting caspases by interacting with other IAPs and also modulates stress pathways by interacting with heat shock proteins ((Altieri 2010)). We observed a rapid reduction in expression of Survivin on treatment with FasL alone or with CHX and FasL (Figure 14D) displaying an onset of mitochondrial stress related apoptosis in MSC which can be linked to generation of mitochondrial ROS by FasL.

FasL also caused the activation of pro-apoptotic pathways in MSC. Upon activation of stress pathways such as JNK and p38 MAPK, Bad becomes de-phosphorylated and binds the

anti-apoptotic proteins Bcl-2 and Bcl-xl, displacing Bax from these proteins, causing the translocation of Bax to the mitochondrial membrane where it forms pores in the membrane, causes decrease in membrane potential and activates the intrinsic apoptotic pathway ((Zha, Harada et al. 1996)). With treatments of FasL alone or CHX and FasL we saw decreased phosphorylation of Bad (Figure 14E). Together these findings suggest a tip in the balance of signaling towards apoptosis.

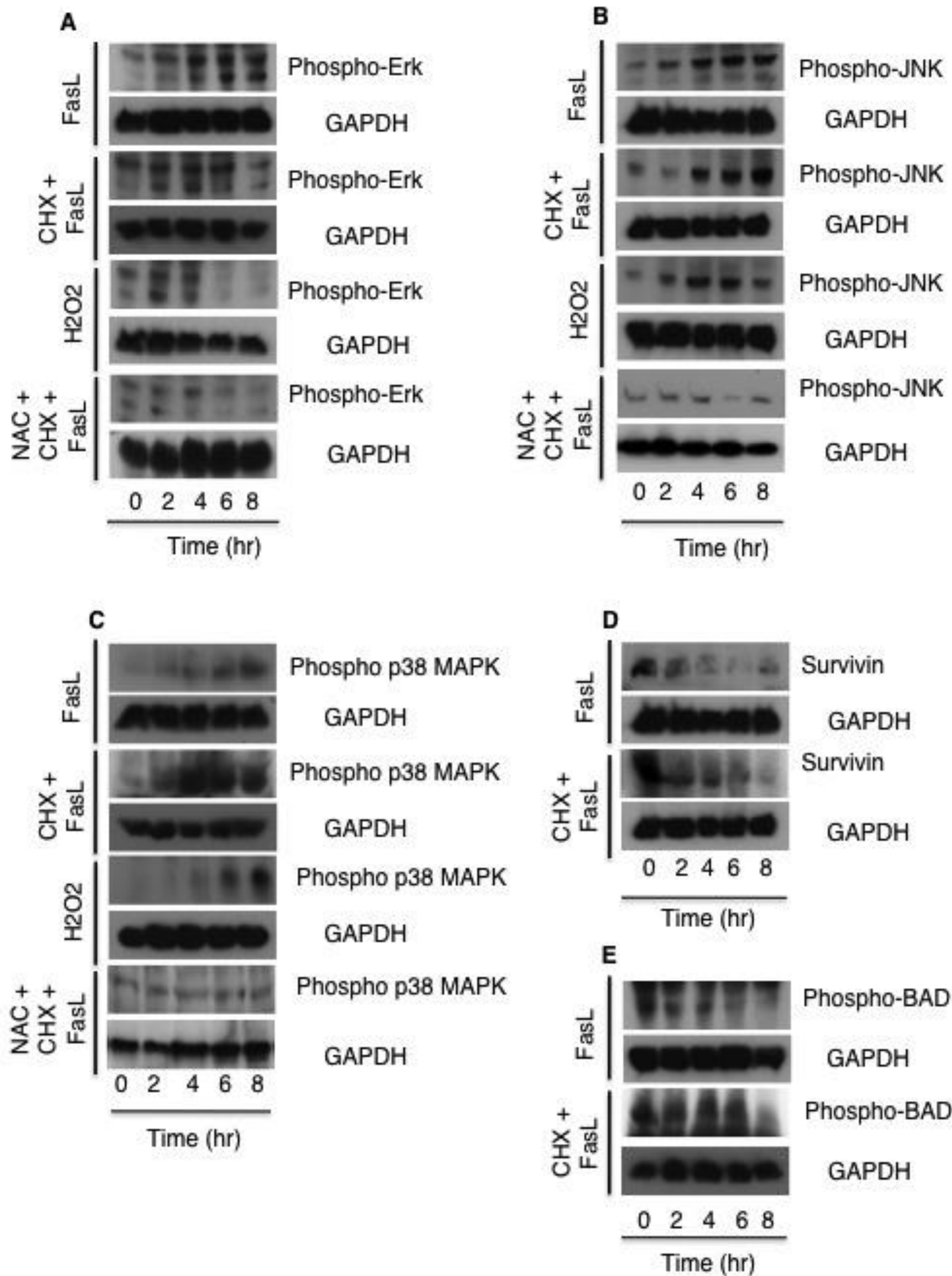


Figure 14 FasL induced ROS activates the MAPK pathways in MSC and proteins of the Mitochondrial Death Pathway. (A) Expression of Phospho-ERK, (B) Phospho-JNK, (C) Phospho-p38 MAPK, (D) Survivin and (E) Phospho-Bad by immunoblot after treatment with indicated agents and times. The immunoblots are representative of at least two experiments.

3.5 DISCUSSION

Considerable attention, including numerous clinical trials, has been devoted to MSC due to the potential of using these cells to regenerate tissue. In addition, MSC have been promoted as being immuno-privileged (Ghannam, Bouffi et al. 2010; Yagi, Soto-Gutierrez et al. 2010)) making them ideal to be used not just by themselves to regenerate tissue, but also with other implanted cells to suppress inflammation. However tests have increasingly demonstrated that grafting of MSC in animal models leads to inflammation and subsequent death of MSC ((Aguirre, Planell et al. 2010)). MSC regenerative therapy has been curtailed by the failure of the cells to permanently engraft to form tissue. Threats to MSC in the wound milieu are multiple: in addition to cytokines and reactive oxygen species in the micro-environment, hypoxia and serum starvation commonly seen during ischemia generate increased ROS due to increased expression of NAD(P)H oxidase and reduced catalase, display lower levels of the pro-survival protein survivin, increased Bcl-2/Bax ratios, causing MSC death ((Peterson, Aly et al. 2011)). It is generally considered that non-specific inflammation induced upon introduction of any new cell or material may contribute to the death. FasL is one such cytokine. FasL is also highly expressed on cytotoxic T-lymphocytes and Natural killer cells, both of which recently have been implicated to promote MSC death after an allogeneic transfer, while NK cells have been shown to promote MSC death on autologous transfer ((Crop, Korevaar et al. 2011)). Thus, we sought to understand the response of MSC to FasL and ROS.

Extracellular ROS when applied to MSC is known to reduce focal adhesions, induce caspase activation and promote death by apoptosis. In addition, advanced glycation products seen in diabetic patients are shown to increase ROS in MSC leading to reduced proliferation and migration of these cells ((Yang, Wang et al. 2010)). These studies suggest that MSC may be

susceptible to oxidative stress and damage. Our results, which suggest that MSC promote their own death by increasing levels of ROS in the presence of inflammation, are in line with this hypothesis. We used FasL as a representative inflammatory cytokine since previous work has demonstrated MSC death in the presence of FasL ((Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011)). We were able to demonstrate that addition of an antioxidant, NAC ((Cotgreave 1997)), reduces FasL-induced ROS production in MSC and is able to suppress MSC apoptosis. However, prolonged exposure to FasL causes MSC to apoptose despite presence of NAC. This later death was due to activation of the extrinsic death pathway by FasL.

The general pro-survival growth factor EGF prevented FasL-induced ROS in MSC and protected from apoptosis for a short time frame, after which the pro-death signals break through. EGF has previously been shown to impair oxidative stress and protect cells from oxidative cell death in other cell types and our results concur with these reports ((Carmona-Cuenca, Herrera et al. 2006)). EGF has also been shown to specifically protect from FasL induced apoptosis in other cell types ((Gibson, Tu et al. 1999)). However, the loss of protection offered by EGF is likely due to receptor internalization and degradation. We have reported that EGF causes a transient activation of downstream pathways due to EGFR internalization and degradation, which prevents it from protecting MSC from FasL induced cell death. However, restraining EGF to the cell surface, thus preventing the EGF-EGFR complex internalization, causes a more prolonged activation of downstream EGFR pathways involved in survival and offers a protective advantage to MSC in the presence of FasL ((Fan, Tamama et al. 2007)).

Uncoupling proteins, present on the inner mitochondrial membrane contribute to the "uncoupling to survive" hypothesis wherein mitochondrial ROS is attenuated by partial uncoupling of the proton gradient while still maintaining ATP production. UCP1 ((Mookerjee,

Divakaruni et al. 2010)), the first identified UCP is mainly found in fat tissue and maintains thermogenesis. UCP2 is ubiquitously expressed, with the mRNA being expressed in all cells, but the protein restricted to only a few tissues. However, our MSC by and large expressed only UCP2, which prompted us to look at changing expression of UCP2 with increases of ROS. UCP2 expression increased in a time-dependent manner with the addition of FasL or CHX + FasL, the pattern of increase following the increasing trend of ROS. Knocking down UCP2 in the presence of FasL or CHX + FasL increased ROS production and quickened the onset of apoptosis to just 4 hours after treatment with FasL proving that UCP2 delayed ROS generation in MSC. UCP2 has a very high turnover time, with a half-life of 1 hour, which might explain rapid increases in protein expression on exposure of MSC to FasL. Stem cells are thought to have low baseline ROS levels compared to other cell types due to relatively higher amounts of anti-oxidants glutathione and superoxide dismutase ((Urish, Vella et al. 2009)). Uncoupling proteins may be another such means by which MSC under normal physiologic conditions maintain their ROS levels.

Muscle-derived stem cells (MDSC) display lower levels of oxidative stress related death compared to their more differentiated phenotype, myoblasts ((Oshima, Payne et al. 2005)). However the reduced baseline ROS does not prevent MDSC from undergoing cell death in the presence of oxidative stress. This has been shown in studies where pretreatment of MDSC with NAC prior to implantation of cells in the heart increases their survival, while on the contrary addition of a pro-oxidant like di-ethyl maleate decreases survival ((Drowley, Okada et al. 2010)). Our results concur with these reports and suggest that addition of NAC to MSC at sites of inflammation will moderate ROS and increase the chance of MSC survival.

In an initial step of linking the Fas receptor to the death pathways, we examined the main MAPK species in MSC, based on reports in other cell types of FasL activating ERK and JNK leading to oxidative damage ((Kefaloyianni, Gaitanaki et al. 2006)). This prompted us to look at FasL-induced ROS activating the MAPK pathways. ROS generated in response to FasL in MSC activated both the MAPK ERK and MAPK stress pathways: p38MAP kinase and JNK. While ERK has traditionally been considered as an anabolic MAPK pathway, supporting mitogenesis, motility and survival, there have been several studies, which highlight ERK signaling in driving cell death ((Cheung and Slack 2004; Ponnusamy, Liu et al. 2011)). ROS activation of ERK is also implicated in leading to DNA damage ((Monks, Xie et al. 2006)). JNK and p38MAPK are canonical stress activated pathways and have been implicated in activating apoptosis. JNK primarily activates mitochondrial apoptosis by modulating the Bcl2 family of proteins and activating Bax and Bad ((Weston and Davis 2007)). Our results show activation of Bad and a link of FasL-induced ROS stimulation of JNK to the mitochondrial death pathway. Activation of p38 MAPK by ROS on the other hand has been shown to kill and limit the population of hematopoietic stem cell in vivo ((Ito, Hirao et al. 2006)).

FasL has previously been described to stimulate ROS in two waves: The first by activation of NADPH oxidase and NO synthase ((Suzuki, Ono et al. 1998; Banki, Hutter et al. 1999; Beltran, Quintero et al. 2002)) and the second via induction of mitochondrial permeability transition ((Sato, Machida et al. 2004)). The first wave does not generally lead to caspase3-mediated apoptosis, the second wave however helps in formation of the apoptosome, mitochondrial permeability transition, release of cytochrome-c and apoptosis. Although we did not check in particular for these ROS waves produced in MSC, our results tilt in the direction of the second wave being responsible for apoptosis in these stromal cells, since we describe the

ROS generated by FasL to be mitochondrially derived. We also see activation of the mitochondrial death pathway in MSC which is the pathway activated by the second ROS wave.

In conclusion, we have demonstrated that MSCs are capable of enhancing their own demise in the presence of inflammation, which is a major hurdle in terms of MSC therapy. In order for translational therapy to be effective, we need to better understand ways in which MSC survival can be promoted. Use of anti-oxidants including NAC offers one such approach to increased survival. Our earlier reports and studies ((Tamama, Fan et al. 2006),(Rodrigues, Griffith et al. 2010)) herein suggest that pro-survival factors such as EGF may hold promise in protecting MSC. However, while EGF appears protective early in the challenge but not over extended time periods, as noted herein, the abbreviated protection may be due to rapid internalization and downregulation of the few EGFR present on these cells ((Tamama, Kawasaki et al. 2010)). Thus, extended signaling ligands, including tethering ((Fan, Tamama et al. 2007),(Tamama, Kawasaki et al. 2010)) or low affinity ((Iyer, Tran et al. 2007; Iyer, Tran et al. 2008)) EGFR ligands, may be useful in protecting implanted MSC from para- and auto-crine signaled apoptosis.

4.0 MULTIPOTENTIAL STROMAL CELLS/ MESENCHYMAL STEM CELLS REMAIN IN A STATE OF 'PRE-AUTOPHAGY' WHILE UNDIFFERENTIATED

4.1 ABSTRACT

Multipotential stromal cells/ mesenchymal stem cells (MSC) have the ability to differentiate and reform tissue. To bring about these functions however MSC have to condition to and withstand stressors including low oxygen and nutrient deprivation. We question in this study whether MSC undergo autophagy, a mode of survival upon stress and starvation under low oxygen and nutrient deprivation. Surprisingly we find that MSC grown in proliferation media comprising 5.5mM glucose, mimicking normal glucose concentrations in vivo express LC3 II a marker for autophagosome formation. Upon switching MSC to hyperglycemic media with 25mM glucose, over four days, the expression of these markers reduces and is completely abolished. However reducing oxygen concentrations from 20% to 4% or 1%, or serum concentrations from 10% to 0.5% does not induce autophagy. To investigate autophagy in MSC under conditions of 5.5mM glucose, we looked at these cells under transmission electron microscopy and saw MSC entrapped with large number of autophagosomes, but none of the autophagosomes were fused with lysosomes or showed residual bodies. This made us hypothesize that autophagosomes formed in MSC are not broken down or recycled under normal conditions of metabolism. To test this we used an mRFP-GFP-LC3 plasmid to track the turnover

of autophagosomes. The plasmid expresses LC3 with both mRFP and GFP and works on the principle that GFP gets quenched under harsh lysosomal conditions, while RFP does not. Thus LC3 will be labeled with a mix of GFP and RFP when localized to early and mid-stage autophagosomes, but will be labeled with RFP alone when fused to lysosomes. Consistent with our hypothesis, we find that MSC cultured under low glucose have autophagosomes that do not fuse to lysosomes unless stimulated to do so with rapamycin. Further we see that MSC lose LC3 II expression as they differentiate into osteoblasts under conditions of low glucose and this happens faster at 4% than 21% oxygen due to osteoblast differentiation occurring faster at 4% oxygen. Taken together our findings suggest that MSC while undifferentiated entrap organelles in autophagosomes and remain in a state of "pre-autophagy", which is lost during differentiation.

4.2 INTRODUCTION

Macroautophagy is a conserved form of autophagy, a process of 'self-eating' or cannibalism wherein starving cells fuel themselves by forming double membranous vacuoles called 'autophagosomes' that sequester and degrade cytoplasmic material by fusion with lysosomes. Autophagosome formation is prevalent in cells under conditions of stress such as nutrient deprivation and hypoxia (Papandreou, Lim et al. 2008; Rabinowitz and White 2010). Macroautophagy (called autophagy hereafter) also plays an important role in cellular differentiation such as mitochondrial clearance during erythrocyte differentiation or fat droplet deposition during adipocyte differentiation (Mizushima and Levine 2010).

There are about 15 autophagy related proteins that act in a hierarchical manner to bring about initiation, nucleation, elongation and recycling of the autophagosomes (Codogno, Mehrpour et al. 2012). The mammalian microtubule associated protein-1 light chain 3 (LC3) is part of the elongation-closure machinery of autophagosomes. It is either present in a form unconjugated to lipids called LC3 I, widely distributed in the cytoplasm or in a form conjugated to the lipid phosphotidylethanolamine (PE) and bound to both membranes of the mature autophagosome called LC3 II (Kabeya, Mizushima et al. 2000). LC3 II is a commonly used marker for autophagy since it migrates to a lower Mr position during electrophoresis (Klionsky, Abeliovich et al. 2008).

MSCs are cells with the ability to self-renew and differentiate, bringing about tissue regeneration post injury and have been tested widely for regeneration of non-healing wounds. However the sites wherein MSC are implanted are wound beds devoid of nutrients and oxygen. Studies with MSC implantation in vivo in small animals have consistently shown that most of the implanted MSC for purposes of regenerative therapy, are lost within 2 weeks of delivery at the wound due to non-specific inflammation, oxidative stress, nutrient deprivation and low oxygen (Rodrigues, Griffith et al. 2012). Our group has previously studied role of death cytokines on MSC survival. We find that FasL is the most detrimental to MSC (Fan, Tamama et al. 2007) and causes cell death by upregulating reactive oxygen species in MSC (Rodrigues, Turner et al. 2012), which otherwise are maintained at low levels in these cells. In this study we wanted to investigate if MSC exploited autophagy for purposes of survival under harsh conditions of low nutrients and oxygen, and whether the autophagic process sustained as MSCs differentiated.

We used LC3 II as a marker for determining mature autophagosome formation in MSC under serum deprivation, low oxygen and glucose concentration *in vitro.* We expected to see autophagy in MSC only upon withdrawal of glucose from media, however MSC displayed a very high expression of LC3 II at normal physiologic levels of 5.5mM glucose. On investigating these autophagosomes in MSC, we found that undifferentiated MSC exist in a “pre-autophagic” state, where the autophagosomes are only formed and entrapped but not being degraded. Such a pre-autophagic state immediately sets cells up for autophagy if needed and maybe a hallmark of a stem cell.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

DMEM 1 g/L glucose [10-014-CV] and 4.5 g/L glucose [10-013-CM] was obtained from CellGro (Mediatech, Manassas, VA). FBS [100-106] for cell culture was from Gemini Bio-Products (Sacramento, CA). RNase Minikit [74104] and Quantitect Reverse Transcription Kit [205311] were obtained from Qiagen (Valencia, CA), and Brilliant SYBR Green qPCR Master Mix [600548] was from Agilent Technologies (Santa Clara, CA). Bafilomycin A1 from *S. griseus* [B1793] was obtained from Sigma, and Tamoxifen citrate [54965-24-1] from ICN Biomed.

For immunoblotting, rabbit polyclonal LC3 antibody [NB100-2331] from Novus Biologicals (Littleton, CO) and goat anti-rabbit IgG secondary antibody [A9169] was from Sigma-Aldrich (St. Louis, MO). Housekeeping genes anti-actin produced in rabbit [A2668] was

from Sigma-Aldrich and anti-a-actinin-1 mouse monoclonal IgG [sc-135819] was from Santa Cruz Biotech (Santa Cruz, CA). Protein ladder for all immunoblots was a Full Range Rainbow marker.

4.3.2 Cell Culture

Immortalized human bone marrow mesenchymal stem cells (ihMSCs) a kind gift from Dr. Junya Toguchida's lab (Kyoto University, Japan) was cultured in proliferation media comprising DMEM with 1 g/l (5.5mM) or 4g/l (25mM) glucose with 10% FBS, containing 1 mM sodium pyruvate, 1 mM L-glutamine, 1 uM non-essential amino acids, and 100 units/mL penicillin-streptomycin to account for changes in glucose concentration. To account for changes in oxygen concentration, cells were either grown in incubators at ambient air condtions (20% oxygen) or incubators at 4% and 1% oxygen. Experiments for cells at 4% and 1% oxygen were done in the Biospehrix chamber. To observe changes under varying serum concentrations, proliferation media was prepared with either 10% FBS or 0.5% dialyzed FBS. For osteogenic differentiation media, proliferation media was supplanted with 100 nM dexamethasone, 100 uM β -glycerophosphate, and 50 uM L-ascorbic acid.

4.3.3 Immunoblotting

Cells grown in 5.5mM glucose were switched to 25mM glucose for 96 hours and vice versa. Similarly cells grown in 21% oxygen were switched to 4% oxygen or 1% oxygen for 96 hours. Cells grown in high serum were switched to low serum for 96 hours. After treatment for

the designated time frame, cells lysates were collected using SDS lysis buffer containing 5% β-mercaptoethanol. Lysates were separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane for 1.5 hours at 350 mA. Membranes were blocked for 1 hour in 5% milk in TBS-T, followed by incubation overnight at 4°C at dilutions of 1:1000 for anti-LC3 and 1:2000 for anti-actin or anti-a-actinin, in 1% milk in TBS-T. Following incubation, blots were washed in TBS-T and incubated in HRP conjugated secondary antibody for 1 hour, treated with HRP substrate for 1 minute, and subjected to autoradiography analysis.

4.3.4 Transfection

Lipofectamine 2000 was used to trasfect MSC with tfLC3 plasmid, a kind gift from Dr. Tamotsu Yoshimori's lab (Kimura, Noda et al. 2007), Osaka University, Japan. 24 hours post transfection, cells were left untreated or treated with 5μM rapamycin (LC Laboratories, Woburn, MA) for 15 minutes, 1 hr or 3 hr and imaged for RFP and GFP fluorescence. The numbers of red and green puncta were counted for each cell. While yellow (merge of GFP and RFP) puncta indicate early autophagosomes, red puncta indicate late autophagosomes, when autophagosomes merge with lysosomes and all the GFP is degraded (Gusdon, Zhu et al. 2012).

4.3.5 Probing for Osteogenesis

Immortalized hMSC were incubated in osteogenic DMEM media for 30 days at 20% or 4% oxygen. After 0, 10, 20, and 30 days, hydroxyapatite deposition was probed for using von Kossa stain. Samples were washed with PBS without calcium or magnesium, fixed in 4% paraformaldehyde, treated with 1% silver nitrate solution and incubated under UV light for 10

minutes. Wells were washed with sodium thiosulfate overnight and imaged under transmitted light.

4.3.6 Quantitative PCR

RNA was isolated from MSC grown in osteogenic media on Days 0, 10, 20 and 30 using RNeasy kit (Qiagen, Valencia, CA). After treatment with genomic DNA wipeout buffer, 1 ug template RNA was reverse transcribed into cDNA using Quantitect cDNA kit (Qiagen, Valencia, CA). Quantitative PCR was performed using 1 ul cDNA for all samples with GAPDH as control, 12.5 uL of Brilliant SYBYR green master mix (Agilent Technologies, Santa Clara, CA) and 3.75 uL forward and reverse primers. Primers for Runx2 and Osteocalcin, markers for early and late osteogenesis, were used to probe for osteogenesis. Forward and reverse primer sequences were:

GAPDH Forward Primer: 5'-GAGTCAACGGATTTGGTCGT-3'

GAPDH Reverse Primer: 5'-TTCATTTTGGAGGGATCTCG-3'

Runx2 Forward Primer 5'-CCTCGGAGAGGTACCAGATG-3'

Runx2 Reverse Primer 5'- TTCCCGAGGTCCATCTACTG-3'

Osteocalcin Forward Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3'

Osteocalcin Reverse Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3'

4.4 RESULTS

4.4.1 MSC display LC3 II under physiologic levels of glucose; low oxygen and low serum do not affect LC3 II expression in MSC

MSC are implanted in wound regions, where nutrients are in low supply due to ruptured blood vessels, pro-death cytokines are high due to non-specific inflammation and oxygen is low. We wanted to test how MSC responded to some of these stressors, the first being glucose and serum concentrations. We expected reduction of glucose levels in MSC to cause autophagy. Surprisingly, MSC grown in 5.5mM glucose, which represents normal blood glucose levels displayed autophagy. When we grew MSC in hyper glycemic media of 25mM glucose for 3 days prior to immunoblotting, MSC showed no trace of LC3 II expression (Figure 15A). This lead us to trace the time-required for loss in LC3 II expression. MSC grown in 5.5mM glucose were swapped with 25mM glucose media for 4, 8, 24, 48 and 96 hours. 24 hours after change to hyperglycemic media, MSC showed visible reduction in LC3 II bands. By 96 hours, there was also a reduction in LC3 I band, displaying a decrease in basal levels of LC3 in MSC (Figure 15B). This significant reduction in LC3 II in MSC is represented by integrated density of LC3 II bands normalized to integrated density of actin bands (Figure 15C). Conversely MSC grown in 25mM glucose and switched to 5.5mM glucose media showed increases in LC3 II starting as early as 4 hours post changing of media (Figure 15D). We next wanted to see if reduction in serum caused LC3 II levels to alter. MSC grown in 25mM glucose media in 10% FBS were switched to 25mM glucose media with 0.5% dialyzed FBS. There was no induction of LC3 II expression over 96 hours (Figure 15E). Finally we looked at change of oxygen concentration on LC3 II expression. Since the MSC are bone marrow derived, and the physiologic levels of

oxygen in the bone marrow are between 4 and 7%, we used 4% oxygen to represent physiologic oxygen levels, 1% oxygen to represent physiologic levels of oxygen stress and 21% to represent ambient air conditions in tissue culture. We saw a decrease in LC3 II levels at 1% oxygen after a period of 96 hours, but no change in LC3 II levels at 4% oxygen (Figure 15F). These results helped us conclude that MSC under physiologic levels of glucose express the autophagosome marker LC3 II, which reduces under physiologic stress of 1% oxygen.

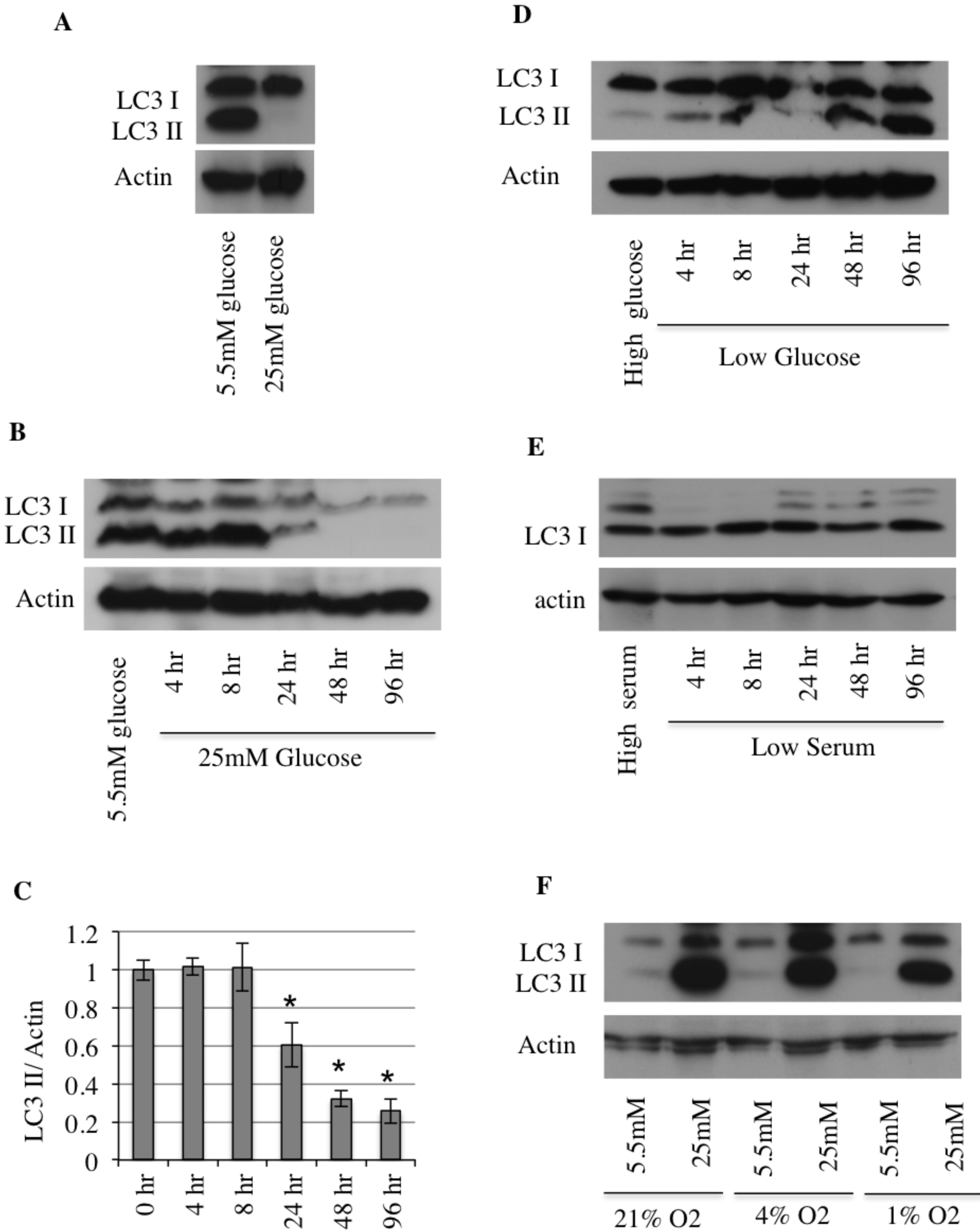


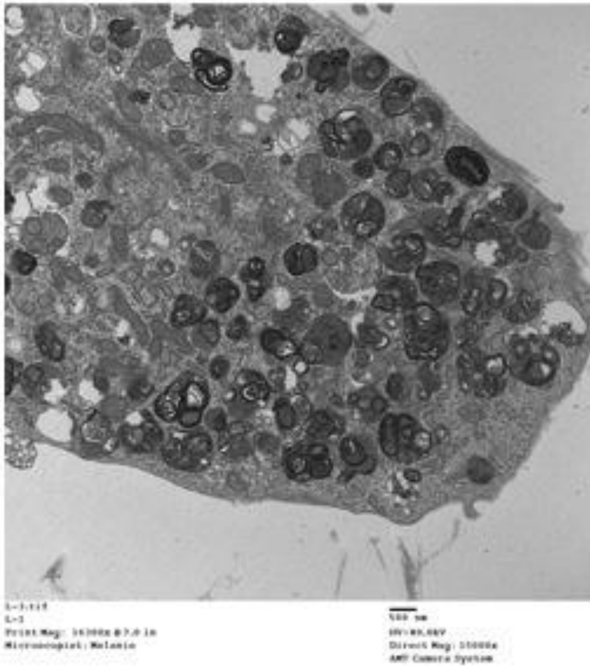
Figure 15 MSC express LC3 II when grown in physiologic glucose concentrations in culture. (A) Immunoblot for the autophagosome marker LC3 in imhMSC grown in 5.5mM and 25mM glucose. (B) Immunoblot for LC3 in imhMSC grown in 5.5mM glucose and switched to 25mM glucose. (C) Integrated density of LC3 II

bands normalized to actin bands obtained by immunblot on growing MSC in 5.5mM glucose and switching to 25mM glucose. (D) Immunoblot for LC3 in imhMSC grown in 25mM glucose and switched to 5.5mM glucose. (E) Immunoblot for LC3 in imhMSC grown in 10% serum and switched to 0.5% dialyzed serum. (F) Immunoblot for LC3 in imhMSC grown in 21%, 4% and 1% oxygen.

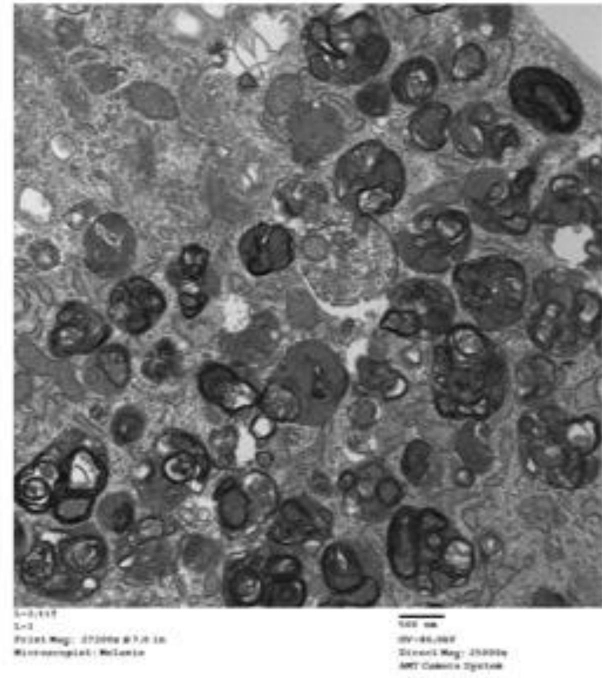
4.4.2 MSC grown in physiologic levels of glucose are filled with autophagosomes which are all early stage

Since we saw MSC express the autophagosome marker LC3 II at 5.5mM glucose, we wanted to look at autophagosome formation by TEM. We found imhMSC cytoplasm to be filled with autophagosomes at a magnification of 15,000X (Figure 16A). The autophagosomes were all early stage. No late autophagosomes, showing degraded organelles and empty spaces within the autophagosome space were observed at a magnification of 25,000X (Figure 16B) or 150,000X (Figure 16C). Switching of MSC to media in high glucose took away all autophagosomes diaplying tiny mitochondria (Figure 16D). Since we were seeing early autophagosomes in the presence of 5.5mM glucose, conditions at which MSC normally reside in the bone marrow, we hypothesized that MSC might metabolically exist in a state of “pre-autophagy” where autophagosomes are formed, but do not fuse with lysosomes to facilitate degradation.

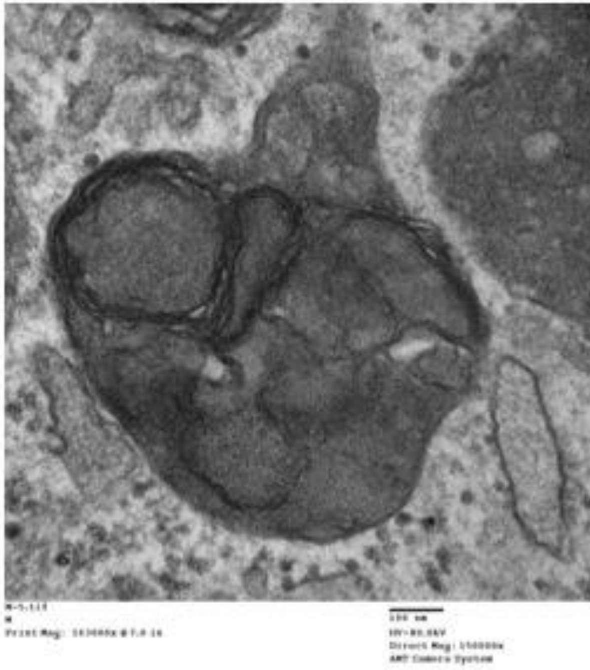
A



B



C



D

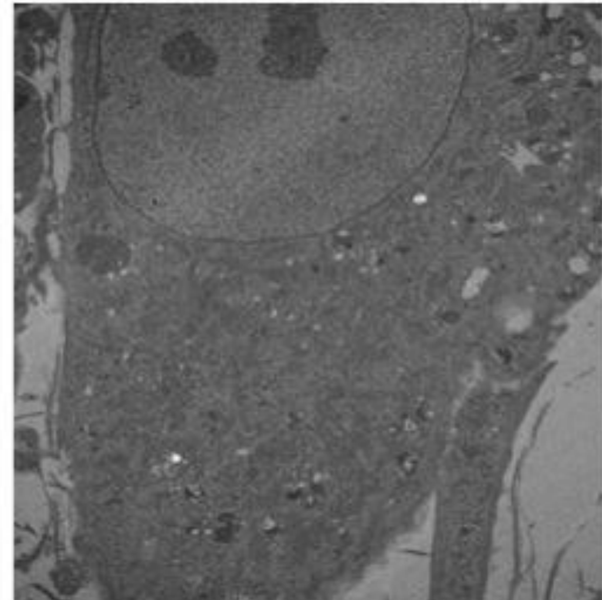


Figure 16 MSCs display large numbers of early autophagosomes but no late autophagosomes in the presence of 5mM glucose. TEM images of imhMSC at 15000X (A), 25000X (B) and 150000X (C).

4.4.3 MSCs exist in a state of pre-autophagy

To test our hypothesis that MSC are being entrapped in autophagosomes, but are not being degraded, we transfected imhMSC with tfLC3, and followed the turnover of LC3 in MSC. MSC grown in 5.5mM glucose displayed both GFP and RFP puncta, but no RFP puncta alone, indicating that there were no late-autophagosomes formed by fusion of autophagosomes with lysososomes. When these MSC were treated with 5μM rapamycin, a drug that inhibits mTOR and activated autophagy, there were RFP puncta without GFP fluorescence starting at 15 minutes, and within 1 hour of treatment, cells were predominantly stained with RFP only. Conversely when MSC were treated with 5nM bafilomycin, a drug that prevents fusion of autophagosomes with lysosomes by preventing lysosomal acidification, after 1 hour the cells were filled with RFP and GFP co-stained puncta, similar to that of untreated cells indicating no degradation of the autophagosomes (Figure 17).

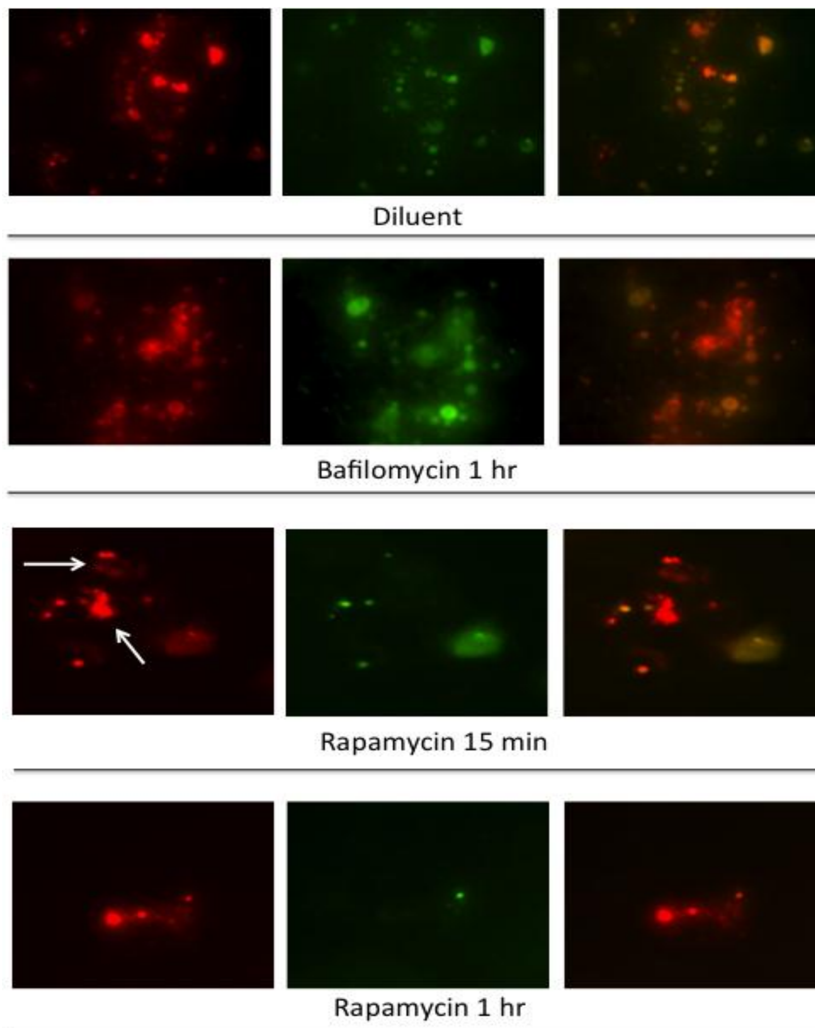


Figure 17 imhMSC exist in a state of pre-autophagy. imhMSC transfected with tfLC3 and visualized for RFP (red) or GFP (green) fluorescence in the presence of diluent, bafilomycin an inhibitor of late autophagosome formation or rapamycin a stimulator of autophagy. Arrows denote loss of GFP fluorescence and maintenance of RFP fluorescence.

4.4.4 MSC are dependent on glycolysis for ATP synthesis

Since we see a great deal of dependence of MSC on glucose levels and it has been previously reported that MSC exhibit increased glycolytic metabolism, we questioned if our cells exhibiting pre-autophagy metabolically produced energy via glycolysis or oxidative phosphorylation. To test this, we blocked glycolysis using 2Deoxyglucose or different stages of oxidative phosphorylation: oligomycin is an ATP synthase inhibitor, FCCP is an uncoupler of oxidative phosphorylation, and rotenone blocks transfer of electrons from complex I to ubiquinone. We found that blocking glycolysis but not oxidative phosphorylation inhibits ATP production in MSC, indicating that most of the ATP produced in these cells is via glycolysis (Figure 18)

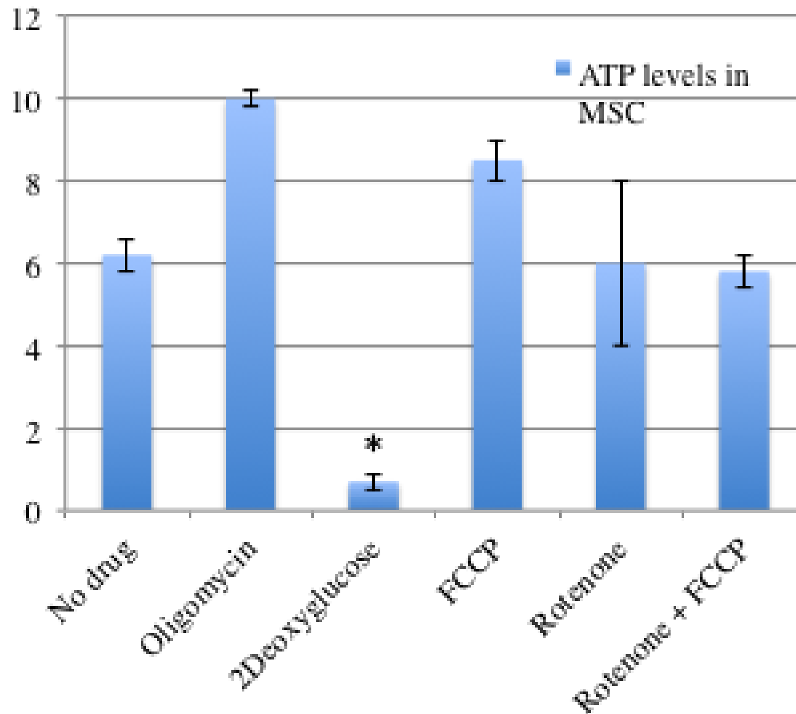


Figure 18 MSC produce ATP via glycolysis. Levels of ATP in imhMSC post treatment with drugs inhibiting glycolysis or various stages of oxidative phosphorylation. * $p<0.01$ compared to cells with no drug.

4.4.5 MSC lose LC3 II expression on differentiation into osteoblasts

We were curious to find if MSC remain in the state of pre-autophagy only when undifferentiated or also when they differentiate. If MSC do retain early autophagosomes on differentiation, the case of pre-autophagy could just be a characteristic of the cell line we use in culture and need not necessarily be a characteristic of a stem cell. MSC were grown to confluence in proliferation media (Day 0) and then switched to osteogenic differentiation media. At Day 0, MSC grown in 21% and 4% oxygen displayed LC3 II, as seen earlier in Figure 1.

However at Day 10 there was no LC3 II band in MSC differentiating at 4% oxygen (Figure 4A) and at Day 20 there was no LC3 II band in cells differentiating in both 21% and 4% oxygen. When we checked the cells for osteogenic differentiation, we found that MSC at 4% oxygen had already started differentiating into osteoblasts at Day 10 based on the increase in the early osteogenesis marker Runx2 (Figure 4B) and late osteogenesis marker Osteocalcin (Figure 4C) by qPCR, as well as von Kossa staining for hydroxyapatite (Figure 4D). MSC grown at 20% oxygen show osteogenesis at Day 20 and loss of LC3 II with the onset of osteogenesis. These results indicate that pre-autophagy is a condition exhibited only by undifferentiated MSC.

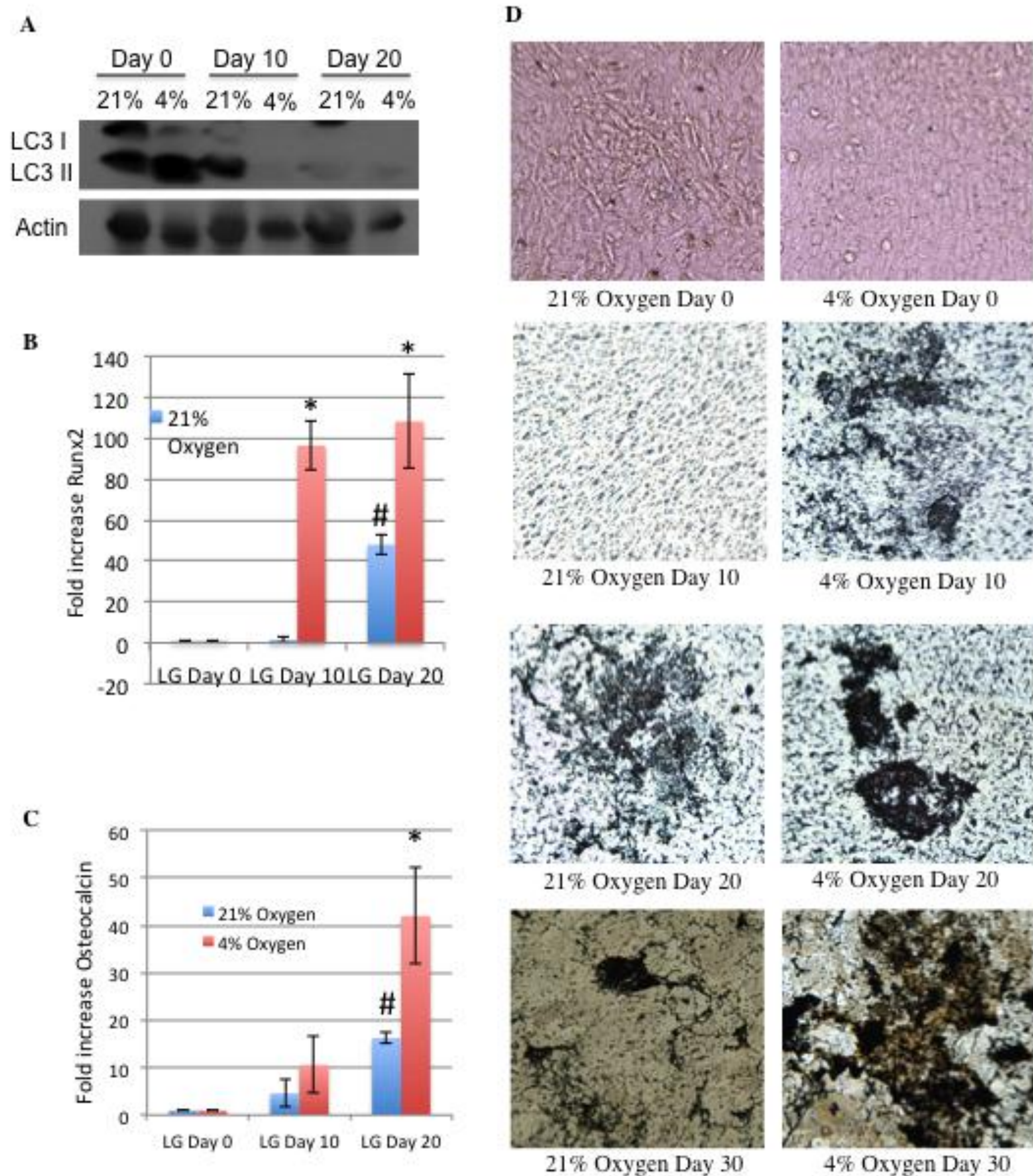


Figure 19 MSC lose LC3 II expression on osteogenic differentiation. (A) imMSC placed in osteogenic differentiation media probed for LC3 II expression under 21% and 4% oxygen. (B) qPCR for Runx2, an early osteogenic marker in imhMSC grown at 21% and 4% oxygen (*, # indicates $p<0.01$ compared to Day 0). (C) qPCR for Oseocalcin, a late osteogenic marker in imhMSC grown at 21% and 4% oxygen (*, # indicates $p<0.01$ compared to Day 0). (D) von Kossa staining of imhMSC cultures grown at 21% and 4% oxygen.

4.5 DISCUSSION

We show in these studies for the first time that MSC hold large numbers of early autophagosomes under conditions of physiologic glucose and the expression of the autophagosomes does not change when MSC are cultured either at ambient conditions of 21% or physiologic normoxia of 4% indicating that the autophagosome formation is independent of oxygen concentrations in culture. MSC however are highly sensitive to glucose concentrations and significantly reduce autophagosome formation when hyperglycemic glucose concentrations are used. Further, when we look at turnover of autophagosomes using a tfLC3 in MSC we find that the cells are trapped in early autophagy and do not proceed into late autophagy by fusing to lysosomes and degrading. The tfLC3 expresses both GFP and RFP when autophagosomes are early and not fused to lysosomses, and expresses only RFP when autophagosomes are fused to lysosomes since GFP cannot withstand the low pH of lysosomes, while RFP remains stable under these conditions. Since glucose has such a dramatic effect on autophagosome formation in MSC, we block different stages of glycolysis and oxidative phosphorylation and find that MSC are highly glycolytic and generate most of their ATP via glycolysis. We also find that differentiation of MSC into osteoblasts causes the loss of autophagosome formation in MSC, leading us to conclude that MSC exist in a state of pre-autophagy.

Pre-autophagy in MSC might be important for 3 main functions. Firstly, for scavenging organelles especially mitochondria to keep MSC glycolytic. Secondly for aiding in MSC differentiation and thirdly for helping MSC stay in a state of preparedness, to rapidly break down autophagosomes on encountering nutrient deprivation in a region of injury. There have been indications in literature for the occurrence of the first two events in other stem cell types, but no study actually identifying and studying these phenomena of autophagy.

The mammalian target of rapamycin (mTOR) is a core regulator of autophagy. Under low nutrient situations, mTOR repression causes recycling of cellular components and shifts cellular metabolism towards autophagy (Cecconi and Levine 2008). Inhibiting mTOR signaling using rapamycin in mouse embryonic fibroblasts promotes somatic reprogramming and formation of induced pluripotent stem cells (iPSC) (Chen, Shen et al. 2011). One of the reasons for the switch to pluripotent cell formation is the conversion of energy generation from mitochondrial oxidative phosphorylation to glycolysis, which rapamycin establishes (Menendez, Vellon et al. 2011). MSC like pluripotent cells are glycolytic (Samudio, Fiegl et al. 2008; Panopoulos and Izpisua Belmonte 2011). It is shown that MSC are physiologically glycolytic and the switch to oxidative phosphorylation occurs due to shifting the culture of these cells to 21% oxygen on isolation rather than culturing cells at physiologic relevant conditions of 4-7% oxygen (Estrada, Albo et al. 2012). Our results indicate that MSC are filled with large numbers of early autophagosomes, which do not turn over unless stimulated by rapamycin. Further these MSC display a glycolytic phenotype. Taken together, one of the reasons for MSC to exist in a state of pre-autophagy maybe for maintenance of a glycolytic phenotype.

Autophagy is involved in several cell processes, including differentiation. Recent reports show the need for autophagy in embryonic stem cells to undergo cardiomyocyte differentiation (Zhang, Liu et al. 2012). Conversion of brown fat to white fat during adipocyte development and collection of fat droplets are also processes dependent on autophagy (Singh, Xiang et al. 2009; Zhang, Goldman et al. 2009). Our results indicate that MSC lose LC3 II expression on forming osteoblasts. This indicates that pre-autophagy in MSC must have a role to play in differentiation. The question of to degrade or not to degrade might determine the differentiation lineage of MSC.

We find that reduction of oxygen concentrations from 21% to a physiologically relevant 4% does not bring about changes in autophagosome formation. This indicates that it is not the culture conditions that cause pre-autophagy of MSC. Glucose concentrations however greatly impact expression of the autophagosome marker LC3 II and MSC are found to be glycolytic. Glycolysis has previously been found to have pro-survival functions in MSC apart from energy metabolism, allowing for MSC to live under ischemia much longer than other cell types (Mylotte, Duffy et al. 2008). The glycolytic metabolism and pre-autophagic state in MSC therefore seem to be interdependent, having effects on cell survival.

This leads us to the final and most important role that pre-autophagy must have a play in the canonical role of survival under nutrient deprivation and stress. We find that rapamycin added to MSC initiates degradation of the autophagosomes as early as 15 minutes after treatment of cells. We think that MSC may be conditioned to react to stressful conditions such as those encountered in the wound by pre-autophagy, where rapid degradation can fuel turnover and differentiation in a hostile nutrient environment.

In conclusion pre-autophagy seems to be a characteristic of undifferentiated MSC having effects on metabolism, differentiation and survival of MSC.

5.0 GROWTH FACTOR REGULATION OF PROLIFERATION AND SURVIVAL IN MULTIPOTENTIAL STROMAL CELLS

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5.1 ABSTRACT

Multipotential stromal cells (MSCs) have been touted to provide an alternative to conservative procedures of therapy, be it heart transplants, bone reconstruction, kidney grafts, or skin, neuronal and cartilage repair. A wide gap exists, however, between the number of MSCs that can be obtained from the donor site and the number of MSCs needed for implantation to regenerate tissue. Standard methods of MSC expansion being followed in laboratories are not fully suitable due to time and age-related constraints for autologous therapies, and transplant issues leave questions for allogenic therapies. Beyond these issues of sufficient numbers, there also exists a problem of MSC survival at the graft. Experiments in small animals have shown that MSCs do not persist well in the graft environment. Either there is no incorporation into the host tissue, or, if there is incorporation, most of the cells are lost within a month. The use of growth and other trophic factors may be helpful in counteracting these twin issues of MSC expansion and death. Growth factors are known to influence cell proliferation, motility, survival and morphogenesis. In the case of MSCs, it would be beneficial that the growth factor does not induce differentiation at an early stage since the number of early-differentiating progenitors would be very low. The present review looks at the effect of and downstream signaling of various growth factors on proliferation and survival in MSCs.

5.2 INTRODUCTION

There is a growing need for new ways to regenerate and repair injuries in organs. Most organs have limited inherent regenerative capacity, with scarring preventing full organ

functioning. For instance, myocardial infarction is often followed by the myocardium being replaced with noncontractile scar tissue, which can further result in congestive heart failure(Fonarow 2000; Lloyd-Jones 2001). In the case of bone, metabolic disorders such as osteoporosis cause abnormal bone loss and traumatic injuries lead to large lesions, which are incapable of self-regeneration. The search has therefore turned to novel ways to stimulate the original organogenic process and regenerate normal tissue.

Use of multipotential stromal cells (MSCs) or mesenchymal stem cells to reconstruct tissue looks extremely promising due to their trans-differentiation potential. MSCs have the ability to form cells of the connective tissue, muscle, heart, blood vessels and nerves (Kopen, Prockop et al. 1999; Makino, Fukuda et al. 1999; Pittenger, Mackay et al. 1999; Caplan 2007). These cells are easy to isolate from almost all individuals; these cells are relatively safe as they rarely form teratomas (Kumar, Chanda et al. 2008). In addition, these stromal cells offer several advantages over conventional therapy. MSCs respond to their environment by differentiating into the needed lineages. These cells will therefore grow, remodel and adapt to changes in tissue functions over time. As MSCs derive from bone marrow, these can be isolated from most adults with the potential of autologous transplantation, not requiring immunosuppressive agents. This procedure is in contrast to traditional methods of transplantation that lead to infection, immune rejection or simply not enough material for large-scale grafts.

Preclinical animal studies have shown promise of using MSCs for tissue regeneration. Application of these cells has led to the formation of bone, the regain of ventricular function, and the restoration of renal tubular function in rodents (Orlic, Kajstura et al. 2001; Hofstetter, Schwarz et al. 2002; Morigi, Imberti et al. 2004; Akahane, Shigematsu et al. 2010). Mice rendered paraplegic by spinal cord injury have recovered on MSC treatment (Osaka, Honmou et

al. 2010). The use of MSCs is limited, however, by their scarceness in the bone marrow, as they constitute only 0.001 to 0.01% of the bone marrow population. Since regeneration of large tissues requires around 10^7 to 10^8 MSCs (Tsutsumi, Shimazu et al. 2001), there exists a need for MSCs to be expanded prior to tissue regeneration. In culture conditions, however, proliferation of these cells is highly inconsistent – which subsequently impacts differentiation.

Even if the desired cell numbers are obtained, there is another hurdle to be crossed before differentiation of MSCs begins: the incorporation of MSCs into regenerating tissue. MSCs applied to regenerate porcine hearts following an infarct display only 5% survival in a 14-day period (Pittenger and Martin 2004; Freyman, Polin et al. 2006; Tamama, Kawasaki et al. 2010). Similar results are seen on implantation of MSCs into mouse hearts with infarcts. While MSCs injected into nonischemic hearts survive better initially, one cannot find viable cells after 4 weeks (Zhang, Methot et al. 2001; Tang, Tang et al. 2005; van der Bogt, Schrepfer et al. 2009). In rat brains with cerebral artery occlusion, allogenic and human MSC transplants also show very low survival (Yang, Wang et al. 2010). The failure of these cells to regenerate tissue may thus simply be that they do not survive to contribute to the new tissue.

The reasoning behind low incorporation of MSCs may be attributed to poor viability of cells caused by ischemia, anoikis, loss of trophic factors or inflammation at the graft site (Song, Song et al. 2010). To test whether nonspecific inflammation induced death of MSCs, Griffith and colleagues subjected human MSCs to various nonspecific inflammatory cytokines *in vitro*. MSCs were extremely susceptible to FasL-induced cell death and also died in the presence of TRAIL (Fan, Tamama et al. 2007). Not only does the inflammatory response challenge transplanted MSCs – these cells are also considered for regeneration of tissues with harsh microenvironments. For example, when used to regenerate cartilage, MSCs need to adapt to an avascular, low oxygen

concentration and a low pH microenvironment characteristic of chondrocytes (Bibby, Fairbank et al. 2002). Taken together, the microenvironment in which MSCs are delivered, the presence of inflammation, or the loss of trophic factors may play a role in maintaining a proliferating MSC population at the graft site [Figure 20].

On the contrary, there are reports of improved healing on MSC delivery. In a rat cerebral occlusion model there is significant recovery in motor neuron function after MSC transplantation. Despite the low survival rate of MSCs in ischemic hearts, there is decreased scarring and increased neo-angiogenesis after MSC transplantation (Arminan, Gandia et al. 2010; Li, Turdi et al. 2010). MSC injection has also helped to improve pulmonary emphysema (Zhen, Xue et al. 2010). In all these cases, secretion of various growth factors and cytokines by MSCs is thought to bring about paracrine signaling and revival of endogenous tissue cells or suppression of harmful inflammation (Caplan 2009). The lack of demonstrated persistence of the transplanted MSCs has resulted in these affects being attributed not to MSC integration and regeneration of tissue, but to trophic effects brought about by these unique cells (Caplan and Dennis 2006).

The role of growth factors in increasing proliferation and survival in MSCs has been widely studied over the past few years. Most growth factors are pleiotrophic, causing multiple biological effects. They bring about changes in motility, proliferation, morphogenesis and survival. The search for the ideal growth factor for use with MSCs is still ongoing. While some groups aim at finding a growth factor not affecting differentiation, other groups opt for a growth factor that has differentiation preference towards a specific lineage. All groups, however, attempt to find a factor that improves *ex vivo* expansion and heightens survival on implantation. The

present review explores the effects of various growth factors on MSC expansion and survival and the signaling mechanisms behind these effects.

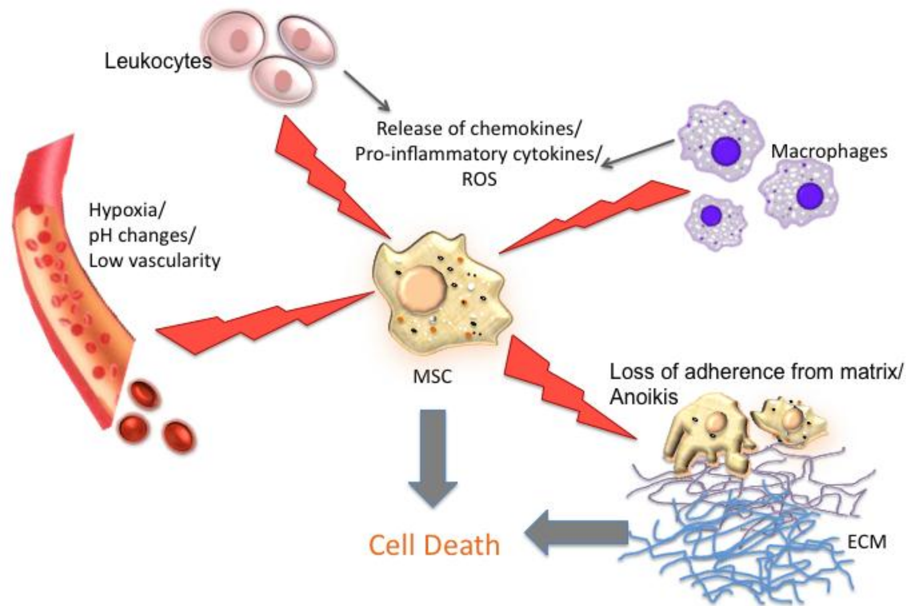


Figure 20 Illustration of the various threats MSC face at the site of delivery leading to loss of cells. Leukocytes and macrophages secrete pro-inflammatory cytokines, chemokines and reactive oxygen species (ROS), which might activate apoptotic cascades. Low vascularity and hypoxia at the site might also cause MSC loss. Also, lack of attachment to extracellular matrix (ECM) may cause cells to detach, undergo anoikis and thus cell loss.

5.3 GROWTH FACTOR SIGNALING BEHIND MSC PROLIFERATION AND POPULATION EXPANSION

5.3.1 Transforming Growth Family β Family of Growth Factors

The choice of growth factors to be used on MSCs was initially determined based on previously existing knowledge about the effect of a particular growth factor on cell morphogenesis. This was done with the dual pursuit of expanding MSCs and causing them to differentiate into the lineage that it was known to favor. Transforming growth factor beta (TGFβ), for example, is known to influence cells from the chondrogenic lineage *in vivo*, promoting initial stages of mesenchymal condensation, prechondrocyte proliferation, production of extracellular matrix and cartilage-specific molecule deposition, while inhibiting terminal differentiation (Canalis, McCarthy et al. 1989). When applied to MSCs *in vitro* to study chondrocyte regeneration, cells show increased proliferation and a bias towards the chondrogenic lineage (Bonewald and Dallas 1994; Longobardi, O'Rear et al. 2006; van der Kraan, Blaney Davidson et al. 2009). TGFβ exists as three isoforms: $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$. While all three isoforms induce proliferation of MSCs and chondrocyte formation, $TGF\beta_3$ has been found to have the most pronounced effect on chondrogenesis and consistently increases proliferation of MSCs (Ogawa, Akazawa et al. 2010; Weiss, Hennig et al. 2010), making it a prime factor for induction of chondrogenesis from implanted MSCs.

Similarly, bone morphogenetic protein (BMP)-2 through BMP-7 – factors belonging to the TGFβ superfamily – are known to affect bone formation. While BMP-2, BMP-4, BMP-6, and BMP-7 induce MSCs to form osteoblasts, BMP-2 has the greatest impact on differentiation

(Luu, Song et al. 2007). MSCs overexpressing BMP-2 and implanted with the extracellular matrix protein collagen I as a hydrogel system increase proliferation of MSC differentiation into bone, and this model has been used to study cranial closures in swine (Lou, Xu et al. 1999; Chang, Chung et al. 2010). Another member of the same family, BMP-3, increases MSC proliferation threefold (Stewart, Guan et al. 2010). Since these factors all affect bone formation at different rates and some have a greater effect on proliferation, synergistic pairs of these growth factors can be used at optimal doses and at specific points during the bone regeneration process. One such search for synergistic pairs led to combination treatment of TGFβ_3 with BMP-2 on MSCs; chondrogenic differentiation was found to be enhanced (Simmons, Alsberg et al. 2004).

TGFβ signaling occurs when TGFβ or factors from the family bind a type II serine–threonine kinase receptor recruiting another such transmembrane protein (receptor I). Receptor I phosphorylates the primary intracellular downstream molecules SMADs, causing their translocation into the nucleus and specific gene transcription. Receptor I can be ALK-1, ALK-2, ALK-3, or ALK-6 that signal SMAD 1, SMAD 5, and SMAD 8, or can be ALK-4, ALK-5, or ALK-7 that signal SMAD 2 and SMAD 3. Signaling via SMAD 1, SMAD 5, or SMAD 8 is required for chondrocyte differentiation while signaling through SMAD 2 or SMAD 3 blocks chondrocyte differentiation (Valcourt, Gouttenoire et al. 2002). TGFβ and members of this growth factor family can also signal via the mitogen-activated protein kinase (MAPK), Rho GTPase and phosphoinositide-3 kinase (PI3K) pathways (Zhang 2009). The effect of BMP-2 on proliferation and osteogenic differentiation of MSCs has been shown to occur via sustained signaling of the MAPK Erk (Lou, Tu et al. 2000). The mitogenic effects of BMP-3, on the other hand, have been found to be mediated by TGFβ/activin signaling and not by any of the MAPK signaling pathways, with ALK-4 and SMAD 2 and SMAD 3 being the key players involved

(Stewart, Guan et al. 2010). Figure 2 shows how signaling via SMAD 2 or SMAD 3 leads to proliferation of MSCs but blocks terminal differentiation into chondrocytes, while signaling via SMAD 1, SMAD 5, or SMAD 8 potentially leads to chondrocyte differentiation in MSCs. Figure 2 also shows how sustained signaling via Erk leads to osteoblast formation.

5.3.2 Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are a family of growth factors involved in wound healing and angiogenesis. Among the various members of this family, FGF-2 or basic fibroblast growth factor (b-FGF) has been used in MSC-related studies showing increased rabbit, canine and human MSC proliferation *in vitro*, with the mitogenic effect being more pronounced when MSCs are seeded at lower densities (Tsutsumi, Shimazu et al. 2001; Yanada, Ochi et al. 2006; Farre, Roura et al. 2007; Stewart, Byron et al. 2007). b-FGF not only maintains MSC proliferation potential, it also retains osteogenic, adipogenic and chondrogenic differentiation potentials through the early mitogenic cycles; eventually, however, all of the MSCs differentiate into the chondrogenic line. There is a report that b-FGF can extend the proliferation of MSCs for at least 80 population doublings, which is in excess of the Hayflick number (Yanada, Ochi et al. 2006). Other reports, however, do not find this extension; rather, b-FGF may just decrease the doubling time (Bianchi, Banfi et al. 2003) with the MSCs observing the Hayflick limitation (Tarte, Gaillard et al. 2010). This slowing and senescence of MSCs follows that seen in other cell types; as the cells reach senescence, their growth factor receptors become downregulated and signal attenuation is highly increased to bring about resistance to the growth factor stimuli (Shiraha, Gupta et al. 2000; Tran, Rusu et al. 2003).

FGF-4, another member of this growth factor family, also increases MSC proliferation at lower densities. In addition to MSC proliferation increasing five times, the number of colony-forming units – indicative of progenitor cell populations – increases by one-half (Battula, Bareiss et al. 2007). This observation suggests not only that growth factors can drive proliferation; they could contribute to stem cell expansion and a greater number of cells undergoing differentiation. FGF signals proliferation through the MAPK cascade in various cell types. From microarray analysis, Tanavde and colleagues determined that MAPK-Erk signaling might be involved in increased growth induction by b-FGF (Ng, Boucher et al. 2008). The schematic mechanism is presented in Figure 21.

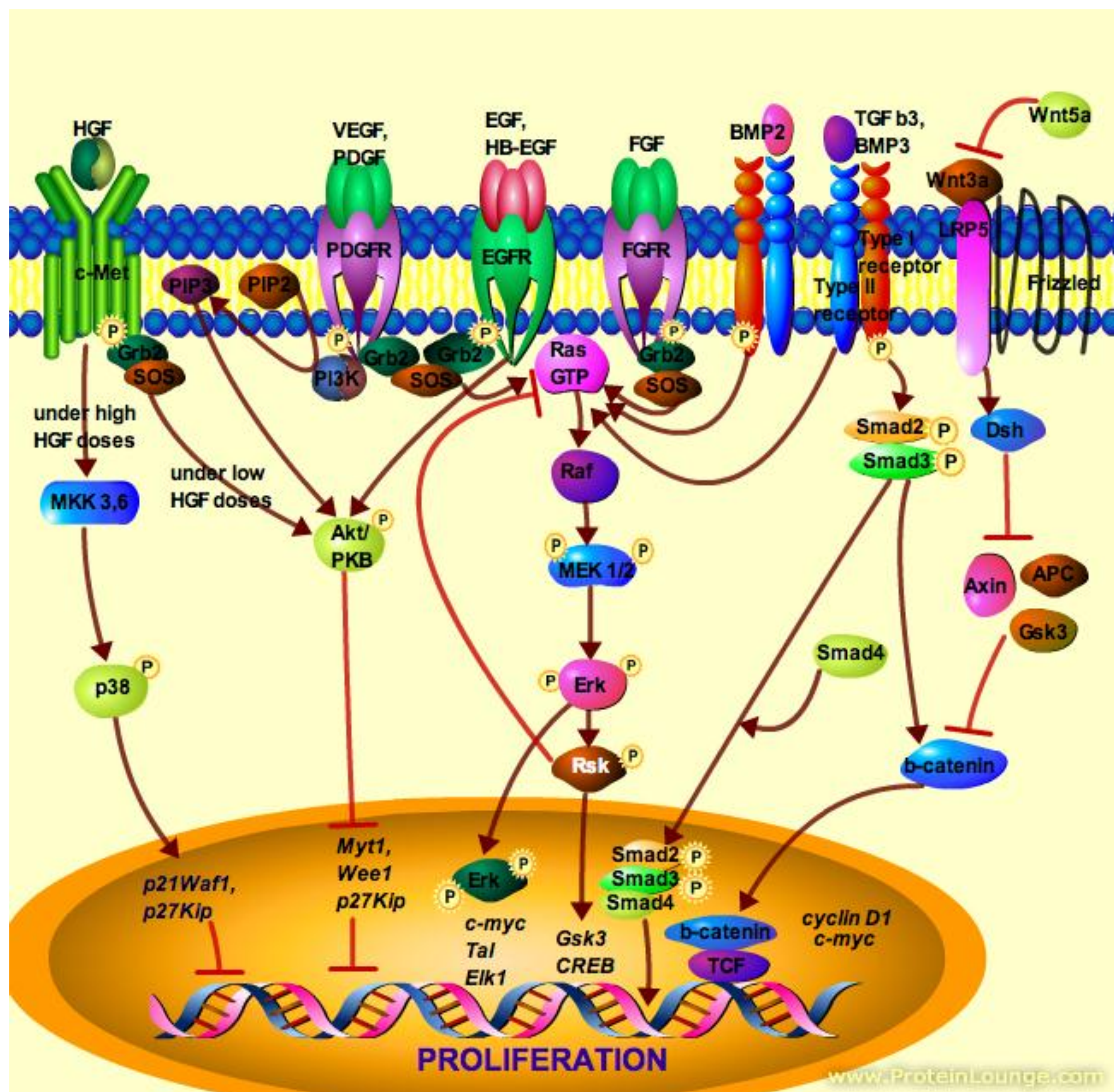


Figure 21 Growth factor signaling pathways mediating proliferation in MSC. Binding of FGF to FGFR, binding of EGF and HB-EGF to EGFR and binding of PDGF and VEGF to PDGFR causes phosphorylation of the respective receptors, recruitment of the adaptor proteins Grb2 SOS and causes activation of downstream pathways primarily PI3K and the MAPK Erk. Phosphoryated Erk either enters the nucleus and activates transcription of cellular proliferation genes such as *c-myc* or activates downstream receptors such as Rsk that then activates proliferation genes. Akt similarly prevents the expression of proteins including Myt1 and Wee1, which are

involved in inhibiting proliferation. BMP2 activates proliferation via the MAPK Erk pathway unlike BMP3 that activates Smad2 and Smad3 via Activin signaling. TGFβ3 is the most potent TGFβ mitogen causing proliferation via activation of Smads2, 3 and 4. Binding of Wnt3a to the Frizzled receptor causes a nuclear influx of β-catenin, activating the cell cycle proteins cyclin D1 and c-myc. TGF-β also causes an influx of β-catenin through a Smad3 dependent manner. Binding of HGF to c-Met under low doses causes activation of Erk and Akt, but under higher doses it inhibits proliferation by activating the p38 MAPK pathway and causing the expression of cell cylce progression inhibitors p21Waf1 and p27Kip.

5.3.3 Vascular Endothelial Growth Factor

While investigating ways to better vascularize the MSC transplant site, it was noted that vascular endothelial growth factor (VEGF) increased MSC proliferation on its own (Pons, Huang et al. 2008). Both endogenous and exogenously secreted VEGF has been found in porcine MSCs (Wang, Hu et al. 2006) but the amounts are too low for autocrine signaling. For *in vivo* transplantation studies, therefore, MSCs have been either adenovirally transduced with the VEGF gene or injected with a VEGF peptide to bring about increased cell counts (Gao, He et al. 2007). Some signaling studies imply that MSCs do not express the VEGF receptor. This could imply that VEGF stimulates MSC proliferation by activation and downstream signaling of the platelet-derived growth factor (PDGF) receptors (Ball, Shuttleworth et al. 2007).

5.3.4 Platelet Derived Growth Factor

PDGFs are potent mitogens of MSCs (Kang, Jeon et al. 2005)and these stromal cells express all forms of the growth factor: PDGF-A and PDGF-C at higher levels and PDGF-B and PDGF-D at lower levels. Both receptors PDGFRα and PDGFRβ are also expressed (Tokunaga, Oya et al. 2008). The two receptors homodimerize or heterodimerize to generate overlapping but distinct cellular signals: PDGFRαα binds PDGF-AA, PDGF-BB, PDGF-CC and PDGF-AB; PDGFRββ binds PDGF-BB and PDGF-DD; and PDGFRαβ binds PDGF-BB, PDGF-CC and PDGF-AB. Several groups have found PDGF-BB to induce both proliferation and migration in MSCs (Kratchmarova, Blagoev et al. 2005; Tamama, Fan et al. 2006; Fierro, Illmer et al. 2007). While PDGFRβ inhibits osteogenesis, however, PDGFRα has been observed to induce osteogenesis (Tokunaga, Oya et al. 2008). Akt signaling has been proposed to mediate both the

suppression and induction of osteogenesis by PDGFR signaling (Kratchmarova, Blagoev et al. 2005). As the two receptor isoforms present quantitatively different preferences for pathway activation, due to distinct phosphotyrosine motifs, definition of critical signaling elements will await a system's approach to parse the delicate balance of competing impetuses.

Early studies with PDGF showed Erk to be responsible for MSC proliferation (Tamama, Fan et al. 2006). Recently, however, it was shown that while Erk gets phosphorylated in the presence of PDGF, addition of a PDGFR inhibitor does not change phosphorylation levels of Erk (Ding, Knox et al. 2010) – which might imply that Erk activation occurs not by direct PDGFR signaling but via a secondary pathway. The same group showed that increase of MSC proliferation occurs in a dose-dependent manner due to Akt phosphorylation. Not only was there an increase in proliferation on Akt activation, there was also secretion of VEGF (Ding, Knox et al. 2010). Further, VEGF was found to act as a ligand to PDGFR in MSCs (Ding, Knox et al. 2010). The mitogenic pathways operative downstream of PDGFR activation are thus still uncertain.

5.3.5 Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) and its receptor c-Met are expressed at low levels in mouse MSCs (Neuss, Becher et al. 2004). While the low levels of HGF found in culture media are insufficient to activate the receptor, exogenous addition of HGF to MSCs triggers the activation of receptor, affecting proliferation, migration and differentiation. Interestingly, short-term exposure to HGF in MSCs activates Ras-ERK and PI3K-Akt; these are the main pathways activated by HGF in other cell types (Furge, Zhang et al. 2000). Despite activation of these pathways, long-term exposure to the growth factor inhibits mitogenesis. In addition, exposure

brings about cytoskeletal rearragement, cell migration and expression of cardiac markers. The inhibition of proliferation probably occurs by activation of p38 MAPK and blockade of G_0–G_1 phase transition. This signaling also induces the universal cell cycle progression inhibitor p21waf1 and p27kip proteins (Forte, Minieri et al. 2006). HGF therefore does not seem to be an ideal factor for use with MSCs. Table 2 summarizes the effects of the various growth factors on MSCs.

5.3.6 Epidermal Growth Factor and Heparin-binding Epidermal Growth Factor

The growth factors described above facilitate MSC proliferation but bias differentiation into a particular lineage. This is helpful in generating specifically differentiated cells in culture. When injected into the body or when implanted at the site of requirement for a different or multiple lineage, however, this bias can be counterproductive. Adding to this, if the growth factors initiate differentiation, this competes with expansion and thus there may be insufficient cell numbers to completely regenerate the desired tissue. The search for a growth factor that does not cause MSC differentiation led to investigation of growth factors from the near-ubiquitous prototypal growth factor receptor family of ErbB1/epidermal growth factor receptor (EGFR). EGFR signaling induces proliferation, motility and survival of MSCs. Two of the receptor's ligands, epidermal growth factor (EGF) and heparin-binding EGF, promote *ex vivo* expansion of MSCs without triggering differentiation into any specific lineage (Krampera, Pasini et al. 2005; Tamama, Fan et al. 2006). In addition to its mitogenic effect on MSCs, EGF also increases the number of colony-forming units by 25% (Tamama, Kawasaki et al. 2010). This observation indicates that treatment with EGF would also be beneficial for the maintenance of early progenitor cells.

Classical growth factors, upon binding to their cognate receptors with intrinsic tyrosine kinase activity, activate several downstream pathways that lead to proliferation: Ras GTPase through Raf and MEK to the ERK MAPKs, PI3K activation of Akt/PKB, and the STAT pathways. Tamama and colleagues showed that EGF does not activate STAT3 for proliferation in MSCs, but rather triggers ERK strongly (Tamama, Fan et al. 2006). Heparin-binding EGF, the other EGFR ligand implicated in MSC proliferation, shows activation of ERK1/2 as well as phosphorylation of Akt, but the activation of Akt is significantly lower than that by EGF. With activation of EGFR signaling, therefore, the overall population of both MSCs and their early progenitors will be high, leading to enough cells for tissue formation.

5.3.7 Wnt Family

There have been several conflicting findings concerning Wnt signaling proliferation in MSCs. One set of studies suggests that canonical Wnt signaling maintains stem cells in an undifferentiated but self-renewing state. Addition of Wnt3a by activating the canonical Wnt pathway increases both proliferation and survival while preventing differentiation into the osteoblastic lineage in MSCs (Boland, Perkins et al. 2004). Frizzled 1 and Frizzled 4 are present on undifferentiated MSCs and are responsible for canonical Wnt transduction via Wnt3a. In addition, Wnt3a also increases the survival rate of MSCs. Wnt5a, a noncanonical Wnt, competes for Wnt3a binding to the Frizzled receptor and negates the positive effect of Wnt3a on MSC proliferation (Boland, Perkins et al. 2004). The cell cycle progression factors cyclin D_1 and c-*myc* have been implied in both these signaling mechanisms (Baksh and Tuan 2007). Studies with Wnt4, another noncanonical Wnt, show no change in MSC proliferation (Chang, Sonoyama et al. 2007). The other set of findings connotes that canonical signaling initiated by Wnt3a inhibits

human MSC proliferation (Qiu, Andersen et al. 2007). A third set of studies, however, proposes that canonical Wnt signaling at low levels promotes proliferation while at higher levels inhibits MSC proliferation (de Boer, Siddappa et al. 2004).

Part of the controversy surrounding Wnt signaling is the extensive crosstalk between Wnts and other signaling pathways that affect the fate of MSCs. $TGF\beta_1$, for example, causes rapid nuclear translocation of β-catenin in a Smad3-dependent manner, causing enhanced proliferation and suppression of osteogenesis. Table 2 summarizes the roles of various growth factors on MSC.

Table 2. Growth Factors and their effects on proliferation and survival of MSC

Growth factor family	Growth factor	Receptor/signaling modulator	Effects on proliferation /survival/morphogenesis
TGF-β	TGF-β3	ALK 1, 2, 3, 6 (Valcourt, Gouttenoire et al. 2002)	Increases chondrogenesis (Weiss, Hennig et al. 2010)
		ALK 4, 5, 7 (Valcourt, Gouttenoire et al. 2002)	Increases proliferation (Longobardi, O'Rear et al. 2006)
	BMP2	Erk (Lou, Tu et al. 2000)	Increases osteogenesis (Luu, Song et al. 2007), Increases proliferation (Lou, Xu et al. 1999; Chang, Chung et al. 2010)
	BMP3	ALK4/SMAD2, 3 (Stewart, Guan et al. 2010)	Increases proliferation (Stewart, Guan et al. 2010)
FGF	FGF2	FGFR/Erk (Ng, Boucher et al. 2008)	Bias towards chondrogenesis on prolonged exposure (Tsutsumi, Shimazu et al. 2001), Increases proliferation (Tsutsumi, Shimazu et al. 2001; Stewart, Byron et al. 2007)
	FGF4	FGFR/ Erk (putative)	Increases proliferation (Farre, Roura et al. 2007)
VEGF	VEGF-a	VEGFR/PDGFR (Ball, Shuttleworth et al. 2007; Ball, Shuttleworth et al. 2007)/ Erk (Tamama, Fan et al. 2006)	Increases proliferation (Wang, Hu et al. 2006; Pons, Huang et al. 2008)
		VEGFR/PDGFR/ PI-3K (Tamama, Fan et al. 2006)	Increases survival (Tang, Wang et al. 2010)
PDGF	PDGF-bb	PDGFR/ Erk (Tamama, Fan et al. 2006)	Increases proliferation (Chase, Lakshmipathy et al. 2010)
		PDGFR/Erk (Tamama, Fan et al. 2006)	Increases survival (Krausgrill, Vantler et al. 2009)

EGF	sEGF	EGFR/ transient Erk (Fan, Tamama et al. 2007; Platt, Roman et al. 2009)	No effect on differentiation (Tamama, Fan et al. 2006), Increases proliferation (Tamama, Fan et al. 2006)
	tEGF	EGFR/ sustained Erk (Fan, Tamama et al. 2007; Platt, Roman et al. 2009)	Increases spreading and survival (Fan, Tamama et al. 2007)
	HB-EGF	EGFR/ Erk (Forte, Minieri et al. 2006)	No effect on differentiation (Krampera, Pasini et al. 2005), Increases proliferation (Krampera, Pasini et al. 2005)
HGF	HGF	c-Met/p38 MAPK (Forte, Minieri et al. 2006)	Enhances survival (Forte, Minieri et al. 2006)
		c-Met/PI-3K (Forte, Minieri et al. 2006)	Inhibits proliferation (Forte, Minieri et al. 2006)
Wnt	Wnt3a	β-catenin	Enhances proliferation and survival (Boland, Perkins et al. 2004)

5.4 MSC SURVIVAL AND ROLE OF GROWTH FACTORS

A second cell behavior critical for the successful use of MSCs in regenerative repair is the survival of transplanted cells. Various growth factors – trophic factors as they are called in other cell types – have been queried for promoting this survival. VEGF is one factor that has been extensively used in MSC survival studies. MSCs treated with VEGF *in vitro* and MSCs carrying the VEGF gene *in vivo* have been shown to increase survival in these stromal cells. Rodent hearts that have undergone myocardial infarction and have been injected with MSCs along with the VEGF peptide show a higher number of MSCs at the site of injection (Pons, Huang et al. 2008). The surge in survival is attributed to an increase in Akt signaling causing a

reduction in infarct size, lesser fibrosis, increased vascularity and thicker ventricular walls (Wang, Hu et al. 2006; Tang, Wang et al. 2010). Akt signaling in other cell types causes increased expression of prosurvival proteins XIAP, Bcl2 and Bcl-xl, and decreased levels of caspases and apoptotic proteins Bad, Bax and Bim. Akt signaling is also known to inhibit the transcription factors FOX01, FOX02 and FOX03 involved in causing cell cycle arrest and apoptosis (Brunet, Bonni et al. 1999).

MSCs pretreated with transforming growth factor alpha and implanted at the ischemic site after a myocardial infarction show increased survival. This improvement is also attributed to VEGF signaling, although direct signaling through the EGFR receptor cannot be discounted. Transforming growth factor alpha increases VEGF production via the p38 MAPK pathway and enhances recovery (Herrmann, Wang et al. 2010). For the ischemic cardiac tissue, MSCs supplanted with VEGF have so far been the best choice for increased survival, leading to improved vascularity in ischemic cardiac tissue and isolated islets. The current issue of debate, however, is whether VEGF causes greater incorporation of MSCs and succeeding survival, or whether it brings about paracrine effects on surrounding endothelial cells, increasing angiogenesis and formation of more vessels.

Several other growth factors have proven to increase MSC survival. MSC transplantation with brain-derived neurotrophic factor and nerve growth factor into rodents after traumatic brain injury has shown a significantly higher number of engrafted cells compared with MSCs transplanted without any growth factor (Mahmood, Lu et al. 2002). PDGF-BB has been found to reduce the 46% loss of cells by apoptosis seen between days 5 and week 3 in rats following acute myocardial infarction (Krausgrill, Vantler et al. 2009). Contrary to its limiting effect on MSC

expansion, HGF causes a slight increase in MSC survival. PI3K signaling is implicated in this increase (Forte, Minieri et al. 2006).

Another major growth factor studied for its effects on MSC survival is EGF. Since initial studies showed that EGF in the soluble state did not cause differentiation of MSCs but enhanced expansion, it was hypothesized that soluble EGF would similarly enhance survival of MSCs subjected to prodeath cytokines such as FasL and TRAIL *in vitro*. Contrary to what was expected, soluble EGF did not protect MSCs, but increased cell death in the presence of FasL (Fan, Tamama et al. 2007). Fan and colleagues then presented EGF to MSCs tethered to a biomaterial substratum (tEGF). This mode of presentation of the same growth factor enhanced survival of MSCs in the presence of the proinflammatory cytokines. In addition to limiting cell death, tEGF also increased cell attachment and spreading, which might limit cell death by anoikis (Fan, Tamama et al. 2007). The survival brought about by tEGF was found to be mainly due to sustained levels of Erk activation, as opposed to transient Erk activation with soluble EGF. Furthermore, tEGF restricts the subcellular localization of activated EGFR. Unlike soluble EGF that causes the internalization and finally the degradation of EGFR, tEGF restricts EGFR and EGFR signaling to the plasma membrane, and thereby changes the spatiotemporal balance of intracellular signaling pathways (Iyer, Tran et al. 2007). tEGF did not interfere with subsequent differentiation into osteoblasts under inducing conditions while increasing the efficiency and the number of osteoid colonies (Platt, Roman et al. 2009). As this was the first study to directly challenge MSCs with proapoptotic inflammatory stimuli, the technique holds promise as a quantal advance in protecting MSCs from death *in vivo*. The cross–signaling of survival by various growth factors is represented in Figure 22.

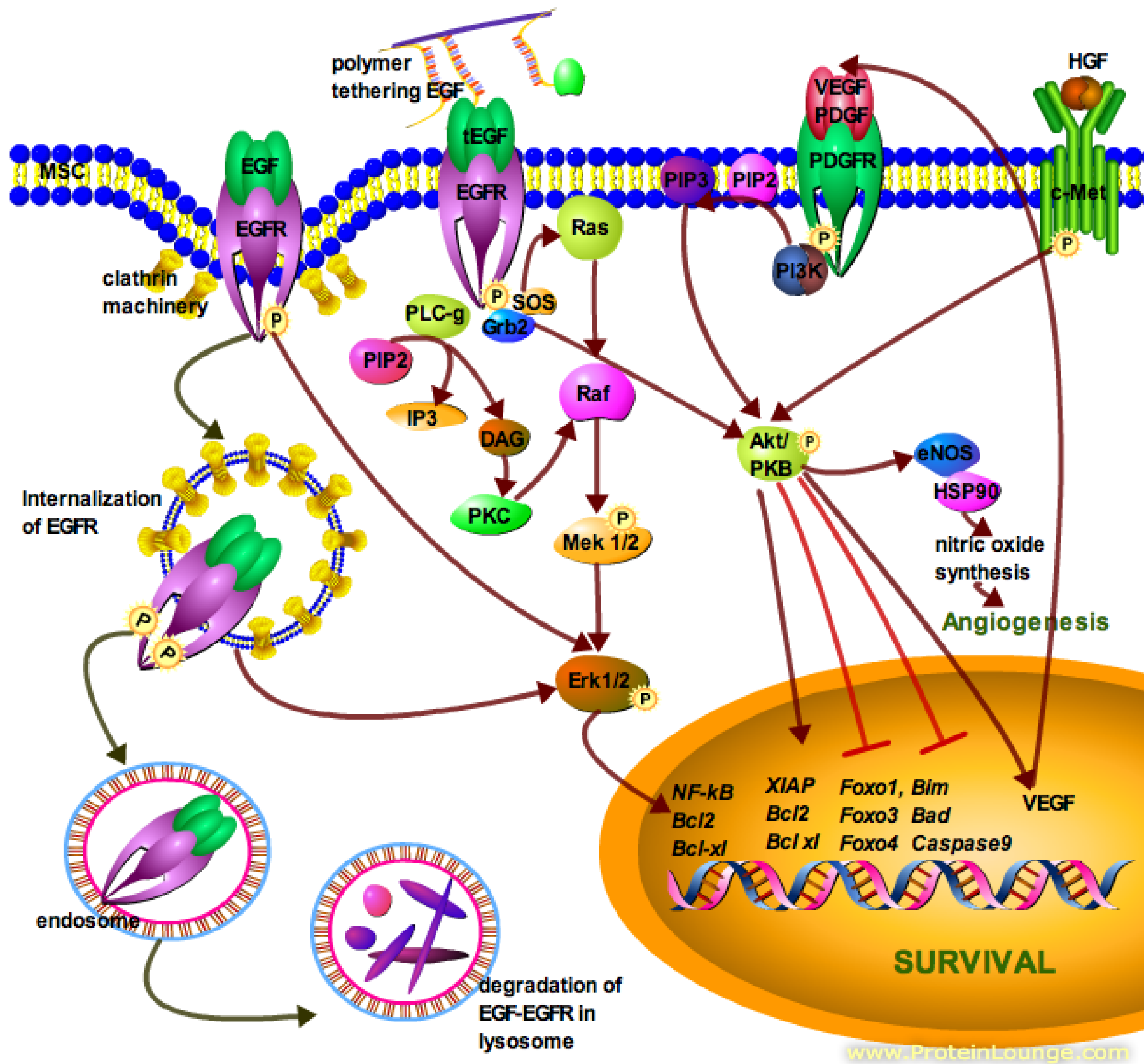


Figure 22 Growth factor signaling pathways mediating survival in MSC. EGF binds EGFR, FGF binds FGFR and PDGF binds PDGFR leading to the activation of Akt and Erk. All these activated receptors however are quickly internalized by clathrin machinery or by alternate internalization mechanisms. Signaling continues while moving through the cytoplasm in the endosome, but once inside the lysosome, the receptor along with the ligand completely degrades and the signal is lost. Both the Akt and the Erk signal generated in this manner is acute and transient. Tethering of growth factors near the membrane, as in the case of EGF however causes a much more sustained signaling of Erk and Akt, leading to MSC survival for a more prolonged time period. Binding of PDGF to PDGFR causes synthesis of VEGF which signals through binding of PDGFR and leads to angiogenesis. This leads

to the possibility of reducing stress and hypoxia at the wound region. Activation of Akt and Erk causes inhibition of pro-death proteins including Bim, Bad and Caspase9, Akt also inhibits the Fork head family of transcription factors inhibiting cell death. Pro-survival proteins such as XIAP, Bcl2 and Bcl-xl are upregulated.

5.5 CLINICAL ISSUES OF USING GROWTH FACTORS WITH MSC

Current limitations to using MSCs for regeneration include providing sufficient numbers of these stromal cells in a timely manner in the challenging *in vivo* milieu. To bring about MSC expansion, fetal bovine serum (FBS) is currently employed since human serum does not fully support growth of MSCs *in vitro*. Complications arise in use of FBS for MSC transplants *in vivo*, however, since FBS contains undefined elements that can vary in inducing proliferation. More importantly, contaminants in FBS can cause infections, and, being of nonhuman origin, the components can trigger host immune reactions (Platt, Roman et al. 2009). On the commercial front, companies have developed serum-free and animal supplement-free MSC media. These media products are comprised of synthetic supplements that are meant to replace serum, thereby reducing variability. The companies claim that MSCs grow as well in the media as in media supplanted with FBS. The proprietary composition of these products, however, goes against them for clinical use. Details of these products are summarized in Table 3. The use of growth factors as culture supplements instead of FBS therefore offers the most promising alternative (Meuleman, Tondreau et al. 2006). Search for a serum-free media to expand MSCs has led to combination treatments with PDGF-BB, FGF-2 and $TGF\beta_1$ showing the most encouraging results. This treatment has not only brought about a synergistic effect on MSC proliferation, but has also retained the phenotype, differentiation and colony-forming potential of these cells (Chase, Lakshmipathy et al. 2010).

In addition to using combined treatment of growth factors to improve proliferation, MSCs have been pretreated with a blend of growth factors to boost survival. Pretreatment of these stromal cells with FGF-2, BMP-2 and insulin-like growth factor-1 before delivery into the ischemic heart has shown enhanced rates of survival (Hahn, Cho et al. 2008).

One of the reasons MSCs are preferred for regenerative use is their genetic stability. MSCs are shown to maintain their diploid karyotype without aneuploidy, polyploidy or chromosomal structural abnormalities (Zhang, Guan et al. 2007). There has also been a report, however, of MSCs displaying localized genetic alterations in the presence of FBS or autologous serum. The same report states that platelet lysate expands cytogenetically normal MSC colonies and that this effect may be due to the presence of growth factors such as EGF, PDGF and FGF in the platelet lysate (Dahl, Duggal et al. 2008). In short, growth factors should be chosen not only based on expansion potential but also on not altering the MSC genome.

There still remains the scare that while growth factors increase proliferation, this proliferation and the added protection offered might let MSCs and surrounding cells escape control and lead to tumor growth. It is therefore important to have proper modes of growth factor delivery, which is localized, controlled and of a time-limited nature. Controlled release of growth factors or presentation of the growth factor in bioengineered forms, such as tEGF, are some of the ways in which this can be achieved (Richardson, Peters et al. 2001; Muschler, Nakamoto et al. 2004; Simmons, Alsberg et al. 2004; Platt, Roman et al. 2009), discussion of which lies beyond the scope of the present review.

Table 3. Commercially available serum free media for ecpansion of MSC

	Serum free Media	Company	Properties	Drawbacks
1	STEMPRO([R])MSC SFM	Invitrogen	Serum-free, xeno-free. Maintains MSC for up to 9 passages as compared to 5 passages with MSC in MEM + 10% FBS. Cells are smaller in size. (Agata, Watanabe et al. 2009)	Marketed as a research product only. Proprietary composition makes it difficult to be used for pre-clinical and clinical purposes.
2	Mesencult	Stem Cell Technologies	Serum free, xeno-free. Causes rapid expansion of cells in the first passage, higher than any other media (Pal, Hanwate et al. 2009).	MSC fail to maintain similar growth rate beyond the first passage and stop growing altogether after the 6th passage, while MSC grown in DMEM-KO and DMEM F12 supplanted with 10% FBS proliferate for up to 25 passages (Pal, Hanwate et al. 2009).
3	Mesengro	StemRD	Chemically defined, serum free and xeno-free. The company claims that the growth rate of MSC in this media is the same as that of MSC supplanted with 10% FBS for up to 9 passages *in vitro*.	No published data using this media as yet.

5.6 CONCLUSIONS AND FUTURE DIRECTIONS

Growth factors are a promising adjuvant to MSCs to circumvent problems of MSC proliferation and expansion, and survival *in vivo*. The choice of growth factor(s), however, depends on three major criteria. First, the growth factor needs to prolong proliferation for several population doublings, to generate a considerable number of MSCs before these cells are to be differentiated into the desired tissue. Second, the growth factor should be able to completely replace the use of animal serum for proliferation purposes, to eliminate the use of xenographic substances and reduce variability. Finally, there need to be modes of localized and controlled delivery, which will help present the mitogenic and protective signals in sustained forms without letting MSCs escape into uncontrolled proliferation. While individual growth factors like b-FGF have advantages in steering MSCs down a select lineage after several population doublings, combination treatments of growth factors currently seem to be drawing a lot of attention due to their synergistic effect on MSCs. Composite treatment with PDGF, b-FGF and TGFβ_1 appears to be a good alternative for proliferation *in vitro* to replace serum. More studies need to be performed, however, to look into whether such a combination would accentuate survival and encourage grafting of cells in the wound microenvironment. There also need to be ways by which such combinations can be delivered at the wound region.

While there have been several groups looking into the proliferation effects of growth factors and their effects on morphogenesis, much less attention has been paid to growth factor signaling for survival. This might partly be because MSCs were for a very long time considered

to be cells with the advantage of survival. When MSCs were not observed in the body on delivery, the absence was attributed more to cells migrating away rather than to cells dying at the site. Only recently have there been studies showing that MSCs are susceptible to death by proinflammatory cytokines and reactive oxygen species, which might be the major reason for their loss at the site of delivery.

The advantage with the EGFR family of ligands is that work has been carried out on almost all aspects of MSC biology: effects on survival, proliferation, differentiation, migration and modes of delivery have been studied. This family of ligands appears to be generalized expanders and survival adjuvants while not affecting MSC differentiation. Moreover, presenting EGF in a tethered form has been studied with respect to sustained signaling, making it one of the factors of foremost importance. Taken together, the right choice of growth factors with proper modes of their delivery will help bridge the gap in MSC regenerative therapy and exploit the full potential of MSCs to regenerate tissue in the near future.

6.0 SURFACE TETHERED EPIDERMAL GROWTH FACTOR PROTECTS PROLFERATING AND DIFFERENTIATING MULTIPOTENTIAL STROMAL CELLS FROM FASL INDUCED APOPTOSIS

Melanie Rodrigues, Harry Blair, Linda Stockdale, Linda Griffith, Alan Wells

6.1 ABSTRACT

Multipotential stromal cells, or mesenchymal stem cells, (MSC) have been proposed to aid in regeneration of bone and adipose tissues, as these cells can form osteoblasts and adipocytes. A major obstacle to this use of MSC transplantation is the initial loss of cells post-implantation. This cell death is due, at least in part, to ubiquitous non-specific inflammatory cytokines such as FasL generated in the wound environment. Our group previously found that epidermal growth factor (EGF) promotes MSC expansion. Further we showed that this factor when tethered (tEGF) onto a two-dimensional substratum, but not when soluble (sEGF) was added, protects MSC from FasL-induced death. However, for this to be useful in tissue regeneration, we needed to determine whether this presentation prevented MSC differentiation and also protected the emerging osteoblasts and adipocytes from apoptosis. The tEGF did not block induced differentiation of the MSCs. MSC-derived pre-osteoblasts showed an increase in Fas levels and became more susceptible to FasL-induced death which tEGF prevented; differentiating adipocytes underwent a reduction in Fas expression and became resistant to FasL-induced death with tEGF having no further survival effect. tEGF was also able to protect undifferentiated MSC from combined insults of FasL, serum deprivation and physiologic hypoxia comprising 1% oxygen. Our results suggest that MSCs and differentiating osteoblasts need protective signals to survive in the inflammatory wound milieu and that tEGF can serve this function.

6.2 INTRODUCTION

Adult human MSC have the potential in vitro to form a variety of cell types including osteoblasts, chondrocytes and adipocytes (Pittenger, Mackay et al. 1999; Jones and Yang 2011), but clinical success in regenerating connective tissues from these cells has been relatively elusive. Although the use of freshly-aspirated marrow during bone grafting material has shown improvement, many studies have shown that transplanted cells likely do not contribute to the bone growth. In regenerative applications in animal models, most of the positive effects seen during MSC delivery in experiments in vivo occur due to trophic effects secondary to MSC release of growth factors. These MSC-derived growth factors support development of extracellular matrix and new blood vessels in cells intrinsic to the wounded tissue; but most evidence suggests that differentiated cells in the regenerated tissue do not arise from transplanted MSC (Caplan and Dennis 2006; Caplan 2009; Uccelli and Prockop 2010). This is in part because most of the implanted MSC are lost within 48 hours and less than 1% of the implanted cells are present after 7 days (van der Bogt, Schrepfer et al. 2009; Rodrigues, Griffith et al. 2010; Semont, Mouiseddine et al. 2010; Zimmermann, Gierloff et al. 2011). The loss in cell numbers can be due to several adverse factors in the ischemic wound environment including ROS (Wei, Li et al. 2010), hypoxia (Zhu, Chen et al. 2006), nutrient deprivation (Kim, Meliton et al. 2010) and presence of first-phase inflammatory cytokines like FasL and TRAIL to which MSC are most susceptible (Fan, Tamama et al. 2007).

We previously reported that ligands for the epidermal growth factor receptor (EGFR) could be used to increase MSC numbers while not affecting differentiation of these cells in the absence or presence of external differentiation cues (Tamama, Fan et al. 2006). However, when challenged with pro-apoptotic cytokines, EGF if anything enhanced death, likely due to its

mitogenic stimulus (Fan, Tamama et al. 2007). Surface-restricted EGFR with persistent low-level of activation is known to signal in a qualitatively distinct manner for motility and survival (Fan, Tamama et al. 2007; Iyer, Tran et al. 2007). To accomplish this, EGF was tethered using a polyethylene oxide based polymer onto a two-dimensional glass surface on which MSC were grown. This restriction of EGFR to the MSC membrane, caused prolonged downstream EGFR signaling and increased survival in the presence of death signals like FasL (Fan, Tamama et al. 2007; Platt, Roman et al. 2009). Thus, this approach became a candidate for promoting survival of implanted MSCs in the wound milieu.

Survival of implanted MSC in an undifferentiated state is only part of the story; the MSCs must subsequently differentiate into the desired tissues, and do so within this inflammatory field. Thus, in this study we investigated whether MSC in the process of differentiating toward a specific lineage were similar to or divergent from undifferentiated MSC in terms of susceptibility to inflammatory signals, since receptors and protein composition in cells undergo major changes during the progress of differentiation. We looked specifically at differentiating osteoblasts and adipocytes with a long-term perspective of cranio-facial reconstruction and healing of critical sized defects in long bones. FasL was used as a representative cytokine since undifferentiated MSC die the most when its receptor Fas is activated, either directly or by transactivation (Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011; Liu, Wang et al. 2011)(Rodrigues, Turner et al. 2012). We found not only that differentiating osteoblasts needed external survival cues, but also that the tEGF signal from the engineered surfaces persisted for 30 days and protected both pre-osteoblasts and osteoblasts in the presence of FasL. Further we threatened MSC with low oxygen, serum deprivation and FasL,

and found that tEGF provided a survival advantage to the cells under these unfavorable conditions.

6.3 MATERIALS AND METHODS

6.3.1 Reagents

α-MEM without ribonucleotides or deoxyribonucleotides was obtained from Gibco (Carlsbad, CA), DMEM 1g/l glucose was from Cellgro (Mediatech, Washington, DC), fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA) and adipogenic differentiation media was from Lonza (Walkersville MD). Human recombinant epidermal growth factor [354052] was from BD BioSciences (Franklin Lakes, NJ), murine epidermal growth factor was from Peprotech [#315-09] (Rocky Hill, NJ) and human soluble recombinant SuperFasL [ALX-522-020-3005] was from Enzo Lifesciences (Plymouth Meeting, PA). RNeasy Minikit and QuantiTect Reverse Transcription kit were from Qiagen (Valencia, CA). Brilliant SYBR Green qPCR Master Mix was from Stratagene (Santa Clara, CA). Poly ADP ribose polymerase (PARP) [9542] and Fas [4233] antibodies were from Cell Signaling (Boston, MA), fatty acid binding protein 4 (FABP4) [SAB2104636] and GAPDH [G9545] antibodies were from Sigma Aldrich (St. Louis, MO), EGFR antibody [sc-365829] was from Santa Cruz Biotechnology (Santa Cruz, CA), and Caspase inhibitor Z-VAD-FMK [G7231] was from Promega (Madison, WI).

6.3.2 Cell Culture

Two human bone marrow derived MSC cell preparations were used for the study. The first is an immortalized human multipotent stromal cell line (imhMSC) (Okamoto, Aoyama et al. 2002), a kind gift from Dr. Junya Toguchida, Kyoto University, Japan; these cells were immortalized by expression of hTERT. These cells were cultured in proliferation media comprising DMEM 1g/l glucose with 10% FBS, 1mM Sodium pyruvate, 1μM non-essential amino acids and 100units/ml penicillin-streptomycin. The second is a primary human bone marrow multipotent stromal cell isolate (prhMSC) provided by Dr. Darwin Prockop (Texas A&M). Proliferation media for these cells was composed of α-MEM without ribonucleotides or deoxyribonucleotides with 16.5% FBS, and 2mM L-glutamine. Cells between 6 and 15 passages were used for experiments. Proliferation media maintains the two MSC cell lines in the undifferentiated state and these cells are referred to as Day 0. When cells at Day 0 established complete cell-cell contact on the culture surface, media was changed to either osteogenic differentiation media comprising proliferation media plus 50μM ascorbic acid, 100 μM glycerol-2-phosphate and 100nM dexamethasone or adipogenic differentiation media from Lonza consisting of differentiation inducers indomethacin, isobutyl methyl xanthine and insulin. Cells were grown either on tissue culture plastic plates, two-dimensional mock or tEGF surfaces. For culture of cells at 4% and 1% oxygen, the Biospherix (Lacona, NY) workstation and incubators were used.

6.3.3 Mock and tEGF Surface Preparation

tEGF was attached to surfaces composed of a silanized glass coverslip spin-coated with poly (methyl methacrylate)-graft-poly ethylene oxide (PMMA-g-PEO) polymer in two different weight percentages as described (Fan, Tamama et al. 2007; Wu, Wells et al. 2011). The 33% PEO component of the polymer covalently binds EGF through an activated 4-nitrophenyl choloroformate group at its free apical end, while the 22% PEO component increases adhesiveness of MSC to the polymer coated glass surface by allowing for adsorption of proteins. The higher weight polymer was mixed with the lower weight polymer in a ratio of 60:40 to achieve a concentration of 5000-7000 EGF per μm^2 of the glass coverslip while allowing for cell attachment and spread. This concentration of EGF allows for saturation of EGFR found in low numbers in MSC: as low as 7,300 in imhMSC (Tamama, Fan et al. 2006). Mock surfaces are made up of PMMA-g-PEO polymer spin-coated onto silanized glass coverslips with no activated 4-nitrophenylcholoroformate groups or attached murine EGF. Murine EGF is used due to the presence of only one terminal amine at the N-terminus that helps link EGF based on amine chemistry. It has been shown to induce downstream EGFR pathways similar to human EGF in human MSC (Fan, Tamama et al. 2007). Mock or tEGF surfaces were placed in wells of a 12-well plate, coated with 3µg/ml of Collagen I in PBS for 2 hours, followed by blocking with 1% BSA in PBS and UV sterilization for 30 minutes before MSC were seeded.

6.3.4 Immunoblots

After treatment of cells with cytokines at defined time points, both floating cells in the media and cells left on the culture plate were lysed in SDS lysis buffer (0.1 M Tris-HCl, 4%

SDS, 0.2% Bromophenol Blue and 5% □-mercaptoethanol). Cell lysates were separated by electrophoresis on polyacrylamide gels in SDS-Laemmli buffers, and transferred electrophoretically to polyvinylidine difluoride-derivatized nylon. After the blots were blocked with 5% milk in PBS, target proteins were probed with primary antibodies at 1:1000 for PARP, 1: 10000 for GAPDH, 1: 500 for EGFR, 1:2000 for Fas in 1% milk in PBS, followed by horseradish peroxidase-conjugated secondary antibodies, and the bound peroxidase was visualized by enhanced chemiluminescence (WBKLS0500, Millipore, Billerica, MA).

6.3.5 FLICA (Flurochrome Inhibitor of Apoptosis Assay)

FLICA consists of cell permeable and non-cytotoxic inhibitors of caspase-3 bound to sulforhodamine, which emits red fluroscence when bound to active caspase-3. MSC were grown on Lab-tek 8 chamber slides. 30 minutes prior to the lapse of time treatment with cytokines, FLICA reagent (#APT 503, Millipore, Billerica, MA) and Hoescht 33342 dye was added to cells in media and incubated for 30 minutes at 37□C. Media were aspirated, cells washed and live cells imaged for fluorescence. Red fluorescence, indicative of active caspase3 was measured by Image J analysis software (National Institutes of Health). To account for cell loss, intensity of FLICA was normalized to the number of cells remaining on the chamber slide.

6.3.6 Real Time Quantitative PCR Analysis

Total RNA from MSC was isolated using guanidine isothiocyanate denaturation followed by silica gel binding (RNeasy, Qiagen, Valencia, CA). 1□g of RNA was reverse transcribed into first strand c-DNA using QuantiTect Reverse Transcription kit (Qiagen). Real time PCR was

performed on a MX3000P instrument (Stratagene) using 1□l of c-DNA and 12.5µl of Brilliant SYBR Green qPCR Master Mix in a total volume of 21µl with 1nM of forward and reverse primers:

GAPDH Forward Primer: 5'-GAGTCAACGGATTTGGTCGT-3';

GAPDH Reverse Primer: 5'-TTCATTTTGGAGGGATCTCG-3';

Fas Forward Primer: 5'-TGAAGGACATGGCTTAGAAGTG-3';

Fas Reverse Primer: 5'-GGTGCAAGGGTCACAGTGTT-3';

EGFR Forward Primer 5'-AGGACCAAGCAACATGGTCA-3';

EGFR Reverse Primer 5'-CCTTGCAGCTGTTTTCACCT-3';

Collagen I Forward Primer 5'-CAATGCTGCCCTTTCTGCTCCTTT-3';

Collagen I Reverse Primer 5'-CACTTGGGTGTTTGAGCATTGCCT-3';

PPARG2 Forward Primer 5'-CACAAGAACAGATCCAGTGGTTGC-3';

PPARG2 Reverse Primer 5'-CAATTGCCATGAGGGAGTTGGAAG-3';

Osteocalcin Forward Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3';

Osteocalcin Reverse Primer 5'-GTTTATTTGGGAGCAGCTGGGATG-3';

Runx2 Forward Primer 5'-CCTCGGAGAGGTACCAGATG-3';

Runx2 Reverse Primer 5'- TTCCCGAGGTCCATCTACTG-3'

The GAPDH primers were the normalization controls, at an annealing temperatures of 55°C and 45 cycles. All reactions were performed in triplicates and the fold change in transcript levels was calculated based on □Ct method with GAPDH as reference. Product size was confirmed using agarose gel electrophoresis.

6.3.7 Differentiation Assays

Oil Red O Assay was used to stain adipocytes. Cells were washed in PBS, fixed in 4% formaldehyde for 20 minutes, placed in 60% iso-propanol for 5 minutes, followed by addition of 3mg/ml Oil Red O in 3:2 isopropanol: water for 10 minutes. Cells were washed and photographed in bright field.

For silver (von Kossa) stain of hydroxyapatite, cells were fixed in 4% formaldehyde for 20 minutes and placed in a 1% silver nitrate in water under UV light for 10 minutes. Cells were washed in sodium thiosulphate and photographed in transmitted light.

6.3.8 Statistical Analysis

Cell apoptosis was measured using paired t test. $P < 0.05$ was stated as statistically significant.

6.4 RESULTS

6.4.1 MSCs remain susceptible to FasL induced cell death as the cells differentiatie into osteoblasts

Our long-term aim is to use MSC to reconstruct critical sized defects in bone that do not heal on their own by combining a source of MSC with a scaffold. For regeneration of bone to occur, both proliferating MSC and differentiating pre-osteoblasts derived from MSC must survive exposure to inflammatory cytokines present at the wound site. To test if differentiating

osteoblasts died in the presence of FasL, we first established culture medium conditions that drove cells toward the osteogenic phenotype as determined by accumulation of hydroxyapatite (assessed via von Kossa staining) and expression of osteogenic markers (as assessed by real-time qPCR for Runx2 and Osteocalcin) after 30 days (Figure 23A-B and Figure 24A). Immortalized hMSC on tissue culture plastic (data not shown), imhMSC on mock surfaces or prhMSC on tissue culture plastic differentiating into osteoblasts, displayed hydroxyapatite deposition after 15 and 30 days in osteogenic differentiation media (Figure 1A). Real-time PCR showed increases in the early osteogenic marker Runx2 and late osteogenic marker Osteocalcin in imhMSC on mock surfaces on Day 6 and Day 21 (Figure 23B) as well as prhMSC on tissue culture plastic on Days 15 and 30 of osteogenic differentiation (Figure 24A).

After defining the osteogenic differentiation protocol, we subjected differentiating imhMSC on tissue culture plastic to FasL cytokine treatment to test for susceptibility to cell death. Differentiating imhMSC on tissue culture plastic were treated with FasL on Day 0, Day 3, Day 6 and Day 12 for 8 hours and apoptosis assessed by the amount of cleaved PARP. The 8 hour time period was chosen based on optimum detection of cells undergoing caspase 3 activation and apoptosis (Figure 25A). On Day 0, while imhMSC were still undifferentiated, treatment with FasL alone caused PARP cleavage. Cell death was enhanced when cells were treated with FasL and cycloheximide (CHX), a protein synthesis inhibitor. sEGF was unable to protect undifferentiated imhMSC from FasL induced cell death as previously observed (Fan, Tamama et al. 2007). The pan-caspase inhibitor zVAD-FMK significantly reduced degradation of PARP in the presence of CHX and FasL, confirming MSCs undergo apoptotic cell death in the presence of FasL. Controls with only CHX or sEGF did not cause imhMSCs to undergo PARP degradation and apoptosis.

Immortalized hMSCs differentiating into osteoblasts continued to undergo apoptosis in the presence of only FasL or CHX and FasL. sEGF did not protect differentiating MSC from FasL or CHX and FasL (Figure 23C). Immortalized hMSC are a true multipotential cell line since they were immortalized from a single clone and all cells of the population behave in a similar manner. prhMSC on the other hand, although isolated to contain mainly cells with stem cell like properties, are a heterogenous stromal cell population; hence, a heterogeneous response to differentiation cues or death signals is expected in this population. To assess possible heterogeneity in response to FasL in this cell population, we used an in situ FLICA protocol after 12 hr of prhMSC treatment with FasL, at Day 0, as well as at Day 15 and Day 30 (Figure 23D). We saw that undifferentiated prhMSC, as well as differentiating pre-osteoblasts and differentiated osteoblasts are susceptible to death with FasL. The 12 hour time-point was chosen for further study of prhMSC death since this time-point showed caspase 3 activation, while still allowing for cell attachment to the culture surface (Figure 25B). We were able to conclude that differentiating osteoblasts are susceptible to FasL-induced cell death similar to undifferentiated MSC.

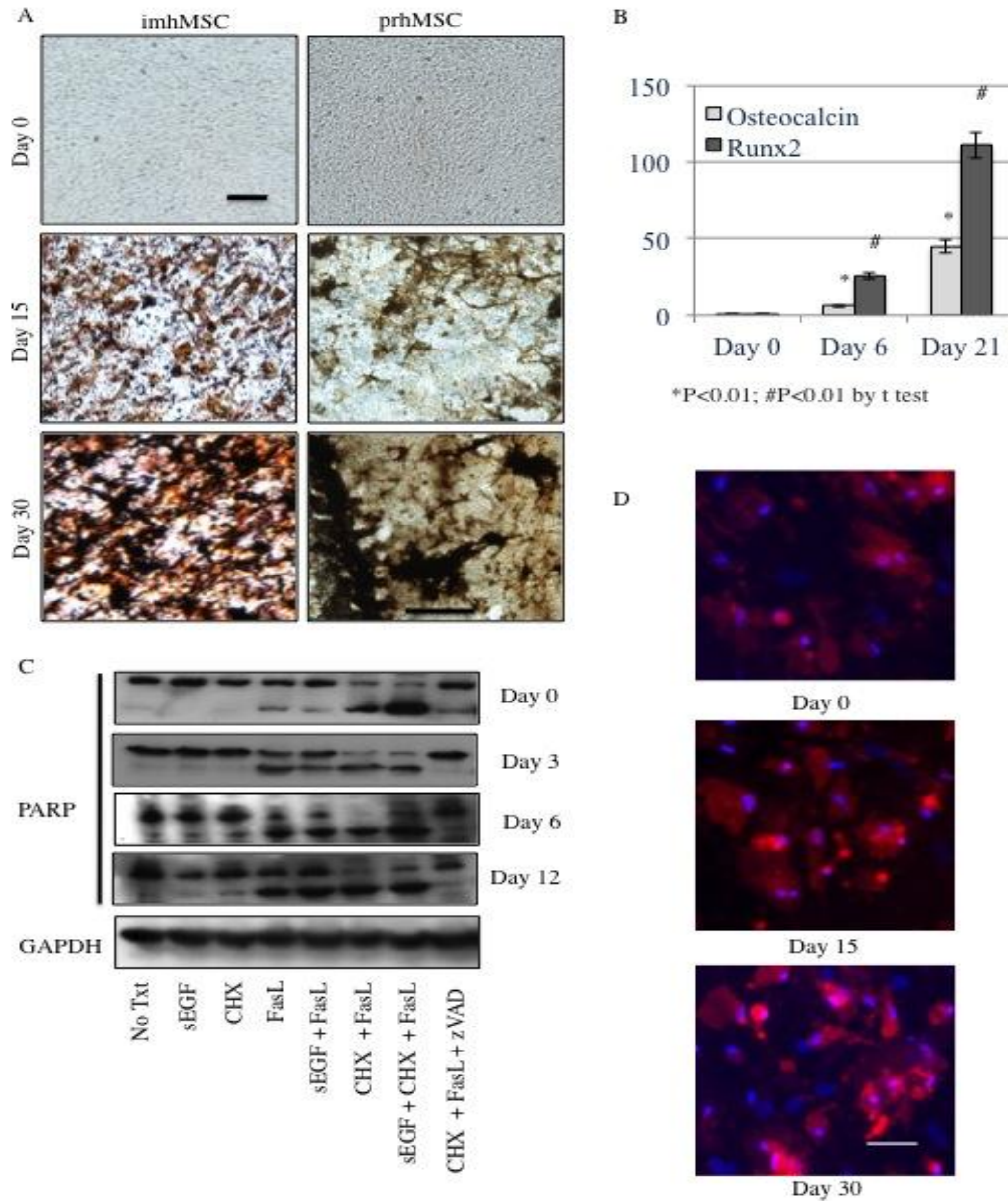


Figure 23 MSC forming osteoblasts remain susceptible to FasL induced cell death. Immortalized hMSC (Magnification 20X, 10m) on mock surface and prhMSC (Magnification 20X, 10m) on tissue culture plastic depositing hydroxyapatite by von Kossa (A). Change in expression of osteogenic markers by qPCR during osteogenic differentiation of imhMSC on mock surfaces (B). Immunoblot for full length and cleaved PARP in differentiating imhMSC grown on tissue culture plastic and treated with cytokines for 8 hours (C). Caspase 3 positive cells (red) and Hoescht 33342 (blue) stained prhMSC during progress of osteogenic differentiation (Magnification 10X, 50m). Shown are representative graphs of mean ± s.e.m and photomicrographs of cells, of two independent experiments.

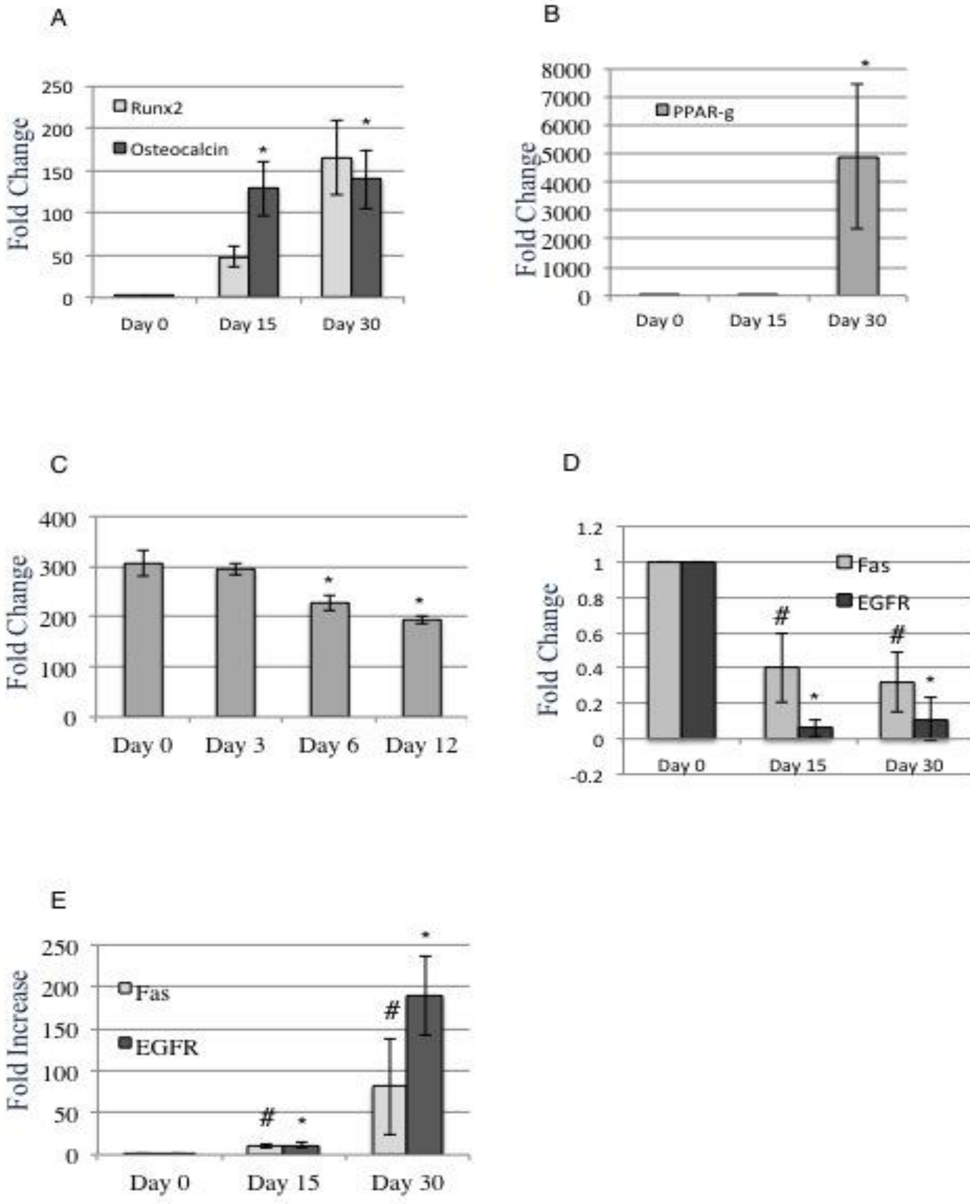


Figure 24 MSC display changes for markers of differentiation as well as cell surface receptors during progress of differentiation. Change in level of adipogenic marker by qPCR during adipogenic differentiation of prhMSC on tissue culture plastic (A). Change in nuclear size during progress of adipogenic differentiation of imhMSC on tissue culture plastic (B). Change in levels of osteogenic markers during progress of prhMSC osteogenic differentiation on tissue culture plastic (C). Change in the expression of EGFR and Fas in prhMSC during adipogenic (D) and osteogenic differentiation (E) on tissue culture plastic.

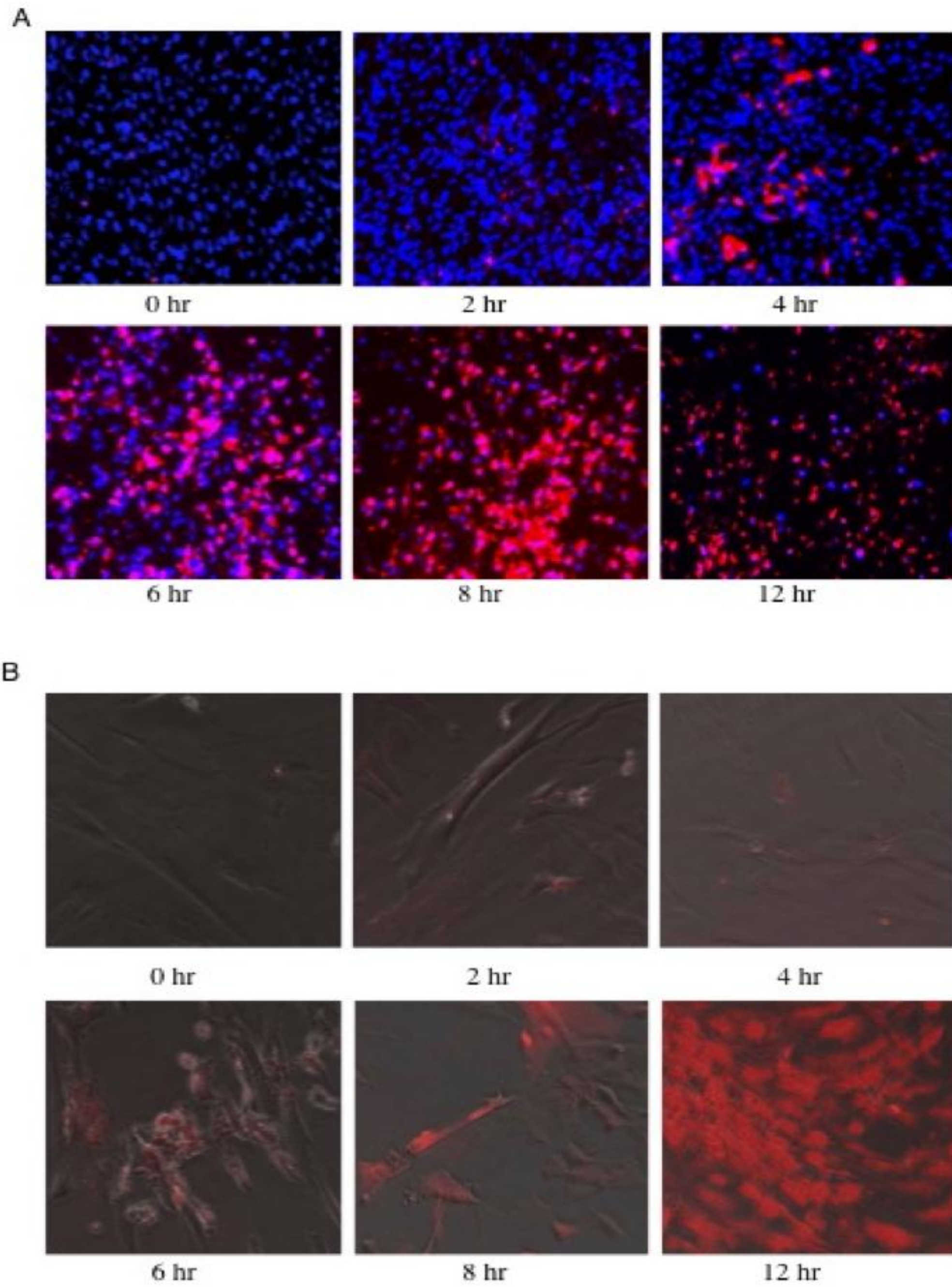
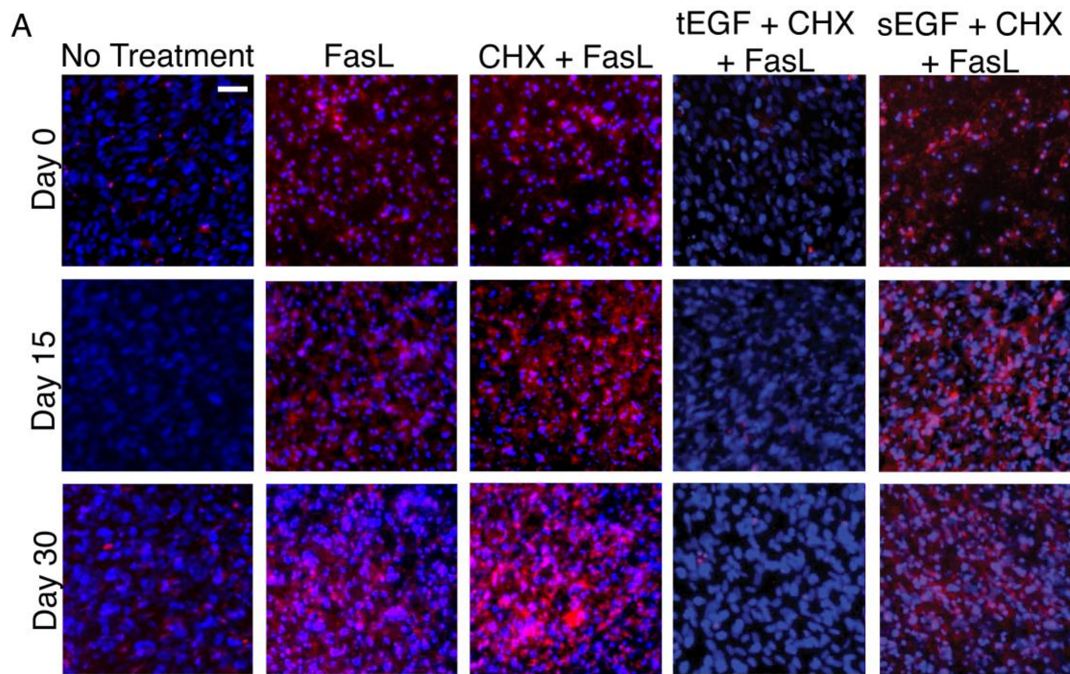


Figure 25 Time course of death in MSC in the presence of FasL. FLICA stained imhMSC (A) and prhMSC (B) after treatment with FasL for defined time-points. Shown is representative photomicrograph of cells of two independent experiments.

6.4.2 tEGF protects differentiating osteoblasts form FasL induced cell death

Although tEGF protected undifferentiated MSC against FasL induced cell death by restricting EGFR to the cell membrane and prolonging downstream EGFR signaling (Fan, Tamama et al. 2007), it was unknown whether the tEGF signal persisted to longer times scales required to protect differentiating MSC-derived osteoblasts from FasL-mediated death. Immortalized hMSC were either grown on mock or tEGF surfaces and were treated with FasL and/or other factors for 8 hours. Tethered EGF protected differentiating osteoblasts at Day 15 and differentiated osteoblasts on Day 30 in the presence of CHX and FasL (Figure 26A). Soluble EGF was unable to protect cells on mock surfaces from CHX and FasL induced apoptosis on Days 15 and Day 30 similar to what was seen on Day 0.

Caspase3 intensity measured in relation to total cell numbers showed continued cell death with FasL alone or CHX and FasL as differentiation into osteoblasts progressed. While sEGF was unable to protect against this death, tEGF significantly reduced FasL-induced apoptosis (Figure 26B). Tethered EGF therefore appears to be an appealing candidate for improving survival in both undifferentiated MSC and in differentiating osteoblasts.



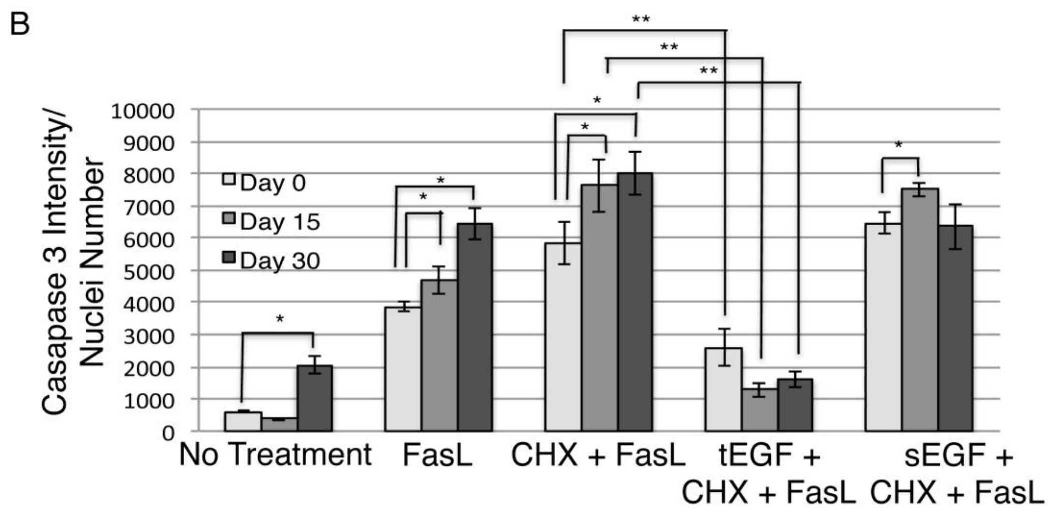


Figure 26 tEGF protects differentiating osteoblasts from FasL induced death. FLICA stained imhMSC (Magnification 10X, 50m) grown on mock/tEGF surfaces under osteogenic conditions, after 8 hours of treatment with cytokines on Day 0/ Day 15/ Day 30 (A). Quantification of FLICA intensity normalized to cell numbers (B). Shown are representative photomicrographs of cells and graphs of mean ± s.e.m of two independent experiments.

6.4.3 MSC susceptibility to FasL induced cell death decreases as cell differentiate into adipocytes

Although stimulation of EGFR with tEGF increased survival of differentiating osteoblasts and did not impair their differentiation, MSC can differentiate down multiple lineages. One of the main concerns for clinical use of MSC is the activation of one of the default pathways of adipocyte formation, hence, we investigated whether differentiating adipocytes also undergo enhanced cell death during the progress of differentiation in the presence of FasL in a manner similar to differentiating osteoblasts. We first established protocols for adipocytic differentiation. Confluent undifferentiated imhMSC and prhMSC placed in adipocyte differentiating media displayed a collection of tiny oil droplets seen by Oil Red O stain on Day 12, which coalesced to form larger droplets in adipocytes on Day 20, indicating white fat cell formation (Figure 27A). Similar progression of oil droplet deposition was seen in imhMSC or prhMSC cells differentiating on tissue culture plastic or mock surfaces (Figure 27A). Real time qPCR of adipogenesis markers peroxisome proliferator activated receptor □ (PPAR□) and alkaline lipid binding protein (ALBP) showed increased expression with the advancement of adipogenic differentiation in both imhMSC grown on mock surfaces (Figure 27C) or prhMSC grown on tissue culture plastic (Figure 24B). Adipogenesis was further confirmed in imhMSC grown on mock surfaces by immunoblot of fatty acid binding protein 4 (FABP4) that increased with progress of differentiation into adipocytes. The average nuclear size of differentiated adipocytes was significantly smaller than that of undifferentiated imhMSC indicating accumulation of fat droplets (Figure 24C).

With adipocytic differentiation protocols established, we next subjected differentiating imhMSC on tissue culture plastic to FasL cytokine treatment to test for susceptibility of these differentiating adipocytes to cell death. Cells on Days 0, 3, 6 and 12 of differentiation regimen were exposed to FasL

for 8 hours and cell death determined by an immunoblot for the caspase-3 substrate PARP. As the imhMSCs underwent differentiation into adipocytes, on Day 3, Day 6 and Day 12 there was lesser cleaved PARP in the presence of both FasL alone or CHX and FasL. Addition of sEGF to FasL or CHX and FasL did not alter the response of pre-adipocytes to FasL. The negative control of zVAD-FMK along with CHX and FasL showed no apoptosis. Treatment with only sEGF or CHX did not cause death of differentiating imhMSC. These results indicate that MSC differentiating into adipocytes become less susceptible to apoptotic death by FasL.

With prhMSC, we hypothesized that not all cells of the prhMSC population might react to differentiation cues or death signals in the same manner. When we tested cell death of differentiating prhMSC by the in situ FLICA assay, we observed that all undifferentiated cells (i.e. day 0) died after 12 hours of treatment, while on Day 15 only cells without oil droplets died, and on Day 30, when most cells had oil droplets, there was very little cell death, occurring only in undifferentiated stromal cells. The 12-hour incubation period for treatment with FasL was chosen based on a time response of prhMSC to FasL (Figure 25). To ensure that the resistance of adipocytes to FasL was not simply a short time window effect, we extended treatment with FasL from 12 hours to 24 hours and looked for apoptosis by FLICA. Even at 24 hours of treatment with FasL, cells with oil droplets did not undergo apoptosis (Figure 28). This confirmed that fat cells were resistant to inflammatory cytokines like FasL.

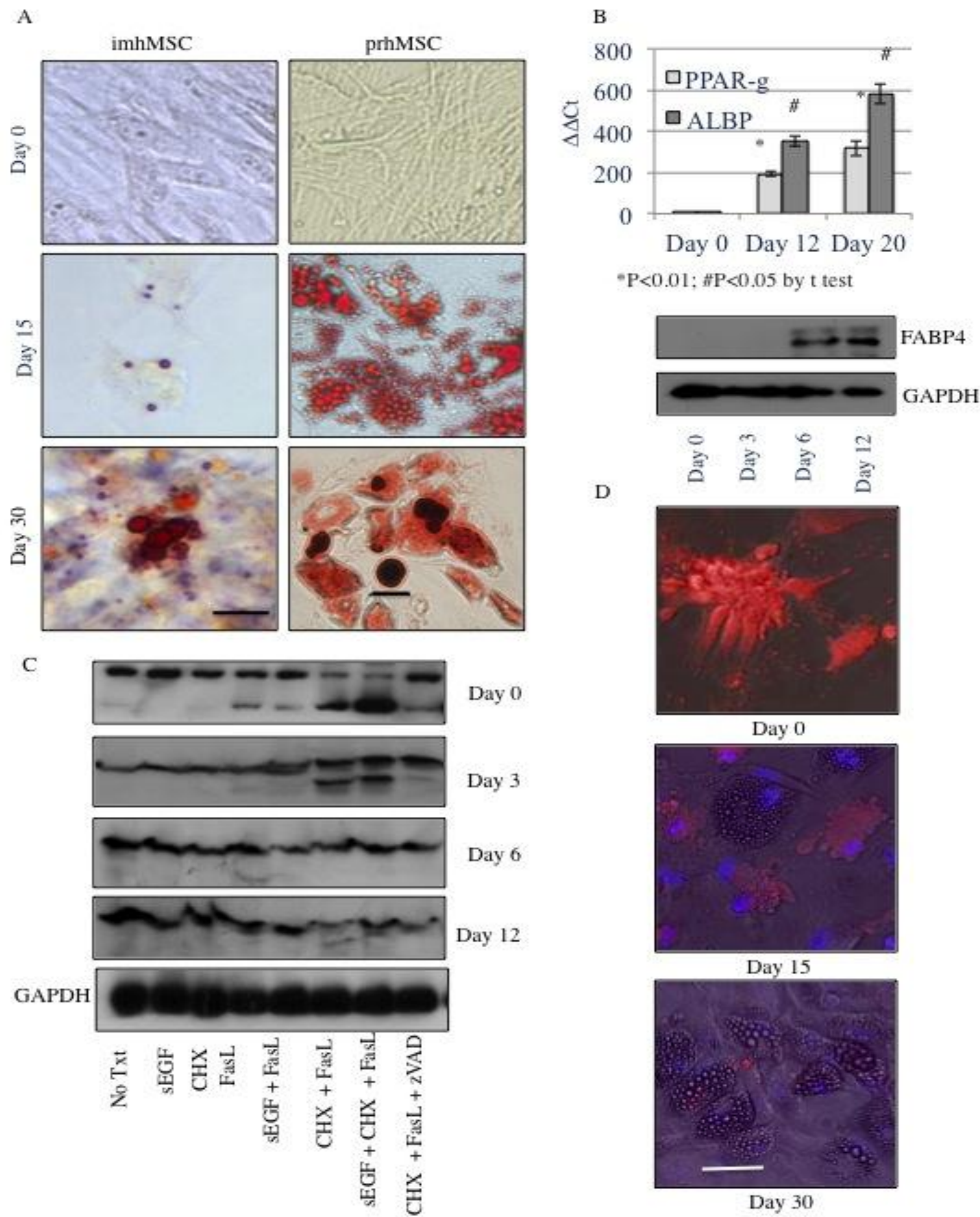


Figure 27 MSC forming adipose cells become resistant to inflammatory cytokines like FasL. Immortalized MSC (imhMSC) (Magnification 40X, 10m) on mock surface and isolated pluripotent (prhMSC) (Magnification 20X, 10m) on tissue culture plastic differentiating into adipose cells by Oil Red O (A). Change in expression of adipogenic markers by qPCR and immunoblot during adipogenic differentiation of imhMSC on mock surfaces (B). Immunoblot for full length and cleaved PARP in differentiating imhMSC grown on tissue culture plastic and treated with cytokines for 8 hours (C). Caspase 3 positive cells (red) and Hoescht 33342 (blue) stained overlaid with phase contrast images of prhMSC during progress of adipogenic differentiation (Magnification 10X, 50m). Shown are representative graphs of mean ± s.e.m and photomicrographs of cells, of two independent experiments.

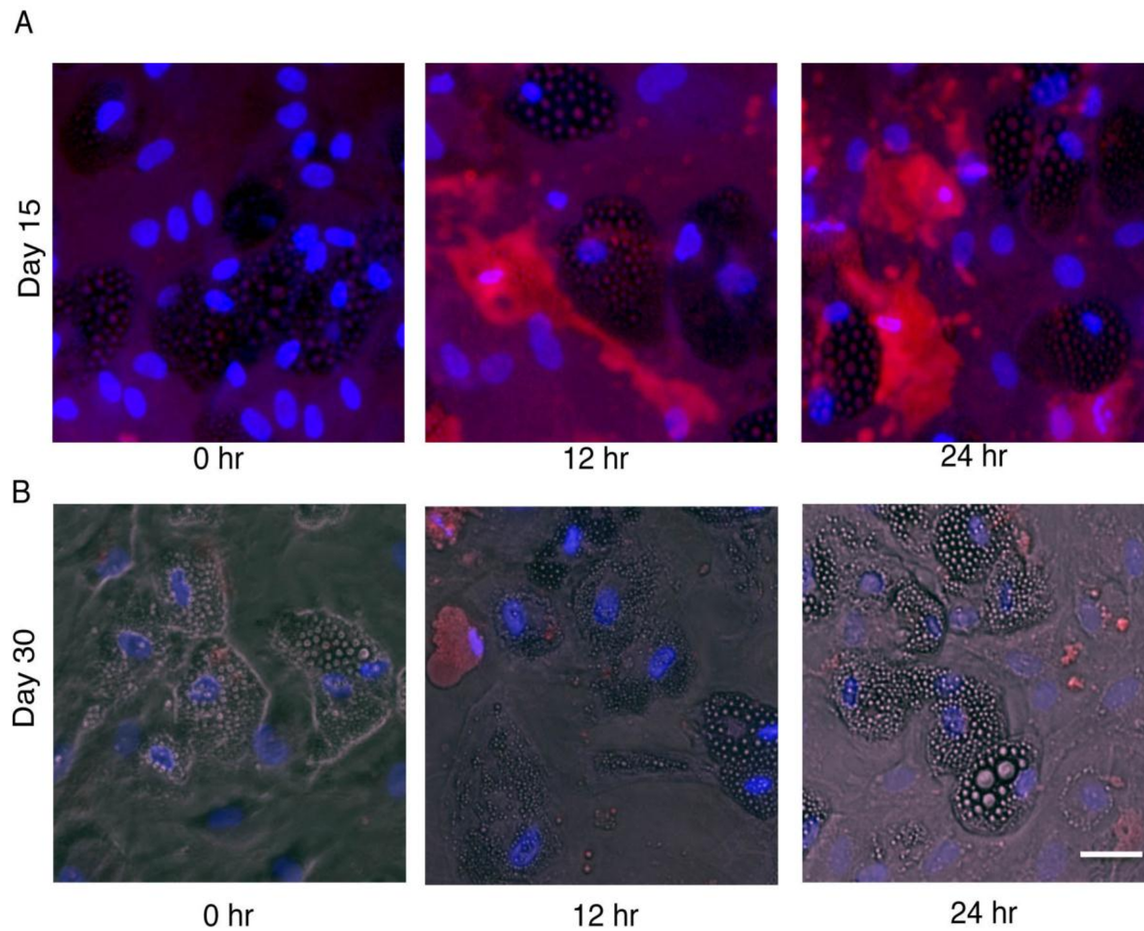
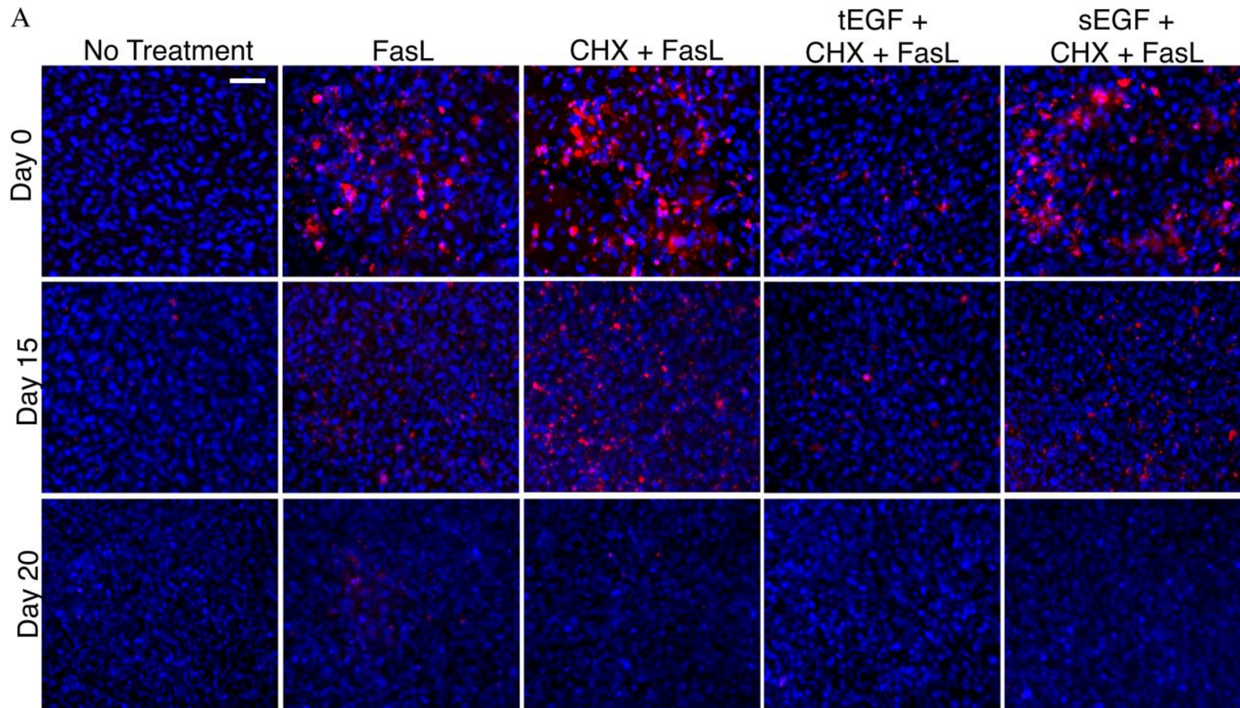
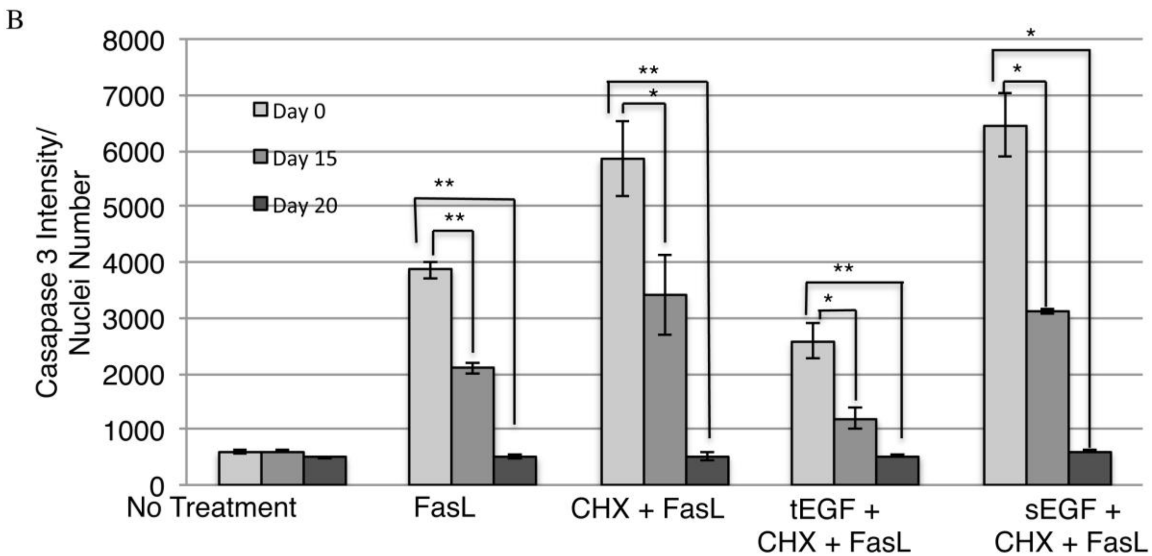


Figure 28 Time course of death of differentiating adipocytes in the presence of FasL FLICA and Hoescht 33342 images of prhMSC in adipogenic media on Day 15 (A) and Day 30, overlaid with phase contrast image to identify fat droplets (B) treated with FasL for 0, 8 and 24 hours. Shown is representative photomicrograph of cells of two independent experiments.

6.4.4 tEGF protects MSC while differentiating adipocytes become resistant to FasL

Since differentiating adipocytes are resistant to FasL we wanted to see if tEGF altered this acquired resistance to cell death in the presence of cytokines. Immortalized hMSC were differentiated on mock or tEGF surfaces and subjected to treatment with FasL for 8 hours. Caspase3 activity was analyzed as a measure of cell apoptosis by FLICA assay. Cells on Day 0 showed caspase3 activation with CHX and FasL which sEGF was unable to prevent. imhMSC grown on tEGF surfaces and treated with CHX and FasL on the other hand were protected from caspase3 activation. Differentiating adipocytes on Day 15 and Day 20 showed lesser caspase3 activation and apoptosis on surfaces similar to outcomes on tissue culture plastic surfaces. Cells on Days 15 and 20 grown on tEGF surfaces did not show a change in cell death in the presence of CHX and FasL (Figure 29A). Intensity of caspase3 fluorescence was normalized to cell numbers to account for the loss of cells nearing late apoptosis and these values across the various treatments represented in Figure 29B. We were able to conclude that MSC differentiating into adipocytes become resistant to death signals as they begin accumulating fat deposits and tEGF signaling from the biomaterial surfaces does not have any effect on this resistance to cell death.





*p<0.05, **p<0.01

Figure 29 tEGF does not alter resistance of adipose cells to death signals. Fluorescent caspase inhibitor (FLICA) stained imhMSC (Magnification 10X, 50m) grown on mock/tEGF surfaces under adipogenic conditions, after 8 hours of treatment with cytokines on Day 0/ Day 15/ Day 20 (A). Quantification of FLICA intensity normalized to cell numbers (B). Shown are representative photomicrographs of cells and graphs of mean ± s.e.m of two independent experiments

6.4.5 Changes in receptor expression during differentiation contributes to differences in susceptibility to death signals.

We initially looked at death in differentiating MSC based on the hypothesis that differentiating cells would react to cytokines and death factors differently from MSC. This is because during the progress of differentiation, MSC will undergo changes in various death factor receptors as well as pro- and anti-apoptotic proteins. Since we used FasL as a representative death cytokine, we looked at expression of its receptor Fas as MSC differentiated into fat and bone in vitro. Fas levels in imhMSC differentiating into adipocytes decreased on Days 0, 6 and 12 by immunoblot (Figure 30A) and real time qPCR (Figure 30B) as well as in adipocytes derived from prhMSC (Figure 24D). Conversely Fas levels in differentiating osteoblasts derived from imhMSC increased as seen by immunoblot (Figure 30C). Quantitative real time PCR showed increases in Fas in differentiating osteoblasts derived from imhMSC (Figure 30D) as well as prhMSC (Figure 24E). These results suggest that changes in death receptor levels alter the susceptibility of differentiating MSC and make adipocytes less susceptible and osteoblasts more vulnerable to the death cytokines FasL.

Since tEGF offered protection to not only MSC but also differentiating osteoblasts in the presence of FasL, we looked at changes in EGFR levels with the progress of MSC differentiation. imhMSC differentiating into adipocytes lost EGFR expression as seen by immunoblot (Figure 5E) and real time qPCR (Figure 30F) as expected (17). Similar loss in EGFR expression was seen in differentiating prhMSC (Figure 24D). The absence of EGFR expression might explain why treatment with tEGF does not affect either adipocyte death or survival in the presence of FasL. imhMSC differentiating into osteoblasts on the other hand show increasing expression of EGFR with advancement of differentiation by immunoblot (Figure 30G) as well as real time qPCR (Figure 30H), as seen in differentiating prhMSC (Figure 24E). Increased EGFR levels allowed for continued protection

offered by tEGF both to pre-osteoblasts and differentiated osteoblasts in the presence of FasL.

6.4.6 tEGF does not block induced osteogenic or adipogenic differentiation, and osteogenic differentiation is faster at 4% oxygen.

All the aforesaid experiments were done at ambient oxygen concentrations of 21%. However oxygen levels in the bone are comparatively lower at 4-7%. We wanted to confirm osteogenesis and adipogenesis of MSC on tEGF surfaces in the presence of differentiation inducers, under ambient oxygen levels as well as physiologic normal levels of 4% oxygen. Immortalized hMSC were grown on tEGF surfaces either in proliferation media alone or osteogenic media for 15 or 30 days, following which von Kossa staining was done on cells. There was osteogenic differentiation on tEGF surfaces in the presence of differentiation media at both 4% and 21% oxygen, however osteogenesis was achieved faster at 4% oxygen as observed by hydroxyapatite deposition on Day 15 (Figure 31A). tEGF by itself in the absence of differentiation inducers did not cause osteogenic differentiation of imhMSC.

Similarly tEGF did not inhibit adipogenic differentiation in the presence of adipogenic differentiation inducers and did not cause adipogenic differentiation in the absence of differentiation inducers as seen by Oil Red O staining (Figure 6B). There was no difference in the rate of fat droplet deposition at 21% oxygen or 4% oxygen.

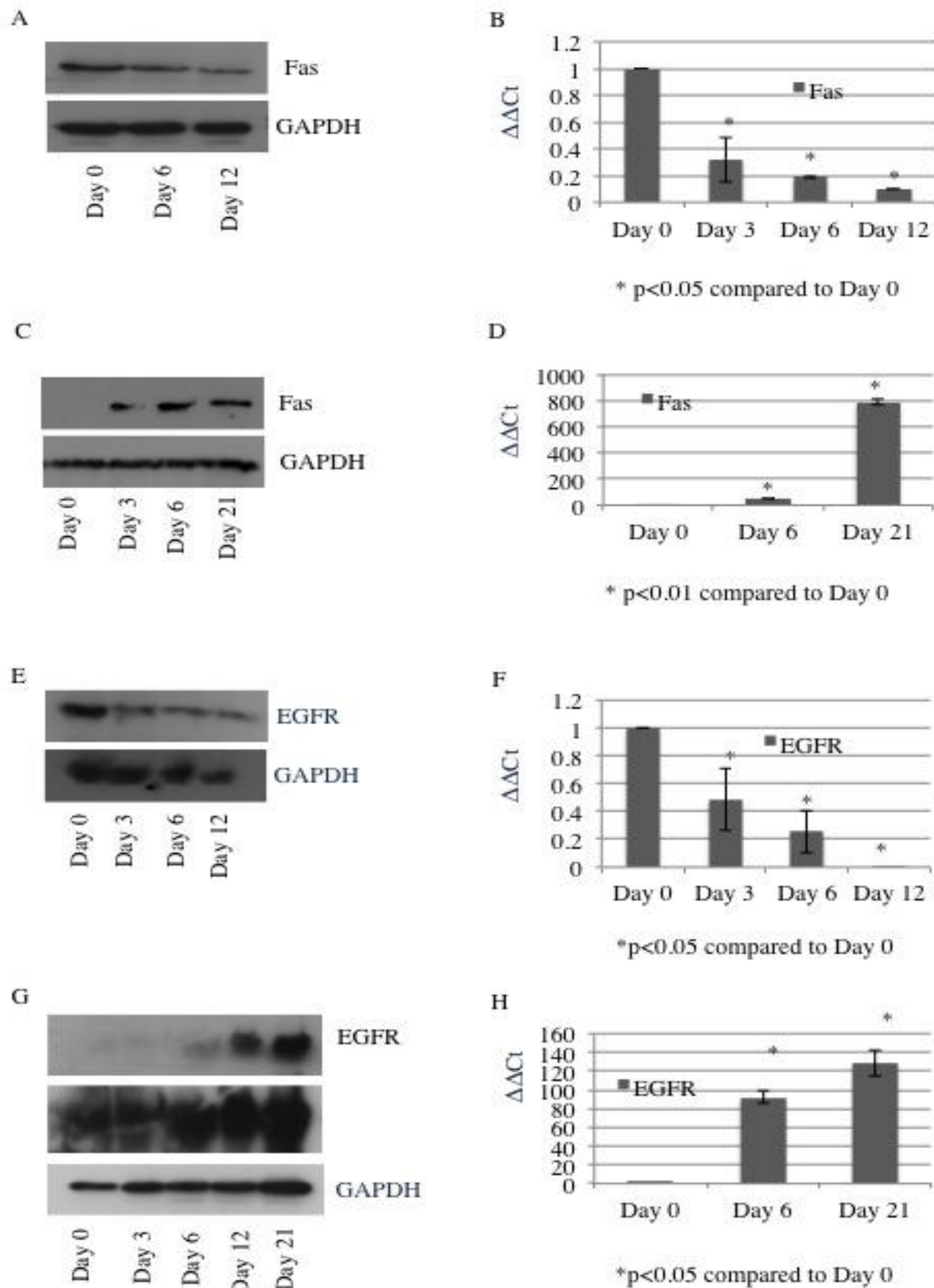


Figure 30 Changes in protein receptor levels during differentiation contribute to altered reactions to death signals. Change in levels of Fas in differentiating adipocytes grown on tissue culture plastic by immunoblot (A) and real time qPCR (B). Change in levels of Fas in differentiating osteoblasts grown on tissue culture plastic by immunoblot (C) and real time qPCR (D). Change in levels of EGFR in differentiating adipocytes grown on tissue culture plastic by immunoblot (E) and real time qPCR (F). Change in levels of EGFR in differentiating osteoblasts grown on tissue culture plastic by immunoblot (E) and real time qPCR (F). Representative immunoblots and graphs of mean ± s.e.m from two independent experiments.

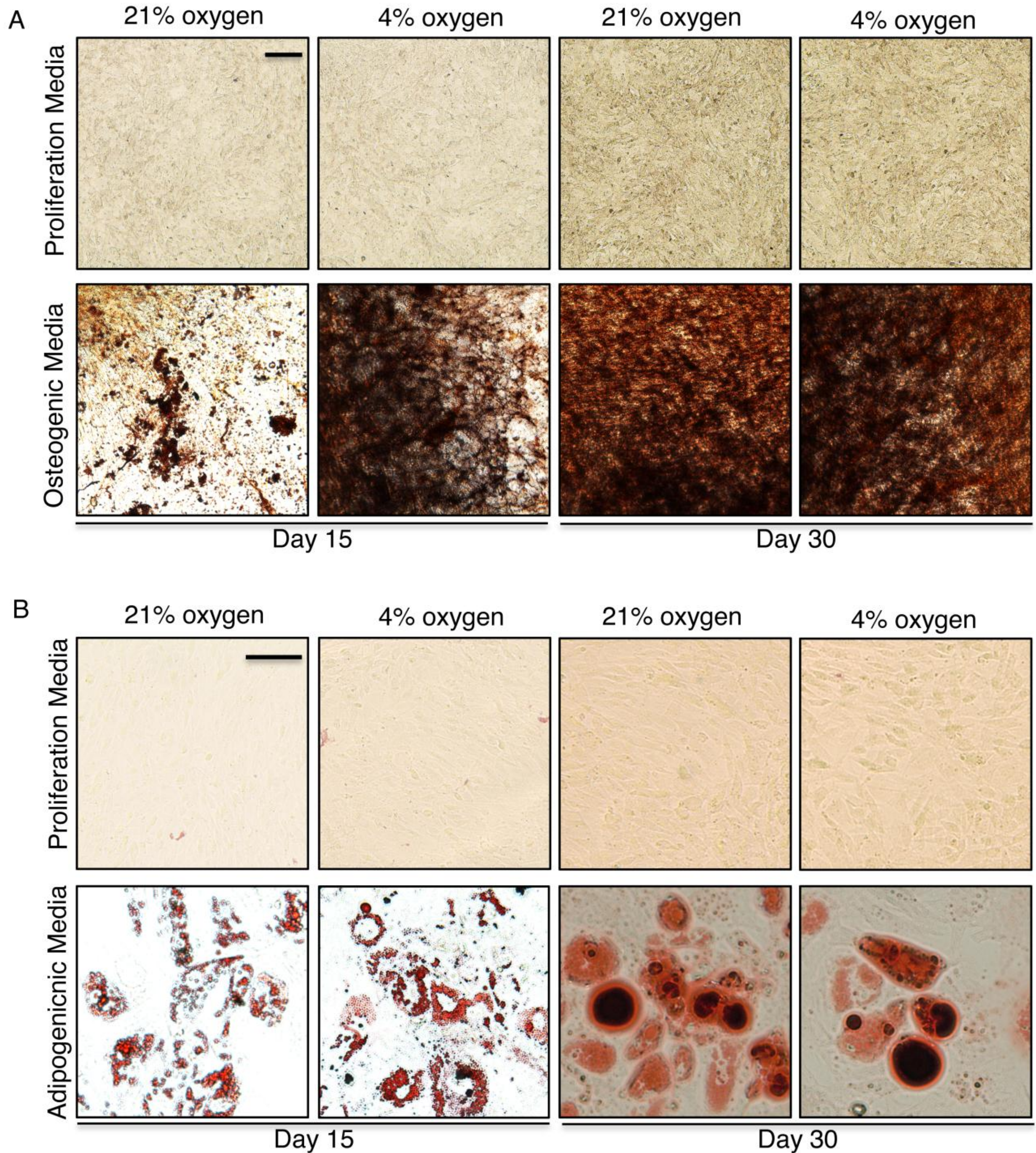


Figure 31 tEGf does not inhibit osteogenesis or adipogenesis in the presence of differentiation media; osteogenic differentiation is faster at 4% oxygen. Immortalized hMSC (Magnification 20X) on tEGF surface in the presence of proliferation or osteogenic media at 21% or 4% oxygen, stained for von Kossa (A). Immortalized hMSC (Magnification 40X) on tEGF surface in the presence of proliferation or adipogenic media at 21% or 4% oxygen, stained for Oil Red O (B)

6.4.7 tEGF protects undifferentiated MSC from combined threats of FasL, low oxygen and serum starvation

Although the Fas pathway has been shown by several groups to be the leading cause of MSC death via cytokine action, there are several other threats like low oxygen and nutrient deprivation that MSC have to encounter in ischemic wounds. To stimulate such conditions in vitro, imhMSC were treated with FasL in the absence or presence of serum under 21%, 4% and 1% oxygen. While 21% oxygen is symbolic of ambient lab conditions, 4% oxygen simulates physiologic normoxia in the bone and 1% oxygen is representative of physiologic hypoxia. We saw that 8 hours after treatment with FasL in the presence or absence of serum, caspase3 activation at 4% oxygen was lesser than caspase3 activation at 21% oxygen (Figure 7). imhMSC at 1% oxygen though were the most susceptible to FasL induced cell death both in the presence or absence of serum. tEGF was able to protect undifferentiated imhMSC from FasL and serum deprivation induced apoptosis at all three concentrations of oxygen.

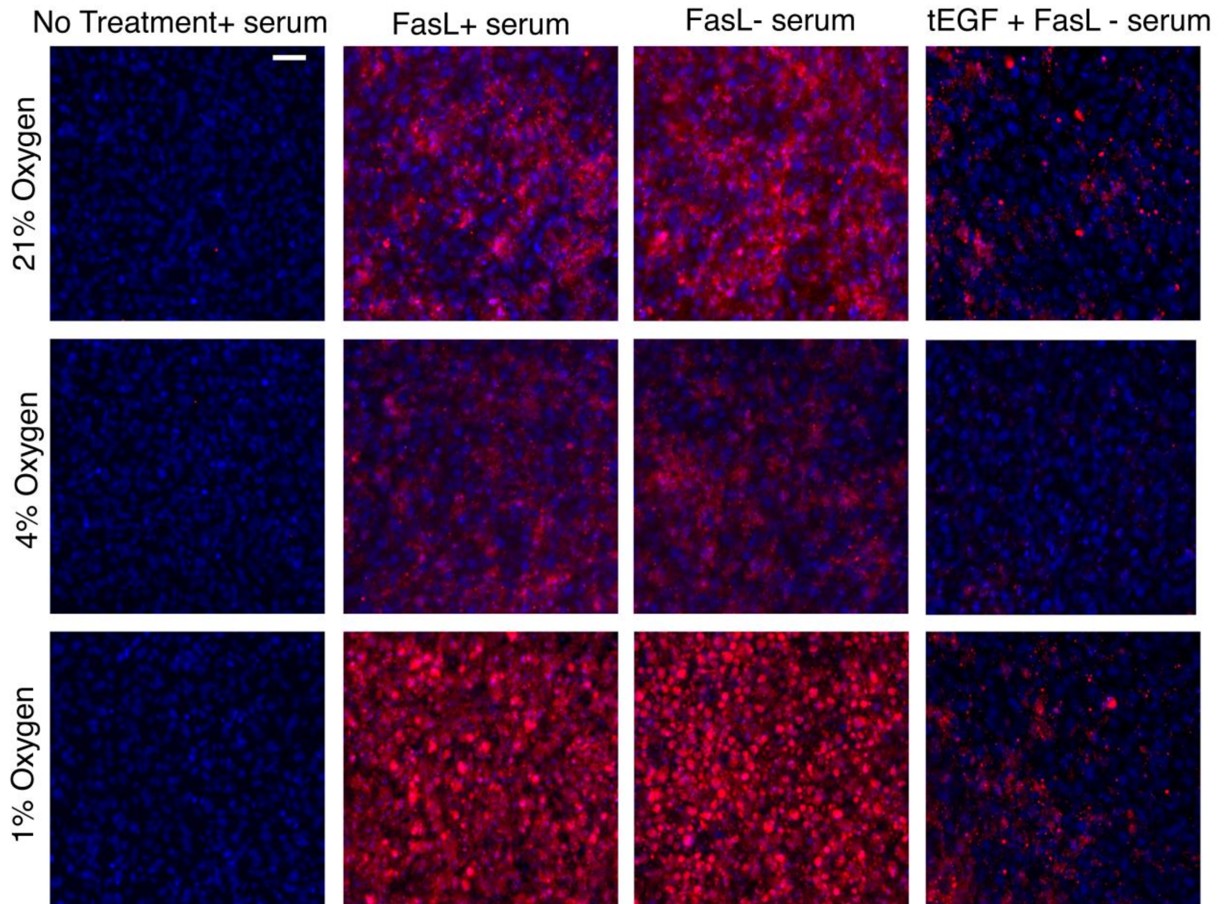


Figure 32 Undifferentiated MSC are more susceptible to FasL and serum deprived death at 1% oxygen which tEGF is able to ablate. Fluorescent caspase inhibitor (FLICA) stained undifferentiated imhMSC (Magnification 10X) grown on tissue culture plastic/tEGF surfaces after 24 hours with or without serum and/ or 8 hours of treatment with FasL.

6.5 DISCUSSION

Successful deployment of MSC in regeneration of tissues such as bone requires incorporation, expansion and finally differentiation of the implanted MSC into osteoblasts, followed by deposition of hydroxyapatite. However, in vivo trials in humans have achieved modest success (Patterson, Kumagai et al. 2008), perhaps due to death of transplanted MSC as observed in animal studies (Rodrigues, Griffith et al. 2010).

Our group has proposed using EGF, a growth factor known to promote proliferation, survival and motility in several cell types (Iwabu, Smith et al. 2004; Leloup, Shao et al. 2010; Shao, Wu et al. 2010) to aid in the course of MSC bone formation (Marcantonio, Boehm et al. 2009). EGF increases MSC proliferation while not affecting differentiation in the absence of differentiation cues (Tamama, Fan et al. 2006). This is advantageous since it would allow for sufficient ex vivo expansion of MSCs that are generally found in scarce numbers of one in a million bone marrow cells. It would also eliminate the use of serum for expansion ex vivo, eliminating variability and immunogenic reactions on implantation (Tamama, Kawasaki et al. 2010). However when we probed MSC survival, we found that MSC were most susceptible to FasL and TRAIL induced cell death (Fan, Tamama et al. 2007); these cytokines are prevalent at wound sites, and thus constitute a threat to MSC survival. EGF delivered in its physiological soluble form was unable to prevent MSC from undergoing death in the presence of these pro-death cytokines. We reasoned that survival signaling via sEGF may not be occurring since EGF would be driving proliferation that sensitizes cells to death signals, and over the longer term would lead to downregulation of EGFR levels limiting the efficacy of survivals signals.

Of interest, differential localization of activated EGFR in the cell can alter the strength of downstream signals to cause varied effects (Wells, Welsh et al. 1990; Haugh, Huang et al. 1999; Wells 1999). EGFR activated Erk when localized to the cell membrane triggers motility preferentially compared to EGFR activated Erk in the cytoplasm which activates cellular proliferation (Chen, Xie et al. 1996). Further, the Erk and AKT signaling elicited from membrane restricted EGFR are more tonic and consistent with survival signaling (Platt, Wilder et al. 2009).

We therefore changed the mode of delivery of EGF based on an earlier designed model system that maintains EGF in both its active conformation as well as a mobile state (Kuhl and Griffith-Cima 1996). The model also increases ligand availability by preventing ligand depletion, a system known to enhance downstream effects such as mitogenesis (Reddy, Wells et al. 1996). EGF was delivered on a PEO tether attached to the underlying polymer susbstrate in a manner that created local clusters of EGF presented to cells in the context of an adhesive surface. tEGF localized EGFR on MSC to the cell surface and promoted survival of undifferentiated MSC in the presence of FasL which sEGF was unable to accomplish (Fan, Tamama et al. 2007).

Addition of sEGF to MSC during the progress of osteogenic differentiation promotes bone formation (Kratchmarova, Blagoev et al. 2005). However cells grown on tEGF in the presence of osteoinductive factors further fastens this differentiation process seen by increased alkaline phosphatase activity at Day 7 and greater mineralization at Day 21 (Platt, Roman et al. 2009). This increased osteogenesis is attributed to increased ratios of phosphorylated EGFR to total EGFR. Tethered EGF caused restriction of phosphorylated EGFR to the cell membrane, where it actively signals, preventing it from being internalized and simultaneously prevented production of new EGFR (Platt, Roman et al. 2009).

The major question that remained in evaluation of our designed biomaterial was whether tEGF would help protect differentiating MSC in the presence of inflammatory cytokines. This is significant since differentiating MSC would not respond the same way to death factors as undifferentiated MSC, which tEGF is known to protect. Others and we have shown that undifferentiated MSC are susceptible to Fas induced cell death one of the major death pathways activated during first phase of inflammation (Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011; Liu, Wang et al. 2011). We have also shown that FasL not only increased MSC death by activating caspases, but promotes production of intracellular ROS in MSC, causing cell death (Rodrigues, Turner et al, 2012).

We addressed the death of MSC differentiating into osteoblasts since our ultimate goal is repairing critical sized bone wounds. We found that MSC differentiating into osteoblasts increased expression of Fas, the receptor for FasL and continued to die significantly like undifferentiated MSC, emphasizing the need for continued protection of MSC differentiating into bone cells. Use of tEGF surfaces as a substratum for growth of MSC differentiating into bone, showed continued protection to both pre-osteoblasts and osteoblasts derived from MSC similar to its protective effect in undifferentiated MSC. Continued protection offered by tEGF correlated with presence of EGFR expression in differentiating osteoblasts. Of note, the decrease in death by day 30 hold promise that fully differentiated osteocytes will not be readily killed by inflammatory cytokines after the supporting materials dissipates in the body. Thus, the tEGF appears to protect through the critical period of engraftment and osteogenic differentiation when the cells are most susceptible to the wound environment.

A major concern during repair is the accumulation of fat droplets in MSC and formation of adipose cells, one of the main default pathways, which may hinder healthy bone development.

When MSC were induced to form adipose cells and tested for death in the presence of FasL, we found MSC became more resistant to FasL induced death. This corresponded with decrease in Fas levels. Tethered EGF did not affect this reduction in cell death since levels of its receptor EGFR decreased with the progress of adipogenic differentiation.

6.6 CONCLUSIONS

We have a two-dimensional biomaterial that uses tethered EGF to restrict signaling of the EGFR receptor to the surface of MSC, altering the balance of signaling of EGFR to make MSC more resistant to inflammatory signals, both in undifferentiated MSC and differentiating MSC, and make the cells more committed to osteogenic differentiation. This biomaterial serves as a foundation for the development of a 3D bioengineered tEGF scaffold that will serve both as a carrier and a signaling device for MSC to aid in the regeneration of non-healing bone wounds.

7.0 THE MATRIKINE TENASCIN-C PROTECTS MULTIPOTENT STROMAL CELLS/MESENCHYMLA STEM CELLS FROM FASL INDUCED APOPTOSIS

Melanie Rodrigues, Linda Griffith, Alan Wells

7.1 ABSTRACT

Multipotential stromal cells / mesenchymal stem cells (MSCs) are ideal candidates for regenerative therapy due to the ability of these cells to differentiate as well as release trophic factors, suppress inflammation and positively influence neighbouring cells. However when faced with non-specific inflammation generated in the wound environment and in response to any implanted foreign body, MSCs are faced with cell death. We have previously shown that sustained and surface-restricted epidermal growth factor receptor (EGFR) signaling by a tethered form of its prototypal ligand epidermal growth factor (EGF) enhances survival of both undifferentiated as well as differentiating MSC; the effects of FasL and other death cytokines are blocked. Interestingly, during wound repair an extracellular matrix (ECM) component Tenascin-C (TNC) is upregulated, and each TNC contains fourteen EGF like (EGFL) repeats which bind as low affinity / high avidity ligands to the EGFR. We queried whether this type of signaling also protects MSC from FasL-induced death. MSCs were grown on TNC and Collagen I since TNC is antiadhesive and cells do not adhere to TNC by itself. MSC grown on TNC and Collagen I had a survival advantage in the presence of FasL. Soluble TNC added to MSC grown on Collagen I-coated surfaces however did not protect from FasL induced cell death. This survival was dependent on TNC activating EGFR and downstream pathways of Erk and Akt through its EGF like repeats; to a lesser extent the fibronectin-like repeats of TNC also contributed to survival. TNC neither sequesters nor neutralizes FasL, rather the effect is via cell signaling. Finally MSCs were found to deposit TNC in proliferative media in culture. Taken together these results suggest that providing MSC with a non-alien ECM moiety like TNC enhances their survival in the presence of death factors via signaling of EGFR and integrins. This matrix can ultimately be

used to supplement MSC delivery on scaffolds to provide a survival advantage against non-specific inflammation in vivo.

7.2 INTRODUCTION

MSCs display immense potential in vitro in differentiating into cells of various lineages, however when implanted in vivo, these cells show marked reduction in cell numbers by the very first week and are unable to survive long enough to differentiate and reform tissue. Although MSCs bring about positive effects such as production of growth factors favoring angiogenesis (Caplan and Correa 2011), increase of anti-apoptotic molecules and suppression of inflammation (Singer and Caplan 2011), it is thought that these positive effects occur in the short time-frame between implantation and death of MSC (Rodrigues, Griffith et al. 2012). The loss in implanted MSC numbers have been seen across tissues, be it injection into the inflammatory bowel, (Semont, Mouiseddine et al. 2010) and infarcted hearts, (van der Bogt, Schrepfer et al. 2009; Noort, Feye et al. 2010) or implantation into injured bone (Zimmermann, Gierloff et al. 2011) and the ischemic kidney (Mias, Trouche et al. 2008). The disappearance of MSC is closely associated with threats like increased reactive oxygen species (Song, Cha et al. 2010), hypoxia (Zhu, Chen et al. 2006) and nutrient deprivation (Deschepper, Oudina et al. 2010) in the wound environment along with non-specific inflammation generated in response to implantation of any foreign material into the body.

The effects of non-specific inflammation on loss of MSC numbers have been studied extensively. While factors like TNF-α (Liu, Wang et al. 2011), INF-γ (Liu, Wang et al. 2011) and TRAIL (Fan, Tamama et al. 2007) have been reported to kill MSC both in vitro and in vivo, the most potent death initiator in MSC is the Fas-FasL pathway (Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011; Liu, Wang et al. 2011) (Rodrigues, Turner et al. 2012),

activated by both natural killer cells (Yamaza, Miura et al. 2008) and T-lymphocytes (Crop, Korevaar et al. 2011; Liu, Wang et al. 2011). To counter this cell death and provide MSC with a survival advantage our group initially used a tethered form of EGF (tEGF), a growth factor found to increase MSC cell numbers but not induce MSC differentiation in the absence of differentiation inducers (Tamama, Fan et al. 2006), to improve MSC survival in the presence of FasL and TRAIL (Fan, Tamama et al. 2007).

TNC is a 6-armed extracellular glycoprotein with the accessible rod like arms of the protein containing EGFL repeats (Engel 1989). Each EGFL repeat is 2nm long, approximately the same length as that between the N and C termini of EGF (Engel 1989) and can bind and signal EGFR (Swindle, Tran et al. 2001). The EGFL repeats of TNC however bind EGFR with a low affinity, three orders of magnitude lower than that of EGF, which is insufficient to cause EGFR internalization and degradation, leading to a cell-surface restricted EGFR signaling (Iyer, Tran et al. 2007). While the expression of TNC in the adult body is spatially and temporally restricted to tissues such as the tendon, smooth muscle and ligaments, it is transiently upregulated during injury and downregulated after wound healing (Midwood and Orend 2009).

Since TNC is a matrix protein abundant during wound healing and since its EGFL repeats can bind and signal EGFR from the cell surface similar to tethered ligands like tEGF, we hypothesized that TNC should be able to promote survival in MSC under influences of threat such as FasL. Moreover it has previously been hypothesized that de-adhesive matrix proteins like TNC by creating an intermediate state of cellular adhesiveness and causing motility promote survival in the presence of death signals (Murphy-Ullrich 2001). However there have been no subsequent studies testing this hypothesis and the survival effects of Tenascin-C in any cell type.

7.3 MATERIALS AND METHODS

7.3.1 Materials

Human Tenascin-C purified protein (CC065) and FLICA (APT503) was obtained from EMD-Millipore (Billerica MA), rat tail collagen I (354236) was from BD Biosciences (San Jose, CA), human recombinant Super FasL (ALX-522-020- 3005) was from Enzo life sceinces (Farmingdale NY).

7.3.2 Cell Culture

Primary human bone marrow derived multipotential stromal cells (prhMSC) were obtained from Dr. Darwin Prockop's lab (Texas A&M) and maintained in an undifferentiated/proliferative state in α-MEM without ribonucleotides or deoxyribonucleotides supplemented with 16.5% FBS (Atlanta Biologicals, Lawrenceville, GA), 100units/ml Pencillin/Streptomycin and 2mM L-glutamine. Human bone marrow derived multipotential stromal cells immortalized by hTERT (imhMSC) were obtained from Junya Toguchida's lab (Kyoto University, Japan). For differentiation of cells into osetoblasts, proliferation media was supplanted with 50μM ascorbic acid, 100 μM glycerol-2-phosphate and 100nM dexamethasone. For differentiation into adipocytes, adipogenic differentiation media from Lonza was used which contained insulin, indomethacin and 3-isobutyl 1-methlxanthine as differentiation supplements. Day 0 represents MSC that have established complete cell-cell contact and are in proliferation media. Day 15 and Day 30 represent 15 days in osetogenic/adipogenic media, as mentioned.

7.3.3 Inverted Centrifugation

To assay for cell adhesion on ECM coated tissue culture plastic, 6 well plates were coated with 1µg/cm^2 Col I, or 1µg/cm^2 Col I and 1µg/cm^2 TNC diluted in PBS for 24 hours, following which 500,000 cells were plated per 6 well dish and allowed to attach and spread for 2 hours. One set of cells was washed, not centrifuged and number of cells counted. The other set of cells underwent inverted centrifugation. In brief, cells in the 6 well plate were washed, plates completely filled with media eliminating air bubbles by inverting plates into a bucket containing media. The plates were sealed in a box designed to fit in the rotor of a centrifuge and centrifuged in inverted position at 1750rpm for 5 minutes. Post centrifugation, media was aspirated and cells remaining on the surface were counted. The ratios of cells remaining on surfaces in centrifuged plates to those in plates that were not centrifuged were calculated.

7.3.4 Detection of Apoptosis

FLICA is a cell permeable and non-cytotoxic inhibitor of caspase-3 bound to sulphorhodamine, which emits red fluorescence when bound to active caspase-3. Lab-tek 8 chamber slides were coated for 24 hours with 1µg/cm^2 Col I, or 1µg/cm^2 Col I and 1µg/cm^2 TNC diluted in PBS, or left uncoated. MSC were grown on these surfaces for 24 hours, followed by treatment with 100ng/ml FasL or 100ng/ml FasL with 20µM cycloheximide (CHX) for 8 hours. At 7 hours post treatment FLICA reagent and Hoescht 33342 dye was added to cells in media and incubated for 1 hour at 37□C. Media were aspirated, cells washed and live cells imaged for caspase 3 fluorescence.

In situ cell death detection kit (fluorescein) (11 684 795 001) from Roche was used for TUNEL assay. After 8 hours of treatment with 100ng/ml FasL or 100ng/ml FasL with 20µM cycloheximide (CHX) for 8 hours, cells were fixed in 4% paraformaldehyde and treated with TUNEL reagent for 1 hour, followed with staining of nuclei with DAPI. Cells were imaged for fluorescein fluorescence.

7.3.5 Immunofluorescence

For immunofluorescence studies, 8 chamber permanox slides were coated with 1µg/cm^2 Col I, or 1µg/cm^2 Col I and 1µg/cm^2 TNC diluted in PBS, or left uncoated for 24 hours prior to seeding of cells. 24 hours after cells were grown in complete proliferation media, media was changed to proliferation media containing 0.5% dialyzed FBS. For the positive control cells were treated with EGF for 15 minutes. All samples were fixed in 4% PFA, followed by blocking in 1%BSA in 0.3% Triton X 100 solution in PBS. Cells were incubated with primary antibody for phosphorylated EGFR sc-23420 from Santa Cruz Biotechnology (Santa Cruz, CA) overnight at 4°C at a dilution of 1:100. Following washes the next morning, cells were placed in secondary Alexa 594 antibody at a dilution of 1:500 for 1 hour, counterstained with DAPI and imaged.

7.4 RESULTS

7.4.1 TNC in combination with Col I provide an adhesive surface for MSC while not causing MSC differentiation

TNC reduces the number of focal adhesions on cells and decreases cell adhesiveness in several cell types (Murphy-Ullrich, Lightner et al. 1991). To test for MSC adhesiveness on TNC, an inverted centrifugation assay was performed 2 hours post-seeding MSC on tissue culture plastic and the number of cells attached after centrifugation was compared to number of cells attached on a similarly coated surface that had not undergone centrifugation (Figure 31 A). As expected MSC grown on TNC detached from surfaces after inverted centrifugation. To increase adhesiveness of cells on TNC we coated the tissue culture surface with a 1:1 mixture of TNC and Col I, a matrix that promotes MSC attachment and spread. Cells on TNC were only half as adhesive as cells on Col I (Figure 31 A, Figure 31 B). Growing cells on a mixed matrix comprising Col I and TNC was able to significantly increase MSC adhesiveness. For all further studies, a combination of Col I and TNC was used.

Ideally we would expect a factor promoting cell survival in MSC to not cause MSC differentiation. This would allow for MSC expansion post-incorporation into tissue, promoting sufficient numbers of cells to form to regenerate the wounded tissue (Tamama, Kawasaki et al. 2010). We wanted to test if Tenascin-C by itself, in the absence of specific differentiation media promotes MSC differentiation. Since our group focuses on regenerating bone from MSC with minimal amounts of fat, we looked for osteoblast and adipose cell formation in the presence of TNC. von Kossa staining of MSC grown for 30 days in proliferation media (Figure 31 C) showed that TNC and Col I, nor Col I alone caused hydroxyapatite deposition. However in the

presence of differentiation media, TNC did not inhibit osteogenic differentiation (Figure 31 C). Similarly TNC did not promote adipocyte formation on its own neither did it prevent adipocyte formation in the presence of adipogenic inducers (Figure 31 D). The matrix mix of TNC and Col I thus supported MSC attachment and growth and did not cause MSC to differentiate into any particular lineage.

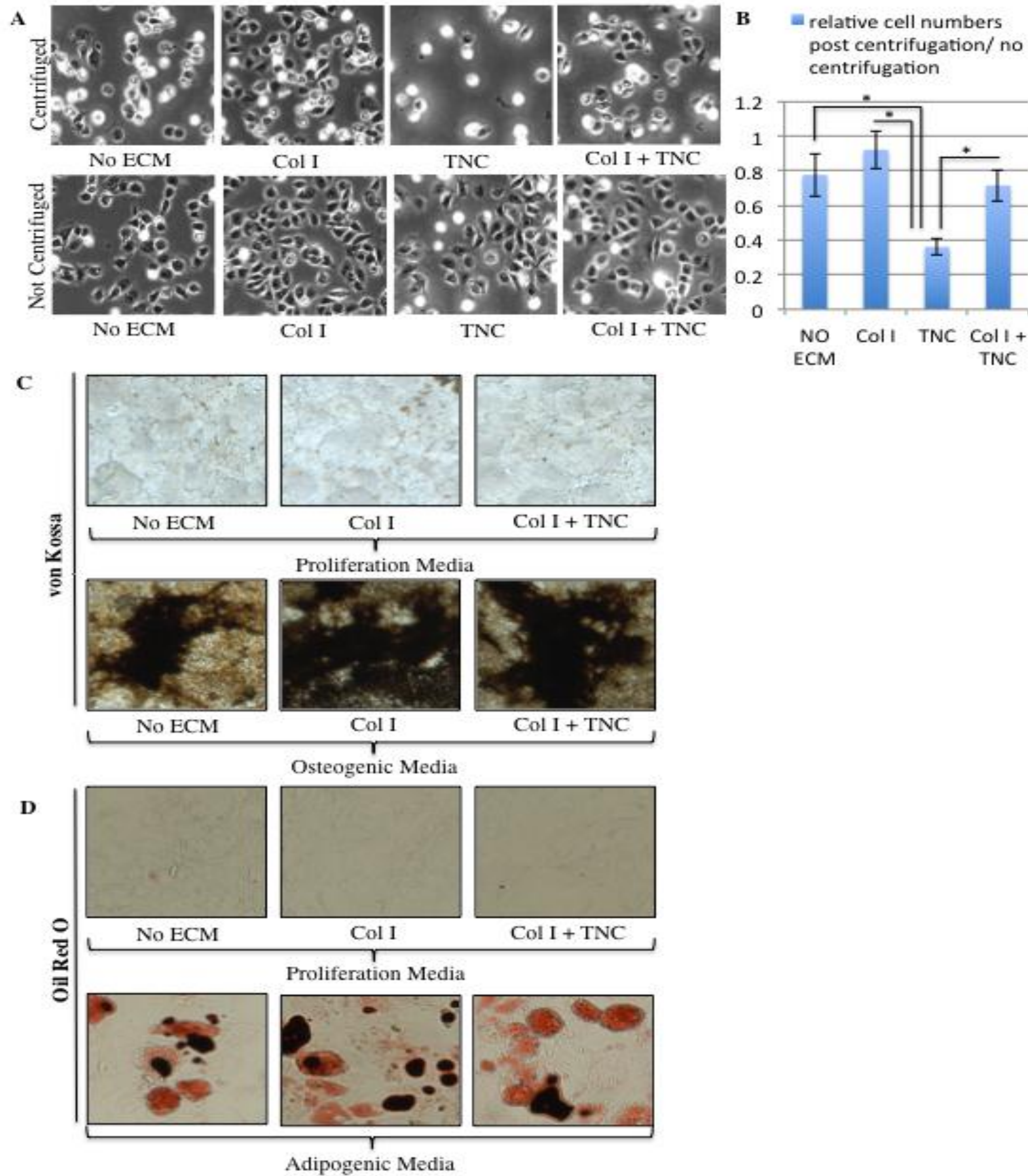


Figure 33 TNC and Col I aids MSC attachment and does not cause MSC differentiation. (A) Representative phase contrast images of imhMSC on indicated surfaces after or without inverted centrigation. (B) Ratio of imhMSC numbers after inverted centrifugation to no centrifugation on indicated surfaces. *$p<0.05$ by Student's t-test. (C) von Kossa staining of prhMSC at Day 30 grown on various surfaces in the presence of proliferation or osteogenic differentiation media. (D) Oil Red O staining of prhMSC at Day 30 grown on various surfaces in the presence of proliferation or adipogenic differentiation media.

7.4.2 TNC protects MSC from FasL induced cell death

Several groups including ours have previously shown that MSC are most susceptible to cell death via the Fas death pathway (Fan, Tamama et al. 2007; Yamaza, Miura et al. 2008; Gotherstrom, Lundqvist et al. 2011; Liu, Wang et al. 2011) (Rodrigues, Turner et al. 2012). We induced cell death in imhMSC (data not shown) and prhMSC and found intense capsase3 activation by FLICA after 8 hours of treatment. Cells were also treated with a low concentration of the protein synthesis inhibitor CHX in addition to FasL to increase stress on cells, imitating cell stress seen in vivo. Cells grown on tissue culture plastic with no matrix displayed caspase3 activation after treatment with FasL or CHX and FasL. Cells grown on Col I and TNC displayed a survival advantage and substantially reduced cell death in the presence of FasL or CHX and FasL (Figure 32 A). We attrtibuted the protective effect to TNC in the matrix since MSC grown on Col I surfaces did not show protection to MSC in the presence of FasL or CHX and FasL.

To confirm that TNC had a protective effect on MSC, we allowed for a longer 12 hour treatment of MSC with FasL and looked for DNA breaks using TUNEL. MSC on plastic surfaces that were uncoated and MSC grown on plastic surfaces coated with Col I showed fluorescein TUNEL positive stain for DNA breaks. MSC grown on TNC and Col I surfaces however did not show DNA breaks at 12 hours post treatment with FasL (Figure 32 B). These results support our hypothesis that TNC plays a role in increasing cell survival in MSC in the presence of threats like FasL.

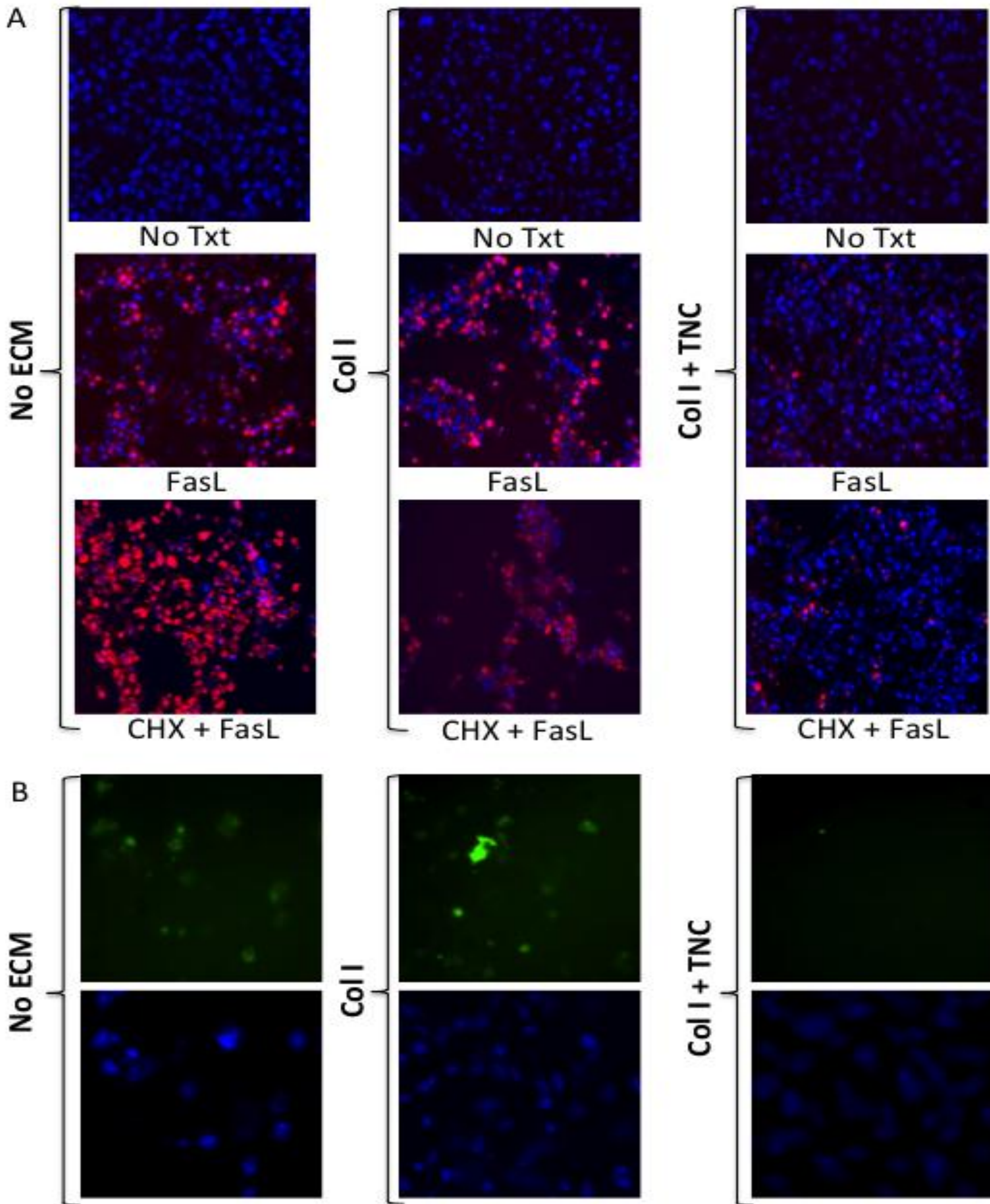


Figure 34 TNC is protective to MSC in the face of FasL induced cell death. (A) Representative images of FLICA and Hoescht stained prhMSC grown on various surfaces and treated with FasL or CHX and FasL for 8 hours. (B) Representative images of TUNEL and DAPI stained prhMSC grown on various surfaces and treated with FasL or CHX and FasL for 12 hours.

7.4.3 TNC activates EGFR on MSC and protects MSC via EGFR signaling

On finding that TNC does have a survival effect on MSC in the presence of FasL, we wanted to test if Tenascin-C was able to activate EGFR and signal for survival via EGFR in MSC. imhMSC were plated on surfaces with Col I alone or Col I and TNC for 24 hours. Immunofluorescence for phospho-EGFR was done on both these samples. imhMSC on Col I and TNC showed phospho-EGFR while cells on Col I alone did not show phospho-EGFR staining (Figure 33 A) . As a positive control, imhMSC seeded on Col I were treated with EGF for 15 minutes prior to fixation, to induce phosphorylation of EGFR. As expected, this treatment stained positive for phospho-EGFR. The intensity of phospho-EGFR on TNC and Col I surfaces however was much lower than phospho-EGFR expression on MSC coated on Col I alone and treated with EGF, indicating lower levels of activation of EGFR by TNC compared to EGF.

We next wanted to test if blocking activation of EGFR in MSC grown on Col I and TNC changes the protective effect that TNC renders to MSC in the presence of FasL. prhMSC were grown on Col I and TNC coated surfaces, and treated with inhibtors of EGFR activation PD153035 or Erk activation PD98059 or Akt activation LY294002 30 minutes prior to addition of FasL for 8 hours. The inhibitors were present during the entire 8 hours of FasL treatment, after which a FLICA assay was done. Blocking of either EGFR or its downstream pathways of Akt and Erk blocked the survival advantage that TNC and Col I provides to MSC in the presence of FasL (Figure 33 B). The controls of only inhibitor treatment PD153035, or PD98059 or LY294002 on MSC grown on Col I and TNC, did not have a toxic effect on cells. As seen earlier MSC grown on Col I and TNC and treated with FasL, or CHX and FasL survived. These results indicate that activation of EGFR and its downstream pathways of Erk and Akt by TNC are necessary for promoting survival in MSC.

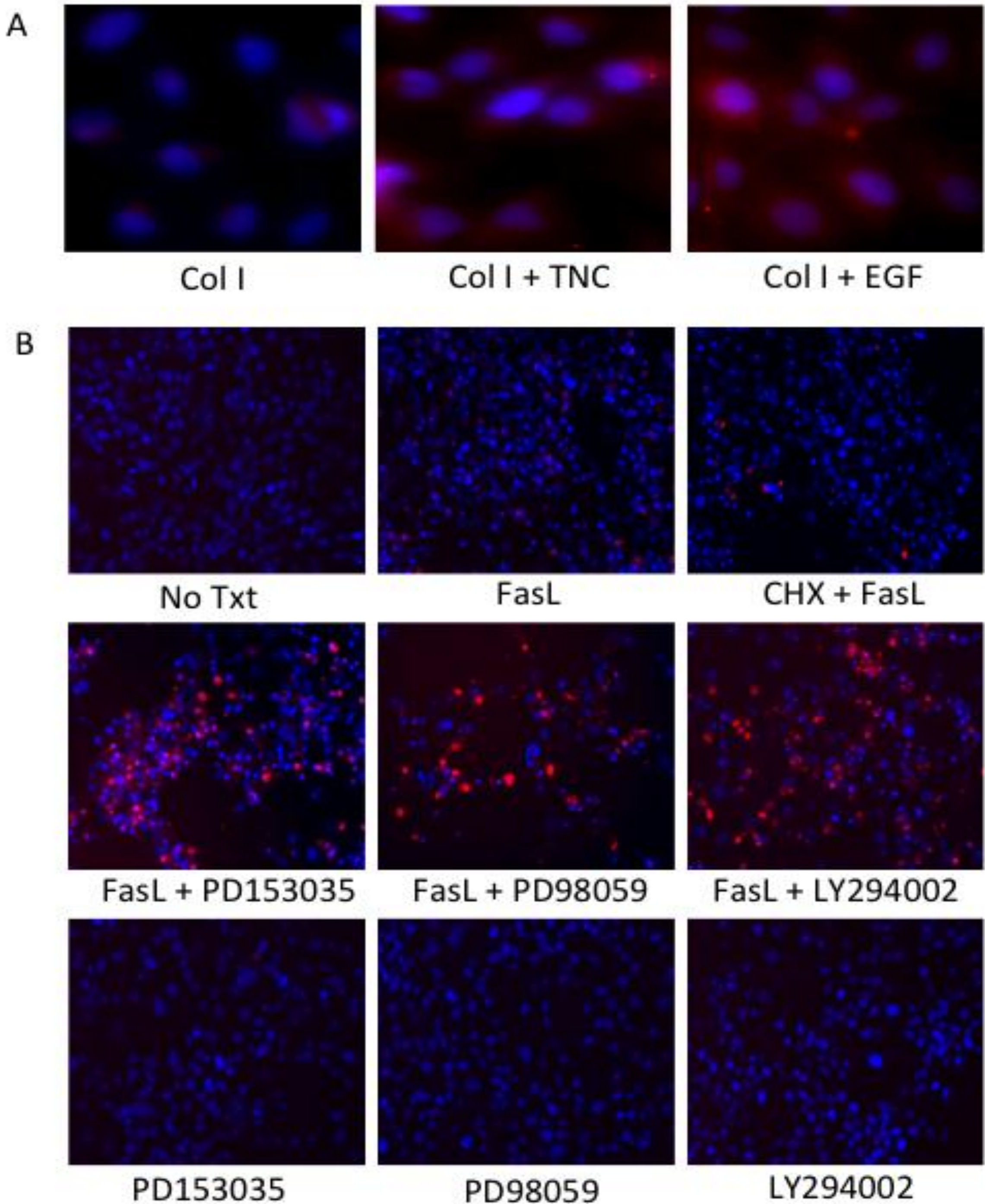


Figure 35 TNC activates EGFR on MSC and EGFR activation is essential for survival effects of TNC. (A) Immunofluorescence for phosphor EGFR on imhMSC grown on indicated surfaces and in the presence of indicated treatments (B) FLICA Assay for active caspase 3 (red fluorescence) in prhMSC grown on Col I and TNC surfaces and undergoing indicated treatment for 8 hours.

7.4.4 The FNL repeats of TNC are required but not sufficient to signal for survival in MSC

Since TNC contains both EGFL like repeats, which can bind and activate EGFR as well as FNL repeats which can bind and signal integrins, we hypothesized that the survival effects of TNC may not be through EGFL alone. To test this, post preparation of coated surfaces and prior to seeding of MSC, neutralizing antibodies to EGFL and FNL were added for 24 hours. imhMSC were then seeded for 24 hours, kept untreated or treated with FasL for 8 hours as done in previous experiments, followed by a FLICA assay to test for caspase3 activation (Figure 34). Presence of neutralizing antibodies for FNL and EGFL was confirmed prior to seeding of cells. Blocking of FNL caused caspase3 activation but to a much lesser extent compared to blocking EGFL (Figure 34). MSC seeded on Col I alone with no neutralizing antibody and treated with FasL showed caspase 3 activation as shown earlier. Addition of neutralizing antibodies did not have a toxic effect on MSC. This was confirmed by keeping MSC on Col I and TNC coated surfaces with neutralizing antibody, untreated, and testing for caspase3 activation. These results indicated that although both EGFL and FNL repeats of TNC are involved in survival signaling in MSC, the dominant survival effect is via EGFL.

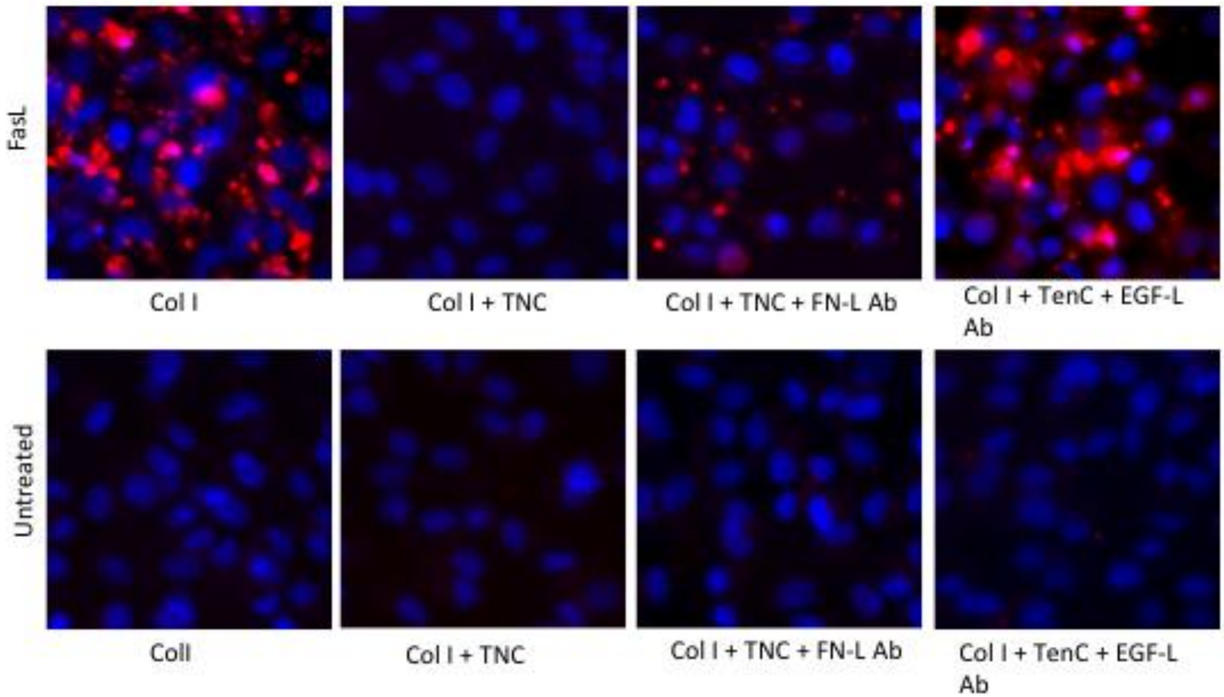


Figure 36 Protection of MSC by TNC is primarily via EGFL. FLICA staining of imhMSC grown with or without indicated ECM surfaces and neutralizing antibodies to EGF-L and FN-L and left untreated or treated with FasL for 8 hours.

7.4.5 TNC does not sequester or neutralize FasL

We next hypothesized that TNC might contribute to survival in MSC not solely through signaling but also by sequestering of FasL and prevention of FasL from binding to its receptor Fas on MSC. To test this we grew MSC inside two cloning cylinders placed in a well of a 6-well dish, enclosing areas without Col I and TNC or Col I and TNC on which imhMSC were grown. One well had Col I and TNC coating surrounding the cloning cylinders, covered with media and FasL, the second well had only media with FasL surrounding the cloning cylinders, with no TNC (Figure 35 A). When the cloning cylinders were taken out and media came in contact with cells within the cloning cylinders, there was comparable cell-death seen in cells not coated with Col I and TNC in both wells (Figure 35 B). Cells coated on TNC did not show caspase3 activation as seen earlier and expected. This indicated that TNC was neither sequestering nor neutralizing FasL and that the protective effects from TNC was purely based on signaling via EGFL and FNL.

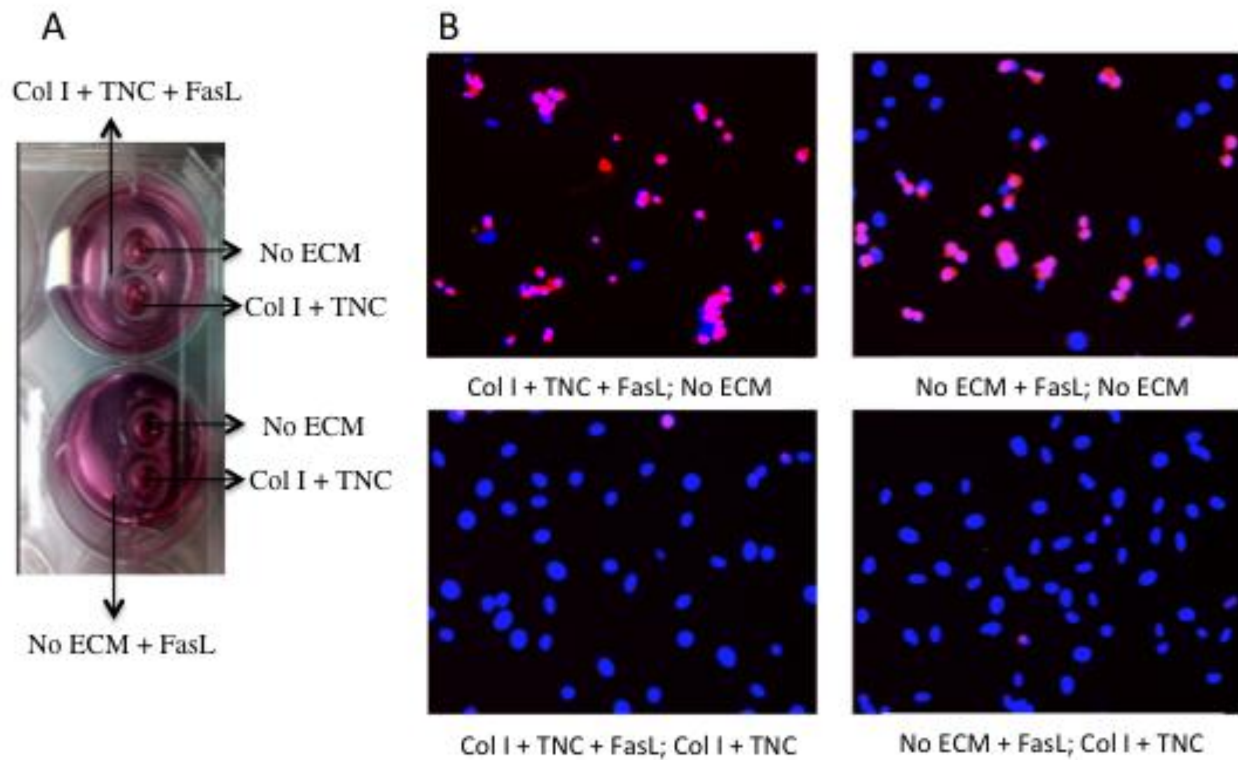


Figure 37 TNC does sequester and neutralize FasL. (A) Image denoting ECM coated surfaces within and outside cloning cylinders. All cloning cylinders have imhMSC growing within. (B) FLICA assay 6 hours after removing cloning cylinders. Treatment outside cloning cylinder are labeled before the semi-colon, treatment within the cloning cylinder are labeled after the semi-colon.

7.5 DISCUSSION

Duration of signaling, sub cellular localization, and intensity of activation are all factors that determine the outcome of growth factor receptor signaling. EGFR binds to various ligands: soluble ligands like EGF and TGFα, bound ligands sequestered in ECM like heparin binding EGF (HB-EGF) or ECM based ligands like TNC, decorin and laminin bringing about diverse effects. Firstly sustained EGFR and Erk activation has been shown to be critical in bringing about wound healing and/or cell survival in several cell types (He, Huang et al. 2004; Bardeesy, Kim et al. 2005; Block, Tolino et al. 2010), including MSC (Fan, Tamama et al. 2007). For example, the matrix bound HB-EGF and not EGF can activate epithelial wound healing due to more sustained activation of EGFR (Tolino, Block et al. 2011). Secondly, differential localization of activated EGFR can alter the strength of downstream signals to cause varied effects (Wells, Welsh et al. 1990; Haugh, Huang et al. 1999; Wells 1999). EGFR localized to the cell membrane and activating Erk triggers motility preferential to EGFR activated Erk in the cytoplasm which activates proliferation (Chen, Xie et al. 1996). Thirdly different ligands bind EGFR with different affinities generating different outcomes (Krall, Beyer et al. 2011). Lower ligand affinities cause slower EGFR internalization, prolonging surface signaling, as well as detach from receptor easily in endosomes causing receptor recycling instead of degradation (Roepstorff, Grandal et al. 2009).

Looking at EGFR based survival signaling in MSC, our group has previously reported that EGF in its soluble form cannot activate survival in MSC due to the short duration of signaling (Fan, Tamama et al. 2007)(Rodrigues, Turner et al. 2012). However sustained, surface restricted EGFR signaling by tEGF protects MSC from FasL induced cell death. In this study we look at the matrikine Tenascin-C, naturally upregulated during wound healing, which consists of

EGF-L presented in multiple valency, and which binds EGFR at the cell membrane with low affinity (Tran, Griffith et al. 2004; Iyer, Tran et al. 2008).

We find that TNC does not promote attachment of MSC as seen in several other cell types, however combination of TNC with Col I, an ECM which unlike Fibronectin does not interfere with TNC signaling, increases MSC attachment (Chiquet-Ehrismann, Kalla et al. 1988). TNC has been shown previously to be associated with chondrogenic and osteogenic differentiation in vivo in chick embryos and has been shown to promote chondrogenesis in wing bud cultures of chick embryo in vitro (Mackie, Thesleff et al. 1987; Murphy, Fischer et al. 2000). However in the presence of proliferation media, the mixed matrix of Col I and TNC does not induce either osteogenic and adipogenic differentiation, and in the presence of differentiation media, does not inhibit osteogenic and adipogenic differentiation. Most importantly we find that the mix of TNC and Col I is able to protect MSC from FasL induced cell death and that the effects of survival are due to TNC and not Col I.

There are limited reports in literature on the effects of TNC on survival, with a major emphasis on TNC based motility signaling. Addition of TNC externally to smooth muscle cells brings about interactions with α_5B_3 integrins, causing rearrangement of the actin cytoskeleton, clustering of activated EGFR on the cell surface near focal adhesion complexes and survival signaling (Jones, Crack et al. 1997). Chondrosarcoma cells grown on TNC display survival under serum deprivation due to Akt activation (Jang and Chung 2005) and TNC has also been shown to have a survival effect on oligodendrocyte precursor cells (Garwood, Garcion et al. 2004). We find that TNC is able to activate EGFR in MSC, to a lesser extent than EGF, and blocking EGFR activation or downstream Akt and Erk activation no longer supports survival of MSC in the presence of FasL. Blocking of different regions on TNC using neutralizing antibodies to either

EGF-L or FN-L shows that the survival effects of TNC on MSC are primarily via EGF-L. The FN-L is required but not sufficient to bring about MSC survival in the presence of FasL. Finally since ECM is known to sequester large numbers of growth factors and cytokines (Schultz and Wysocki 2009), we tested and ruled out the possibility that the TNC and Col I matrix sequesters and neutralizes the effect of TNC.

In conclusion we find that TNC enhances survival of MSC in the presence of apoptotic factors like FasL to which MSC are most susceptible to, and that this survival is via binding and activation of EGF-L to EGFR. Further since TNC does not by itself cause differentiation, but in the presence of differentiation cues does not hinder differentiation, three-dimensional scaffolds of TNC and Col I can be engineered for use with MSC to improve survival of MSC on implantation.

8.0 CONCLUSIONS AND SPECULATIONS

8.1 DEATH PATHWAYS IN MSC

Although most transplantation experiments with MSC see reduction in cell numbers on implantation of MSC in vivo in animals, only few of these studies actually mention the loss of cell numbers. Despite the issue of MSC loss, the number of transplantation studies with MSCs continues to rise exponentially while the number of studies on promoting MSC survival is minimal. Even in cases where specialized and elegant scaffolds are designed for MSC delivery, the primary criteria tested in vitro are adhesion and spread of MSC on the scaffold and MSC proliferation on the scaffold. Most in vivo studies concentrate on designing a delivery method promoting controlled and sustained differentiation of MSC.

The few studies on MSC death show the influence of ROS, hypoxia and nutrient deprivation in mediating apoptosis in MSC (Zhu, Chen et al. 2006; Deschepper, Oudina et al. 2010; Song, Cha et al. 2010). Inflammatory cytokines like TRAIL, FasL, TNFα and INFY have been shown to have an influence on cell death both in vitro and in vivo, mainly through direct activation or transactivation of the Fas death pathway (Fan, Tamama et al. 2007; Gotherstrom, Lundqvist et al. 2011; Liu, Wang et al. 2011) involving NK cells (Yamaza, Miura et al. 2008) and T lymphocytes (Crop, Korevaar et al. 2011; Liu, Wang et al. 2011). The studies in this thesis look at FasL based cell death due to the importance of the Fas pathway in MSCs. We find that

FasL not only activates caspase8 and the extrinsic death pathway, but also causes ROS generation through activation of JNK and P38 MAPK signaling.

Stem cells are thought to have low ROS levels due to the presence of high amounts of anti-oxidants like glutathione and superoxide dismutase (Urish, Vella et al. 2009). In fact the studies in this thesis were the first to show that MSC have high levels of UCP2, an uncoupling protein that keeps mitochondrial ROS levels low. Despite these reduced baseline ROS levels, when faced with a cytokine threat like FasL, MSC upregulate ROS. Increase in ROS causes an increase in UCP2, which is sufficient to delay but not sufficient to prevent cell death.

Further we show that the FasL affects not only undifferentiated MSC but also MSC forming osteoblasts. While MSC forming osteoblasts continue to die in the presence of FasL, MSC forming adipocytes die lesser and do not respond to FasL induced death with the progress of differentiation. These studies have two major implications in bone wound healing. Firstly survival signals ought to last not only while MSC are undifferentiated, but also as MSC form pre-osteoblasts and osteoblasts. Secondly, that it is important for MSC to differentiate and form only osteoblasts, since adipocytes formed will be difficult to kill, leading to unhealthy regenerated bone. The second issue might be more of a challenge. Use of Mx1+ bone marrow cells, which were recently described as cells capable of differentiating into only osteoblasts in vivo, in growing and adult animals, may currently be the best solution to the problem (Park, Spencer et al. 2012).

Although the work in this thesis has focused predominantly on the effect of FasL in determining death in MSC, it is important to establish how all the various threats that MSC could be facing at a wound might come together to bring about death. These factors could include, but are not restricted to low nutrients including serum and glucose, low oxygen- below 4% as

encountered by MSC in the bone marrow, hydrogen peroxide- to stimulate an environment of oxidative stress and death cytokines. The effects of these combinations may not be additive or synergistic and it might prove cumbersome probing undifferentiated and differentiating MSC with a combination of all these factors. Computational models such as Bayesian Networks or Decision Tree models could be used as tools to settle on the most effective combinations.

8.2 SURVIVAL IN MSC

We found during the course of this thesis that MSC grown in culture contain early stage autophagosomes. The levels of these autophagosomes vary between primary MSC and immortalized MSC lines, however MSC show a common characteristic of maintaining these autophagosomes in an early state, without promoting degradation and turnover, which we call "pre-autophagy". On differentiation the state of pre-autophagy is lost and MSC no longer show expression of LC3 II. MSC grown at ambient air conditions of 21% oxygen, or conditions of physiologic normoxia of 4% oxygen, do not show a difference in this state of pre-autophagy. These results prompted us to think that MSC physiologically exist in a state of pre-autophagy, so that when required at a wound, in conditions where nutrients and oxygen are low, when energy cannot be obtained from surrounding blood vessels, the pre-autophagosomes are rapidly broken down to generate energy. This would facilitate proliferation and differentiation of MSC. Although there are no major works in the realm of stem cells and autophagy, there are articles describing inhibition of the mTOR pathway and activation of autophagy to aid in conversion of somatic cells to induced pluripotent stem cells. We suggest that pre-autophagy could aid in self-generation of energy at the wound site and differentiation, and may be a hallmark of stem cells.

We also addressed in this thesis two modes of improving MSC survival in the presence of threats like FasL. The first is a bioengineered growth factor approach, the second an approach using Tenascin-C matrikine signaling. Both approaches are known to lead to surface restricted and sustained EGFR signaling bringing about activation of survival pathways, while not preventing differentiation. These results are of significance when designing a 3dimensional scaffold containing either tethered growth factor or ECM with MSC for repair of tissue, especially bone.

8.3 USE OF TETHERED EGF SCAFFOLDS FOR BONE FORMATION IN VIVO

8.3.1 Exposure to MSC to differentiation inducers prior to implantation

Classical studies looking at bone formation from MSC use osteoinductive scaffolds containing hydroxyapatite (HA) or calcium phosphate, mixed with MSC. As proof of principle, that MSC can form osteoblasts and bone in vivo, most of these studies implant human MSC ectopically in subcutaneous pockets of immunodeficient mice. Positive outcomes have been seen in these subcutaneous transplants. The number of MSC, degree of differentiation of implanted MSC, culture conditions employed for ex vivo expansion are a few factors that play a major role in the outcome of bone formation. To bring about optimal and consistent bone formation it is important to optimize MSC culture conditions before implantation.

One of the most important criteria is the level to which the cells are differentiated or exposed to differentiation inducers prior to delivery of the cells in vivo. Pre-exposing MSC to

differentiation inducers might prime the cells into a particular lineage. Exposing MSC to dexamethasone, ascorbic acid and ß-glcerophosphate for example might completely eliminate MSC forming any adipocytes in vivo.

There are contradictory reports on the effects of pretreating MSC with osteogenic inducers such as dexamethasone prior to implantation in vivo. On one hand, Dennis and Caplan's group report that pre-treatment of MSC with osteogenic media containing dexamethasone for 4-5 weeks prior to implantation in SCID mice leads to enhanced bone formation. They find that pre-treatment with dexamethasone for up to 3 weeks is insufficient for in vivo bone formation (Song, Caplan et al. 2009). They also report that dexamethasone pre-treatment provides a survival advantage to MSC (Song, Caplan et al. 2009). On the other hand Pamela Robey's group reports that pre-treatment of MSC with osteogenic media does not bring about a change in differentiation potential of the cells when implanted in vivo. They find that bone marrow MSC from 22 out of 24 donors do not exhibit changes in osteogenesis in vivo whether pre-cultured in media containining 10^{-8} M dexamethasone or 10^{-4}M ascorbic acid or without any osteogenic inducer. They show that pre-treatment with dexamethasone and ascorbic acid increases MSC proliferation and the number of colonies, which might aid in better osteogenesis (Kuznetsov, Mankani et al. 2011).

The differences may exist because Robey's group did not pre-treat MSC for over 3 weeks with dexamethasone which was the time frame recommended by Caplan's group for observing enhanced osteogenesis. What is surprising in the paper from Pamela Robey's group is the report that dexamethasone promotes increase in CFU, when dexamethasone is the factor known to predominantly enhance alkaline phosphatase activity in MSC and mineralization, while ascorbic acid is known to increase colony forming units (Fiorentini, Granchi et al. 2011).

If we relate our in vitro MSC death studies to these reports, we would expect to see both untreated MSC and MSC pre-treated with osteogenic factors to undergo cell death, if implanted without a material promoting survival pathways in these cells. P3It however might be beneficial to pre-treat MSC for at least 3 weeks, or for a shorter time frame if MSC are cultured at oxygen concentrations close to 4%, in media containing dexamethasone and ascorbic acid, prior to seeding on scaffolds carrying a protective signaling moiety like tEGF or Tenascin-C.

8.3.2 MSC numbers as a factor for in vivo bone formation

Bone formation increases with increasing MSC numbers but the relationship is sigmoidal with a threshold number of BMMSC required for bone formation (Mankani, Kuznetsov et al. 2007). Pamela Robey's group involved in these studies use 1.2 to 3 million human bone marrow MSC, mixed with HA particles. When implanted subcutaneously, bone begins to form at the edges of HA particles starting at 3 weeks, with moderate to abundant lamellar bone at 7 weeks and hematopoiesis at 4.5 weeks (Mankani, Kuznetsov et al. 2006; Kuznetsov, Mankani et al. 2011). There is no new bone growth after 15 weeks. 1.2 million cells is the threshold limit for new bone formation. Use of cells beyond this limit does not bring about a change in the outcome of osteogenesis. Caplan and Dennis's group add 5 million human MSC to a fibronectin coated porous calcium phosphate cube and grow the cells on the scaffolds for 4 weeks in media containing osteogenic inducers:100nM dex, 50uM ascorbate-2-phosphate and 10mM beta-glycerophosphate, prior to implantation in mice. They see extensive osteogenesis after 6 weeks (Song, Caplan et al. 2009). Further there are studies implicating EGF in osteogenesis where MSC pre-treated with EGF for 4 days prior to seeding on HA-tricalcium phosphate scaffolds and

implanted ectopically shows bone formation 5 weeks post implantation (Kratchmarova, Blagoev et al. 2005).

8.3.3 Use of syngenic MSC for transplantation

As mentioned earlier, all classical transplantation studies using MSC are allogeneic transplants of human MSC into immunodeficient mice. The experimental conditions are all proof of principal, not clinically relevant and there is elimination of death of MSC and therefore need for survival. Implanting murine MSC into mice is one option. However murine MSC are easily transformed in vitro and generate osteosarcoma when implanted in mice (Mohseny, Szuhai et al. 2009) with the probability of sarcoma increasing on injection of murine MSC into syngeneic or immunocompromised mice (Tasso, Augello et al. 2009). The transformation events are marked by aneuploidy, translocations and loss of cdkn2 (Mohseny, Szuhai et al. 2009) as well as high chromosomal instability (Josse, Schoemans et al. 2010). Transformation of murine MSC in culture is also not a strain specific or a rare event (Tolar, Nauta et al. 2007).

8.3.4 Suggestions for MSC scaffolds for in vivo testing in mice

Based on studies from this thesis and available literature, it would be best to test for ectopic bone bone formation, subcutaneously in mice using human and not murine MSC. Male human MSC, tracked with a permanent cell tracking dye like CM-DiI can be implanted in female mice, which will allow for tracking of MSC and cells differentiating from MSC based on Y chromosome. For seeding MSC on scaffolds, preferably tricalcium phosphate based scaffolds containing tethered EGF containing at least 500,000 cells/ scaffold can be used. If 70-80% of

cells attach, there will approximately be 350,000-400,000 cells on the scaffold. Cells on tEGF scaffold can be allowed to stay in proliferation media in vitro for 4 days at 4% oxygen to facilitate attachment, spread and expansion on scaffold. Media can then be changed to osteogenic media containing dexamethasone, ascorbic acid and beta-glycero phosphate for 2 weeks at 4% oxygen. Scaffolds can be inserted subcutaneously in pouches created by 4mm punches on the dorsal surface of mice and covered with a steel clip.

Survival of implanted cells can be determined by qPCR and Western blot for SRY present only on implanted male MSC or by detection of permanent tracking dye by immunofluorescence. It might be important to visualize cells as early as Day 3 and Day 7, as well as cells on Day 15, Day 30 and Day 60. Infiltration of inflammatory cells in the first two weeks will be an important factor to visualize and quantitate. Although MSC are immunosuppressive, the implantation will be allogenic causing rejection to occur. Based on our in vitro experiments, tEGF scaffold should circumvent this inflammation and help protect MSC. At Day 30 and Day 60 we would expect to see bone formation. It might also be important to consistently check for tumor formation during the 60 day period. While the tEGF signals must help promote survival and bone formation, we would expect the survival signals to remain contained and not lead to uncontrolled proliferation.

8.4 DESIGN OF HYDROGEL DELIVERY SYSTEMS CONTAINING TNC FOR INCREASED MSC SURVIVAL

There have been advances for delivery of MSC using hydrogels that increase survival and engraftment of MSC on delivery. Pullulan hydrogels quench reactive oxygen species and protect MSC from oxidative stress on implantation in vivo, bringing about cutaneous wound healing (Wong, Rustad et al. 2011; Wong, Rustad et al. 2011). MSC in pullulan based hydrogels remain viable for longer and integrate into the implanted tissue, where they continue to release growth factors like VEGF, causing increased angiogenesis and wound healing (Rustad, Wong et al. 2012). We have shown that MSC grown on TNC and Collagen I matrices promote cell survival in vitro in the presence of FasL. Similarly a matrix mixed scaffold called Integra ® has been used to deliver MSC and ESC to skin burns and bring about increased integration and proliferation of cells in vivo (Hamrahi, Goverman et al. 2012). There are also anisotrophic scaffolds consisting of polyurethaneurea fibers that mimic ECM and which entrap and release IGF-1 in a controlled manner that have been studied for increase in MSC survival in the presence of hypoxia and serum starvation in vitro (Wang, Li et al. 2009).

Collagen scaffolds by themselves have been shown to promote MSC survival for up to 60 days in vitro within the 3D gel structure, causing osteogenic and adipogenic differentiation when placed in differentiation media (Neuss, Stainforth et al. 2008). However this study hasn't tested survival of MSC within these scaffolds under in vivo conditions, or in vitro in the presence of inflammatory cytokines. We find in vitro that MSC grown on ECM comprising TNC and Col I enhances survival of MSC in the presence of death factors like FasL. However MSC attachment

to TNC is essential for survival signaling. MSC delivery systems can therefore be designed with confluent MSC sheets grown on TNC and embedded in Col I gels. Such a delivery system will cause restriction of MSC to the scaffold due to interactions with Col I, but will allow for MSC interaction with TNC, bringing about survival of MSC. Ultimately MSC in such scaffolds will be able to release trophic factors as well as differentiate bringing about wound healing.

ABBREVIATIONS

ATP: adenosine triphosphate

BCA: bicinchoninic acid assay

CM-DiI: 3H-Indolium, 5-[[[4-(chloromethyl)benzoyl]amino]methyl]-2-[3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1-propenyl]-3,3-dimethyl-1-octadecyl-, chloride/ 180854-97-1

CM-H_2DCFDA: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester

CHX: cycloheximide

ECM: extracellular matrix

EGF: epidermal growth factor

EGF-L: EGF like repeats of Tenascin-C

EGFR: epidermal growth factor receptor

ERK: extracellular signal regulated kinase

FasL: Fas ligand

FLICA: Fluorescent inhibitor of caspase assay

FN-L: Fibronectin like repeats of Tenascin-C

JNK: c-Jun N-terminal kinases

HA: Hydroxyapatite

HB-EGF: heparin binding epidermal growth factor

IAP: inhibitor of apoptosis protein

INFγ: Interferon gamma

imhMSC: immortalized human mesenchymal stem cells

LC3: mammalian microtubule associated protein-1 Light chain 3

MAPK: mitogen-activated protein kinase

MSC: multipotential stromal cells/ mesenchymal stem cells

PARP: Poly ADP ribose polymerase

prhMSC: primary human mesenchymal stem cells

NAC: n-acetyl cysteine

PDGF: Platelet-derived growth factor

ROS: reactive oxygen species

sEGF: soluble epidermal growth factor

SRY: sex determining region Y

tEGF: tethered epidermal growth factor

TCP: Tricalcium phosphate

TNC: Tenascin-C

TNFα: tumour necrosis factor α

UCP1: uncoupling protein 1

UCP2: uncoupling protein 2

VEGF: vascular endothelial growth factor

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