EFFECTS OF STRESS-INDUCED EPINEPHRINE
ON STROMAL CELL-DERIVED FACTOR 1’S RECRUITMENT
OF HUMAN MESENCHYMAL STEM CELLS

A Project

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Chuong Minh Nguyen

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Abstract

of

EFFECTS OF STRESS-INDUCED EPINEPHRINE ON STROMAL CELL-DERIVED FACTOR 1’S RECRUITMENT OF HUMAN MESENCHYMAL STEM CELLS

by

Chuong Minh Nguyen

Cellular therapy with mesenchymal stem cells (MSC) is emerging as a promising approach in wound healing. Understanding physiologic cues that modulate MSC migration and recruitment to wound sites is critical to maximizing their clinical use. Stromal cell-derived factor 1 (SDF-1), has been shown to promote MSC chemotactic migration and thus could potentially enhance wound healing. On the other hand, stress-induced epinephrine, whose systemic level can be up to 10-fold elevated in stressed patients, and is generated within the wound, has been shown to impair wound healing. The purpose of this study was to evaluate how stress-induced epinephrine impacts on MSC migratory response to SDF-1. MSC were treated with different concentrations of epinephrine, timolol (β1/2-adrenergic receptor blocker) and/or SDF-1. Individual cells’ images were captured using Volocity software package and manually tracked in OpenLab software and migration rates were analyzed. Immunoblotting and Immunocytochemistry were performed using standard protocols. MSC were seeded and incubated for 36, 60, and 84 hours and stained with Calcein acetoxymethylster (AM) fluorescent dye to label
viable cells and incubated at 37°C for one hour to evaluate proliferation rates. It was our findings that SDF-1 and epinephrine synergistically enhanced MSC proliferation. SDF-1 significantly increased the migratory speed of MSC in a dose-dependent manner. Immunolocalization of actin and vinculin showed cytoskeleton reorganizations and increase in focal adhesions in MSC treated with SDF-1. We also found that MSC migratory speed was significantly reduced by treatment with epinephrine alone. This response was reversed when the β1/2AR antagonist timolol was co-incubated with the cells, demonstrating the βAR specificity of the response. Immunoblotting demonstrated a significant decrease in phosphorylation of the ERK signaling pathway in epinephrine-treated MSC, correlating to the decreased migration. Surprisingly, co-incubation of MSC with both SDF-1 and epinephrine resulted in an increase the migratory speed relative to incubation with SDF-1 alone. This finding is important because it suggests that stress-induced high catecholamine environment at the wound site does not alter the positive effects of SDF-1 on MSC migration. Overall, our study sheds new light on the potential interactions between the two signaling pathways in MSC and provides initial evidence as to how they may be manipulated to improve healing.

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INTRODUCTION

In the United States, approximately two million people suffer from skin burns every year (Brigham & McLoughlin, 1996). Cutaneous wounds resulting from burns may lead to a permanent loss of skin and are a cause of disease or mortality. As these wounds create significant problems for patients, wound healing has become an important topic to medical professionals in the past two decades (Brigham & McLoughlin, 1996). Advances in molecular and cellular biology have brought a better understanding of wound healing and tissue regeneration. In general, the ultimate goal in treating wounds includes rapid surface closure, functional restoration and minimal scar development. However, wound healing is a complex process which requires a well-organized integration of biological events including cell proliferation, migration and extracellular matrix deposition, angiogenesis, and remodeling (Wu, Chen, Scott, & Tredget, 2007). In order to effectively treat wounds, a clearer understanding of the healing process is required to resolve existing limitations within the field.

There is an urgent need for a new approach for the treatment of major burn injuries. Major burn injury, including burns with greater than 20% of body surface area, has been associated with multisystem dysfunction (Sivamani, et al., 2009). Over the past two decades, major burn injury has been treated by surgical removal of damaged soft tissue followed by grafting of tissue from other parts of the body (Herndon & Tompkins, 2004). However, the absence of a protective epithelial layer after tissue removal often causes significant fluid and electrolyte loss as well as wound infection. Moreover, patients who suffer from major burns may not have sufficient remaining tissue for
successful grafting. As a result, the average mortality rate from major burn injury is up to 34% (Brusselaers, Monstrey, Vogelaers, Hoste, & Blot, 2010). Many different approaches have been used to achieve the optimum goal in healing these wounds. Conventional treatments include topical antibiotics, debridement with grafting, and bandages. Advanced methods consist of application of skin substitutes and growth factors to stimulate tissue regeneration. These methods, however, do not yield desirable results due to the high rejection rate of non-autologous skin substitutes at wound sites (Badiavas & Falanga, 2003) and very low rate of wound re-epithelialization and restoration of the protective barrier (Sivamani, et al., 2009).

Stem cell therapy is an emerging and promising approach in wound healing. Multipotent stem cells, by definition, are cells which are capable of unlimited cell cycles and able to differentiate into a variety of specialized cells in tissues or organs (Woodbury, Schwarz, Prockop, & Black, 2000). Human mesenchymal stem cells (MSC) are stem cells that were first collected from human bone marrow tissues before cell differentiation and considered to be adult stem cells (Pittenger, et al., 1999). MSC have recently been isolated from many different organs and tissues other than bone marrow tissues (Meirelles, Chagastelles, & Nardi, 2006). Due to the ability to differentiate into lineages of mesenchymal tissues, and to modulate wound repair, MSC are being studied for therapies of injury and hard-to-heal wounds (Brower, et al., 2011). There is an increasing body of evidence that MSC can serve as therapeutic agents that promote tissue repair and regeneration (Lau, Paus, Tiede, Day, & Bayat, 2009; Oh, et al., 2008).
There are two well-studied pathways by which MSC can act on wounded tissues: differentiation and cellular signaling. MSC can differentiate and directly generate cell lineages needed to replace damaged tissues at wound sites. In the alternate approach by the cellular signaling pathway, MSC regulate cellular responses of their surroundings to promote the wound healing process. Cellular signaling has been proposed as the primary pathway in wound healing since MSC’s differentiation is very limited due to a low rate of MSC survival at injury site as a result of the transit of MSC to and from the wound site (Gnecchi, Zhang, Ni, & Dzau, 2008). MSC cellular signaling regulates cell survival, migration, proliferation and gene expression at wound site. In previous studies, MSC wound treatment in myocardial infarction leads to significant increase in angiogenesis (Gnecchi, et al., 2008; Lau, et al., 2009; Oh, et al., 2008). MSC activities are responsive to their micro-environments. When exposed to the biochemical factors released from injuries, MSC have also been shown to migrate to wound sites and secrete mediators for tissue repair process such as: growth factors, cytokines, and chemokines (Chen, Tredget, Wu, & Wu, 2008; Jackson, Nesti, & Tuan, 2012). There is an increasing amount of evidence that MSC enhance the tissue regeneration process during inflammation, proliferation and extra cellular matrix remodeling phases of wound healing (Jackson, et al., 2012). Either by differentiation or cellular signaling pathway or both, migration of MSC to the wound site appears to be essential for an optimal wound healing process.

Stromal cell-derived factor 1, SDF-1, a pre-B cell growth-stimulating factor that belongs to the CXC subfamily of chemokines, and its G protein-coupled receptor, CXCR4, play an important role in the recruitment of bone marrow-derived MSC
SDF-1 is not only a chemo-attractant to stem cells, but also is critical in their survival and proliferation (Burger & Kipps, 2006; Lataillade, et al., 2000). SDF-1/CXCR4 interaction has been found significant for biologic and physiologic functions of MSC in skin wounds and repair (Ayelet Dar, et al., 2005). The level of SDF-1 was found elevated in recovering dermis layer of human skin (Avniel, et al., 2005) and hypertrophic scars (Ding, et al., 2011) after burns. The up-regulation of SDF-1, at sites of injury recruits circulating or residing CXCR4-expressing MSC which are essential in wound re-epithelization (Ceradini, et al., 2004). Moreover, activation of SDF-1/CXCR4 signaling has been shown to significantly promote MSC migration and eventually wound healing (Haider, Jiang, Idris, & Ashraf, 2008).

In contrast, stress is an important factor that impairs wound healing (Herndon & Tompkins, 2004; Olivera Stojadinovic, 2012). Major burn injuries cause the massive systemic secretion of the physiological stress catecholamine hormones epinephrine and norepinephrine (NE). These stress-induced hormones have been suggested to delay wound re-epithelialization and the repair of skin permeability barrier (Choi, et al., 2005; Choi, et al., 2006). Sivamani et. al. has shown that the elevation of stress-induced epinephrine in response to wounding results in activation of the keratinocyte β2-adrenergic receptor (β2AR) (Sivamani, et al., 2009). Once activated, the β2AR recruits the phosphatase PP2A and extra cellular signal-regulated kinase (ERK) resulting in a significant decrease in ERK phosphorylation which eventually leads to the impairment of cell-motility and wound re-epithelialization. Treatment with β2AR antagonists,
significantly improved keratinocyte migration speed *in vitro* and wound re-
epithelialization *in vivo*.

Our lab’s previous data have indicated the presence of βAR in MSC. In addition, new studies have suggested an interaction between CXCR4 and adrenergic receptor signaling. Pre-treatment of mice with elevated levels of norepinephrine results in up-regulation of expression of CXCR4 in splenic cells (Gruber-Olipitz, et al., 2004). In cardiac myocytes, CXCR4 and β2AR signals interact, with one able to regulate the other by their physical colocalization into a signaling complex (LaRocca, et al., 2010). The possibility of stress mediated cateholamine regulation of MSC migration via SDF-1/CXCR4 signaling is an exciting possibility that was explored in this research investigation.

In this study we extend the laboratory’s investigation of the deleterious effects of epinephrine (E) in stress to determine their role in MSC recruitment to the wound site. The purpose of the study was to assess the regulatory effects of stress-induced epinephrine on MSCs migration in wound healing in the presence of SDF-1. βAR antagonists were used to block the ability of epinephrine to activate these receptors. We hypothesized that the positive effects of SDF-1 on MSCs migration would be reduced by treatment with stress-related catecholamines and this reduction would be reversed in the presence of βAR antagonists in primary cell cultures *in vitro*. 
METHODS

MSC Isolation

MSC were isolated and characterized by the laboratory of Dr. Nolta (Institute of Regenerative Cures, Sacramento, CA) from purchased human bone marrow (Lonza Inc., Allendale, NJ) using established protocols (Fierro, Kalomoiris, Sondergaard, & Nolta, 2011; William Gruenloh, 2011). In brief, bone marrow aspirates were filtered through 70µm cell strainers. The filtered aspirates were then centrifuged with Ficoll solution (GE Healthcare, Pittsburgh, PA) at 700g for 30 minutes. The layer of mononuclear cells at the interphase of plasma-Ficoll solution was separated and plated with minimum essential medium modification- MEMα (Invitrogen, Grand Island, NY) with 10% MSC-qualified FBS (Invitrogen) on plastic cell culture flasks together with the bone spicules left on the filter. Flasks were incubated at 37°C and 5% CO₂. After 48 hours, non-adherent cells were washed off with PBS. The culture medium was changed every day until MSC reach 80% confluence. Flow cytometry with immuno-fluorescent antibodies to CD14, CD31, CD34, CD45, CD73, CD90, CD105, and CD146 MSC markers was used to characterize MSC.

Osteogenic Differentiation

To confirm that the isolated cells were indeed MSC, capable of multilineage differentiation, we tested for their osteogenic ability. To induce osteogenesis, 5 x 10⁴ MSC were plated per well in a 12-well plate in MEMα with 10%FBS and grown until confluence. Medium was changed to osteogenic medium (10%FBS in MEMα, 0.2nM
ascorbic acid, 0.1µM dexamethasone, and 10mM β-glycerolphosphate). The medium was refreshed every three or four days until day 14. At day 14, cells were washed with PBS and fixed with 5% formalin for 15 minutes followed by PBS wash. 400µl/well of 1% Alizarin Red S indicator (Ricca Chemicals Company, Arlington, TX) was used to stain cells for calcium deposits to verify that MSC differentiated into osteoblasts. Each well was washed once with PBS and at least 3 times with water. Individual wells were photographed with Apple Iphone 4S camera (Apple, Cupertino, CA) and images were evaluated for differentiation capability.

Adipogenic Differentiation

The ability of the isolated MSC to differentiate along an adipocyte lineage was also examined. MSC were plated in a 12-well plate in MEMα with 10%FBS and grown until confluence. At 100% confluence, medium was changed to adipogenesis-inducing medium (10%FBS in MEMα, 50µM indomethacin, 0.5µM dexamethasone, and 0.5mM isobutilmethylxantine). Cells were incubated at 37°C and 5% CO₂ for 14 days with medium refreshed every three to four days. At day 14, cells were fixed with 5% formalin for 15 minutes, washed with PBS and adipocytes were stained with Oil Red O (Electron Microscopy Sciences, Hatfield, PA). After two PBS washes, cells were observed under phase contrast microscope (TE2000, Nikon, Melville, NY) and photographed with Apple Iphone 4S camera through microscope eyepieces and images were evaluated for differentiation capability.
Seeding cells for Single Cell Migration assays

MSC were plated on collagen-coated (60µg/ml, Invitrogen) 24-well plates at a density of 3 x 10^3 cells/cm^2. MSC in 0.5% FBS CO_2-Independent medium were seeded to collagen coated plates and incubated at 37°C for two to three hours for attachment. The well plates were placed on the TE-2000 microscope inside an environmental chamber (~48x60x90cm^3) that maintains temperature at 37°C and CO_2 at 5%. Using an imaging software package (Volocity of Perkin Elmer, Waltham, MA), time-lapse movies were recorded with intervals of 1 hour for 12 hours at each of 4 areas (~100µm^2 with up to 20 cells per area) in each well of the 24 well plates. Individual cells were manually tracked in OpenLab imaging software. Trajectory distance/ time was referred as the true speed of individual cells and was used for migration rate analysis.

Transwell migration assay

Boyden chamber (Transwell) migration assay was used to assay for chemotactants. Since cells migrated both on a surface of and down through a 17µm long tunnel within a membrane, the assay may better predict the migratory capacity of MSC within the wound tissue. For the Boyden migration assay, 5x10^3 cells suspended in 100µl of serum-free α-MEM were seeded in the upper chamber of 24-well Transwell plates (8µm pore size filters) (BD Biosciences, Sparks, MD). In the lower chamber, 600 µl of MEM-α supplemented with adrenergic receptor agonists was added. Cells were incubated at 37°C for three hours. Cells retained in the upper chamber were removed by wiping the surface with a cotton swab and those cells that had migrated through the filter were
stained with DAPI, counted under Nikon TE2000 with 20x objective, and imaged using a Retiga Ex camera. Data was analyzed by Volocity software and compared by Student’s t-tests.

SDF-1, AR agonists and antagonists treatments

SDF-1 was purchased from R&D Systems (Minneapolis, MN). SDF-1 was added to supplement MSC growth medium at different concentrations: 1.25nM, 6.25nM, 12.5nM, 25nM. Cell migration was determined as previously mentioned. Epinephrine was purchased from Sigma (St. Louis, MO). MSC’s culture medium was supplemented with epinephrine (at stress-induced or stress-induced super physiologic concentrations: 50nM, 1µM, respectively (Dunser & Hasibeder, 2009)). Cell migration was monitored as previously described. The βAR antagonist timolol was purchased from Tocris BioScience (Minneapolis, MN). MSC growth medium was supplemented with timolol at 10µM.

Western Blotting

MSC were cultured in 10% FBS MEMα medium in 100 mm cell culture dishes until they reached 80% confluence. Cells were then incubated overnight with 0.5% FBS MEMα medium before being treated with SDF-1 and/or Epinephrine in serum free medium for four hours. Cells were lysed with NP40 Cell Lysis Buffer (Invitrogen)/0.1M PMSF (Sigma)/Protease Inhibitor on ice for 15 minutes, mechanically dissociated and collected. Bradford protein assays were done to determine protein concentrations. Samples were stored at -80°C until use. Equal amounts of protein were loaded into 4-12%
gradient polyacrylamide gel. SDS-PAGE assays were run at constant 100mV. IBLOT Transfer Protocols (Invitrogen) were performed to transfer protein onto nitrocellulose membrane. The membranes were then incubated overnight at 4°C with ERK1/2 or pERK1/2 (Cell Signaling Technology, Danvers, MA) primary antibodies with concentrations as suggested by manufacturer. Anti-rabbit or anti-mouse horseradish peroxidase-linked anti-IgG (Cell Signaling Technology) were used to detect immuno-reactive proteins. Immunostained GAPDH (Cell Signaling Technology, Danvers, CA) was used as a loading control. Band visualizations were performed with Enhanced Chemiluminescence (ECL, GE Healthcare) western blotting detection reagents. Scanned images were analyzed for densitometry with ImageJ.

Immunocytochemistry

Immunolocalization of actin and vinculin was done to determine relative concentration of focal adhesion molecules in MSC. MSC were seeded at 5 x 10^5 cells/well in collagen-coated 8-well slides and incubated for 3 hours. Cells were treated with SDF-1 and/or epinephrine. Slides were fixed with 4% paraformaldehyde for 10 minutes, permeablized with 0.1% Triton X-100 for one minute and blocked with 5% BSA PBS for one hour at room temperature. Slides were incubated overnight at 4°C with rabbit monoclonal anti-vinculin primary antibodies (Sigma) and Texas-Red phalloidin conjugated actin antibodies (Sigma) as suggested by manufacturer. Visualizations of focal adhesion molecules, seen as elongated bright circles, were made possible by incubating in goat anti-rabbit Alexa 488 secondary antibodies (Sigma) at concentrations
suggested by manufacturer. Actin staining was identified as filament-like structures in the cytoplasm of individual cells. Negative controls were only incubated with anti-rabbit Alexa 488 secondary goat IgG antibodies. Coverslips were mounted onto slides with Prolong Gold antifade with DAPI mounting medium (Invitrogen). Images were analyzed using Volocity software package on a Nikon TE-2000 microscope.

Proliferation assays

To create a standard curve, MSC were seeded at 0, 2, 4, 6, 8, 10, 12, or 14 x 10^3 cells/well in triplicates on a 24-well plate (Costar, Corning, NY). Plates were incubated at 37°C and 5% CO_2 for three hours to allow adequate time for cells to attach to the plate. After incubation, the plate was washed twice with DPBS (Invitrogen) before cells were treated with 1µg/ml Calcein acetoxymethylester (AM) fluorescent dye (BD Biosciences) to label viable cells and incubated at 37°C for one hour. Fluorescence was measured at 485/520nm (Absorb/Emit). Cell number-versus-fluorescence readings graph was plotted.

For the proliferation experiment, MSC were seeded at 5 x 10^3 cells/well in three 24-well plates in 1%FBS MEMα and incubated at 37°C for three hours to allow for cell attachment to the plate. Cells were treated with 12.5nM SDF-1 30 minutes prior to 1µM Epinephrine treatment. Plates were incubated at 37°C and 5% CO_2 for 36, 60 or 84 hours. At each time point, one plate was washed twice with DPBS before treated with 1µg/ml Calcein AM and incubated at 37°C for one hour. Fluorescence was measured at 485/520. Readings were converted to number of cells in each well. The experiment was repeated
three times with three cell lines from three different donors. Data were normalized to the first set and combined for analysis.

SDF-1 ELISA assays

Supernatant was collected from MSC cell culture under different conditions: 0-0.5\%FBS or 10\%FBS MEM\(\alpha\) for 1 hour, 4 hours, 24 hours or 72 hours and stored at -80\(^\circ\)C until use. Human CXCL12/SDF-1\(\alpha\) Immunoassay kit purchased from R&D Systems was used to measure the amount of SDF-1 present in the collected cell culture supernatant. The procedure and conditions were done as recommended by the manufacturer. In brief, the provided standard SDF-1 samples were diluted 1:2 in seven tubes from 1 \times 10^4 pg/ml to 156 pg/ml. Assay Diluent RD1-55 was first added to each well followed by standards or samples and incubated for two hours at room temperature. Wells were washed 3 times with provided wash buffer. SDF-1\(\alpha\) conjugates were added and incubated for two hours. Plates were washed three times with wash buffer before 30 minutes of Substrate Solution (mixture of hydrogen peroxide and tetramethylbenzidine) light-protected incubation. 2N Sulfuric Acid was used to stop Substrate Solution reactions. Plates were read within 30 minutes at 450nm with correction wavelength at 540nm. A standard curve was generated and absorbance readings from samples were converted back to concentrations of SDF-1.
Statistical Testing

Student’s t-test was used to identify if there is significant difference between the groups of data from different types of treatments. P-values of less than 0.05 were considered statistically significant. $\alpha$-value was modified by Bonferroni correction to control the probability of making type I errors.
RESULTS

_Epinephrine decreased MSC migration speed and norepinephrine increased MSC migration speed._ In order to determine how catecholamines affect migratory speed of MSC, single cell migration assays were done. MSC were treated with either 50nM or 1µM of E or NE one hour before time-lapse movie was recorded. Our results showed that at 50nM epinephrine MSC average migratory speed was reduced 15% and the reduction went up to 23% at 1µM compared to the control group (Figure 1A). In contrast, at 1µM NE treatment, MSC moved 12% faster than the non-treated control (Figure 1B). These results are statistically significant with p-value < 0.001. Since stress-induced epinephrine was shown to be elevated in response to wounding, the fact that it slowed down MSC migration could potential be important in the overall regulation of wound healing process by epinephrine.

**βAR antagonist reversed the migratory effects of epinephrine on MSC.** Since epinephrine was previously shown to activate βAR and slow down migration in keratinocytes, we hypothesized that a similar mechanism might apply to MSC. In order to determine if the effects of epinephrine in decreasing MSC migratory speed was due to activation of βAR, MSC were pretreated with 10µM timolol, a βAR antagonist. The purpose of the timolol treatment was to see if blocking βAR would reverse the effects induced by epinephrine on MSC migration. Our results showed that timolol indeed significantly reversed the speed reduction caused by epinephrine by 89% (Figure 2). As a result, we concluded that the effects of epinephrine on MSC migration were primarily on the βAR signaling pathways.
A.

Figure 1: MSC Single Cell Migration with epinephrine and norepinephrine. The data were normalized and combined from 3 different cell lines (3 donors). Average true speeds of MSC in non-treated control versus A. 50nM and 1µM E and B. 50nM and 1µM NE. * indicates statistical significance (p-value < 0.001). Error bars represent standard errors.
Figure 2: MSC Single Cell Migration with epinephrine and timolol. The data was normalized and combined from 3 cell lines (3 donors). Average true speeds of MSC in non-treated control versus 1µM E and/or timolol-treated groups. * indicates statistical significance against control group (p<0.001). ** indicates statistical significance against epinephrine-treated group (p<0.001). Error bars represent standard errors.
SDF-1 induced a dose-dependent pro-migratory response on MSC. The ability of MSC to cross a membrane through small pores in response to the chemokine SDF-1 in a dose-dependent manner in Transwell migration assays has been shown (Wynn, et al., 2004). However, the effects of SDF-1 on MSC migratory speed on a two dimensional, collagen-coated surface have not been explored. In order to determine the effects of SDF-1 on migratory speed of MSC on a collagen-coated surface, MSC medium was supplemented with SDF-1 at concentrations that were commonly used in prior reported Transwell migration assays (Ryu, et al., 2010; Son, et al., 2006; Wynn, et al., 2004). The data (Figure 3) showed that MSC migratory speeds were significantly enhanced by the treatment of SDF-1 in a dose-dependent manner with the optimal concentration of 12.5nM that induced 24% average true speed increase compared to the control group.
Figure 3: MSC Single Cell Migration with SDF-1 at various doses. Data were normalized and combined from 3 cell lines (3 donors). Migration assays were run on non-treated control MSC versus MSC treated with 1.25nM, 6.25nM, 12.5nM, 25nM SDF-1. * indicates statistical significance (p-value<0.001). Error bars represent standard errors.
Epinephrine did not induce secretion of SDF-1 in MSC. Dar et al. (A. Dar, et al., 2011) showed that the administration of NE increased circulating SDF-1 release and ICI-118551, a β2AR antagonist, reduced SDF-1 concentration in the systemic circulation. In contrast, it has also been shown that hematopoietic progenitor cells release more SDF-1 when treated with ICI in vitro (A. Dar, et al., 2011). Since these results were implications of possible SDF-1 secretion modulated by β2-adrenergic signaling pathway, ELISA assays were run to determine if epinephrine induced secretion of SDF-1 in MSC. Interestingly, both non-treated and 1µM-epinephrine MSC cultured serum-free supernatant after 4 hours and 0.5%FBS supernatant after 24 hours from multiple cell lines showed no SDF-1 secretion (data not shown). These results indicated that in our experimental conditions (0-0.5%FBS medium) epinephrine did not induce secretion of SDF-1. On the other hand, MSC cultured in 10%FBS for 24 hours showed a relatively lower SDF-1 secretion if treated with 1µM epinephrine than non-treated control (Figure 4). This finding, in one donor only, was suggestive. However, further confirmation is needed by testing other donors.
Figure 4: SDF-1 levels in MSC 10% FBS cultures after 24 hours by ELISA. Graphs showed amount of SDF-1 detected at 24-hour time point in non-treated control versus 1µM epinephrine supplemented supernatants. Data shown was observed from one cell line from one donor.
*Epinephrine did not reverse the pro-migratory response of SDF-1 on MSC.* Since SDF-1 increased and epinephrine decreased MSC migratory speed, we tested whether epinephrine would inhibit the positive, pro-migratory effects of SDF-1. MSC were treated with SDF-1 for 30 minutes before epinephrine was added. Interestingly, our results showed that MSC migratory speed in the group treated with SDF-1 and epinephrine was comparable to that of MSC treated with SDF-1 alone (Figure 5). The migratory speed in the combination treated group was significant higher than non-treated control and epinephrine-treated group with p-values <0.001.

*SDF-1 and epinephrine’s synergistic effects enhanced short-term proliferation in MSC.* We examined the effects of SDF-1 and epinephrine on MSC proliferation. MSC showed significant enhancement in proliferation when treated with SDF-1 (p-value=0.015, Figure 6A) and combination of SDF-1 and epinephrine (p-value=0.0006, Figure 6A). Epinephrine, in contrast, did not alter the proliferation rate (Figure 6A). At 60 hours, neither SDF-1 nor epinephrine significantly enhanced MSC proliferation (Figure 6B). However, co-treatments of SDF-1 and epinephrine significantly enhanced proliferation of MSC (p-value=0.01, Figure 6B). Our results showed that SDF-1 and epinephrine produced a synergistic effect in which each agent alone promoted little proliferation but together they significantly increased proliferation of MSC (Figure 6A, B) though the positive effect of each agent or their combination was short-term. At 84 hours, there was no significant difference in proliferation between non-treated and treated groups (Figure 6C).
Figure 5: MSC Single Cell Migration with co-treatments of SDF-1 and epinephrine. MSC were treated with 12.5nM SDF-1 or 1µM epinephrine or combination of 12.5nM SDF-1 and 1µM epinephrine (S+E). Data were normalized and combined from 3 cell lines (3 donors). * indicates statistical significance (p-value <0.05). Error bars represent standard errors.
Figure 6: MSC proliferation with SDF-1 and/or epinephrine treatments. MSC were treated with either 12.5nM SDF-1 or 1μM epinephrine or combination of 12.5nM SDF-1 and 1μM epinephrine (S+E). Cell numbers were recorded at (A) 36-hour, (B) 60-hour and (C) 84-hour time points. Data were normalized and combined from 3 cell lines (3 donors). *, ** indicate statistical significance (*: p-value <0.05, **: p-value <0.001). Error bars represent standard errors.
**SDF-1 induced cytoskeleton re-organization and increased focal adhesion**

*molecules.* Stress fibers are bundles of actin filaments and myosin motors that are held together by cross-linking proteins. Stress fibers are the key mediators of cellular contraction and their formation is critical in migration process (Pellegrin & Mellor, 2007). In addition, the association of cell membrane-spanning integrins to the intracellular actin cytoskeleton by vinculin is also very important in cell migration (Wen, Rubenstein, & DeMali, 2009). In order to understand the mechanism which SDF-1 and the combination of SDF-1 and epinephrine enhanced the migratory speed of MSC, immuno-localization staining of actin and vinculin was performed. In the SDF-1 treated group of MSC, cytoskeleton re-organization was observed with more thick bundles of stress fibers formed to replace the thinner actin filament network in control group (Figure 7). More vinculin adhesion molecules near the edges of cells were observed in SDF-1 treated MSC than in the non-treated control group (Figure 7). Most importantly, the treatment of epinephrine did not alter the effects of SDF-1 on MSC cytoskeleton re-organization nor the number of vinculin adhesion molecules observed (Figure 7).
Figure 7: Representative images of immuno-cytochemistry with actin and vinculin fluorescence staining. MSC were treated with either 12.5nM SDF-1 or 1µM epinephrine or combination of 12.5nM SDF-1 and 1µM epinephrine. Negative controls were incubated with anti-rabbit Alexa 488 secondary only goat IgG antibodies.
Co-treatments of SDF-1 and epinephrine up-regulated phosphorylation of ERK.

Since we previously showed that phosphorylation of ERK correlated with migratory speed in keratinocytes under epinephrine treatments, we then looked for potentially similar effects on MSC. Our results showed that even though SDF-1 induced a very slight change of ERK phosphorylation level and epinephrine critically down-regulated 55% of ERK phosphorylation, the combination of SDF-1 and epinephrine up-regulated phosphorylation of ERK by 27% (Figure 8).

Epinephrine enhanced MSC migratory ability to migrate in the context of Transwell. Since studies have shown that SDF-1 enhanced MSC ability to migrate through membranous pores using Transwell migration assays (Ryu, et al., 2010; Son, et al., 2006; Wynn, et al., 2004), we examined the effects of epinephrine on MSC under the same conditions. Importantly, our results showed that epinephrine in a dose-dependent manner increased MSC migratory ability in Boyden Chamber context (Figure 9).
Figure 8: Western Blotting and densitometry analysis with SDF-1 and/or epinephrine. MSC were treated with either 12.5nM SDF-1 or 1µM epinephrine or combination of 12.5nM SDF-1 and 1µM epinephrine (S+E). Values from ImageJ analysis were recorded and presented as bar graph. Data was normalized and combined from 2 cell lines (2 donors).
Figure 9: MSC Transwell (Boyden Chamber) migration with epinephrine. Data was normalized and combined from 3 cell lines (3 donors). Migration assays were run on non-treated control MSC versus MSC treated with 50nM, 1µM epinephrine. Data were normalized and combined from 3 cell lines (3 donors). * indicates statistical significance (p-value<0.01). Error bars represent standard deviations.
DISCUSSION

The results of the project are integral to the development of a biological bandage by optimizing MSC and utilizing adrenergic receptors agonist or antagonist preconditioning to effectively treat wounds. The effects of adrenergic receptors agonists and antagonists on MSC were evaluated. Our study showed that epinephrine slowed down, while norepinephrine accelerated MSC migratory speed (Figure 1). The epinephrine-induced setback in MSC speed was shown due to activation of βAR since blockade of βAR reversed the effects of epinephrine (Figure 2). Data from our lab showed that in MSC the epinephrine activation was primarily on β2AR and the effect was reversed by ICI- β2AR antagonist. Since norepinephrine has a low affinity for β2AR (Hoffmann, Leitz, Oberdorf-Maass, Lohse, & Klotz, 2004; Molinoff, 1984), it is logical that norepinephrine did not fully activate β2AR signaling pathways and slow down MSC migration as epinephrine did. In addition, it has been shown that β3AR has a 30-fold higher affinity for norepinephrine than epinephrine (Hoffmann, et al., 2004). As a result, norepinephrine may be signaling primarily through the β3AR pathway. However, the expression and functions of β3AR in human MSC have not been fully explored yet. Li et al. (Li, Fong, Chen, Cai, & Yang, 2010a, 2010b) showed the activation of β3AR played a regulatory role in mouse MSC osteogenesis and adipogenesis. In addition, Méndez-Ferrer et al. (Mendez-Ferrer, Lucas, Battista, & Frenette, 2008) showed that norepinephrine triggered β3AR signaling pathway and down-regulated SDF-1 mRNA level in mouse bone marrow stromal cells and activated hematopoietic stem and progenitor cells mobilization. It is our proposal that β3AR signaling pathway plays an important role in
inducing MSC migration. Another alternative is β3AR downstream signals may have created a feedback mechanism that inhibits the activation of β2AR. Additional investigation is required in order to understand the mechanism of norepinephrine in MSC migration.

In contrast to single-cell migration experiments, our Transwell migration assays showed a significant increase in MSC migration toward epinephrine (Figure 9). Spiegel et al. (Spiegel, et al., 2007) also showed similar results in Transwell migration studies with MSC from mice pre-treated with epinephrine. Since Transwell migration assay measures the ability of cells to migrate toward chemo-attractants, these results suggested epinephrine as a potential MSC chemo-attractant. On the other hand, in single-cell migration assays, cells migrate on a surface area which is more similar to the situation at wound site. We proposed that epinephrine acts as a chemo-attractant to recruit MSC to wound site from their niches, but then slows down cell movement at wound site so the MSC would not transit away from the wound. Our hypothesis was supported by the results shown in figures 1 and 9. However, the mechanism of how epinephrine acts differently on MSC in two different migratory assay settings requires further investigation.

Our results showed SDF-1 increased MSC migration in a dose-dependent manner with the optimal concentration at 12.5nM (Figure 3). At 25nM SDF-1, we found a slight decrease in migratory speed of MSC (Figure 3). These effects could have been due to the over-saturation of CXCR4 receptors. Excess amount of SDF-1 might have bound to non-specific cell surface receptors and reduced the migratory speed. The trend in SDF-1
response including in our single cell migration assays was consistent with others’ studies in Transwell assays (Mori, et al., 2004; Wynn, et al., 2004; Xu, et al., 2010). Overall, SDF-1 showed a pro-migratory effect on MSC migration on a collagen-coated surface.

Since SDF-1/CXCR4 and βAR are two independent signaling pathways with opposite effects on MSC migration, we hypothesized that stress-induced epinephrine would reverse the positive effects of SDF-1 on MSC migration. However, our results showed that epinephrine did not reduce the pro-migratory response of MSC to SDF-1 (Figure 6). The Western blotting data was consistent with single cell migration assays. The ERK1/2 signaling pathway has been known for its importance in MSC migration (Gao, Priebe, Glod, & Banerjee, 2009). We were able to show that 4 hours after epinephrine treatment phosphorylation ERK was down-regulated but it was up-regulated if MSC were pre-treated with SDF-1 (Figure 8).

In summary, I believe that these findings are significant because they indicate the therapeutic potential of SDF-1 to override the negative effects of stress in wound healing. Studies have shown that delivery of SDF-1 to wound sites accelerates wound healing in mice, pigs (Rabbany, et al., 2010) and diabetic mice (Gallagher, et al., 2007) models. However, it has been shown that topical administration of AMD3100, a CXCR4 antagonist, also improved wound healing in diabetic mice (Nishimura, et al., 2012). Interestingly, the authors also noted not all the effects occurred through blocking of CXCR4 (Nishimura, et al., 2012). Burns et al. (Burns, et al., 2006) demonstrated an alternative receptor, CXCR7, with specific and high affinity for SDF-1. They also noted that SDF-1 binding to CXCR7 was not blocked by AMD3100 (Burns, et al., 2006).
Together these results indicated there may be a novel pathway in which SDF-1 acts on in wound healing. We hypothesized that this novel pathway included activation of CXCR7 and possibly led to the inhibition of β2AR downstream signals. Our study is one of the first evidences of possible cross-talk between SDF-1/CXCR4 and βAR pathways. Further studies must be done in order to understand the mechanism behind it. Understanding the mechanism of the two pathways’ interactions will be extremely beneficial to wound healing in general and burns specifically since both SDF-1 and epinephrine levels are significantly up-regulated at sites of burn injury (Avniel, et al., 2005; Sivamani, et al., 2009).


