# Stress Dysregulates Inflammatory Gene Expression During Wound Healing

Via miRS

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# THESIS

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**Committee Members** 

Phillip Marucha, Advisor Praveen Gajendrareddy Timothy Koh Lin Tao Xiaofeng Zhou This thesis is dedicated to my beloved and belated grand-parents Jacqueline and Raymond Tymen and to my parents Jocelyne and Pierre Tymen for their love and support along this journey.

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# LIST OF ABBREVIATIONS

Bcl-2	B-cell lymphoma 2
CAM	Classically Activated Macrophage
CCL1	Chemokine (C-C motif) ligand 1
CCL5	Chemokine (C-C motif) 5
CCL22	Chemokine (C-C motif) 22
CD11b	Cluster of Differentiation 11b
CD206	Cluster of Differentiation 206 (Mannose Receptor)
CD68	Cluster of Differentiation 68
CFU	Colony Forming Unit
CXCL10	C-X-C motif chemokine 10 (IP-10)
FGF7	Fibroblast Growth Factor 7
FWD	Food and Water Deprived (Ctrl)
GC	Glucocorticoid
GH	Growth Hormone
GHR	Growth Hormone Receptor
GR	Glucocorticoids Receptor
HPA	Hypothalamic Pituitary Adrenal
IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin 1 β
IL10	Interleukin 10
IRAK1	Interleukin-1 receptor-associated kinase 1
KC	Keratinocyte Chemoattractant
LCM	Laser Capture Microdissection
LNA	Locked Nucleic Acid
LPS	Lipopolysaccharide
MCP-1	Macrophage Chemoattractant Protein 1
MIP-1α	Macrophage Inflammatory Protein 1 $\alpha$
MIP-2	Macrophage Inflammatory Protein 2
miR	microRNA
MyD88	Myeloid differentiation primary response gene (88)
NFκB	Nucelar Factor Kappa-Light –Chain –Enhancer of Activated B cells
NO	Nitric Oxide
PBS	Phosphate Saline Buffer
PGC-1β	PPAR-gamma Coactivator 1β
RELMα	Resistin Like Molecule $\alpha$
ROS	Reactive Oxygen Species
RST	Restraint (Strs)
RST-LNA98	Restraint group treated with LNA-98
RST-LNAscr	Restraint group treated with LNA-scramble
SCD1	Stearoyl-Coenzyme A desaturase 1
SKH-1	Charles River Laboratories Mouse Strain
SNS	Sympathetic Nervous System
SOCS2	Suppressor of cytokine signaling 2
TGF-β	Transforming growth factor beta
TGFβR2	Transforming growth factor beta Receptor 2
TLR4	Toll-like Receptor 4 (CD284)
WHM	Wound-Healing Macrophage

#### SUMMARY

Wound healing is a complex series of events that rely on the successful completion of the previous phase, which means the completion of the inflammatory phase is crucial. Neutrophils and macrophages are recruited during inflammation. They are necessary for bacterial clearance, and their recruitment and functions are regulated by gene expression of cytokines and markers. Previous studies showed that stress delays wound healing and impairs bacteria clearance. Stress dysregulates inflammatory gene expression at the transcriptional and post-transcriptional level. Interestingly, miRs have emerged as potent post-transcriptional regulators. We hypothesis that stress dysregulates expression of genes involved in the inflammatory phase of wound healing, in part via miRs, and alters neutrophil and macrophage recruitment and functions leading to impaired bacterial clearance and wound healing.

In our current study we found that restraint stress induced an excessive recruitment of neutrophils extending the inflammatory phase of healing, and the gene expression of neutrophil attracting chemokines MIP-2 and KC. However, restraint stress did not affect macrophage infiltration. Stress decreased the phagocytic abilities of phagocytic cells, yet it did not affect superoxide production. The cell surface expression of adhesion molecules CD11b and TLR4 were decreased in peripheral blood monocytes in stressed mice. The phenotype of macrophages present at the wound site was also altered. Gene expression of markers of pro-inflammatory classically activated macrophages, CXCL10 and CCL5, were down-regulated; as were markers associated with wound healing macrophages, CCL22, IGF-1, RELM $\alpha$ ; and the regulatory macrophage marker, chemokine CCL1. Restraint stress induced up-regulation of IL10 gene expression. Our study shows that stress did not promote a shift of the macrophage population, but did dysregulate the gene expression of markers of all three of the

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macrophage populations present during wound healing without a significant decrease in macrophage numbers. To modulate macrophage phenotype could provide a new therapeutic approach in the clinical treatment of wound under stress. Stress may impact macrophage activation by inducing the overall dysregulation of macrophage response during tissue repair via differential regulation of activation markers.

Regulation of gene expression can occur at the transcriptional and posttranscriptional level, such as via miR regulation. We identified 230 miRs as differentially regulated during stress-impaired wound healing. This study shows that stress alters miR expression of miR-21, miR-98, miR-132, miR-155, let7b and miR-146b, and affects mRNA levels of miR targets in inflammatory and proliferation phases of wound healing such as CXCL10, TGF $\beta$ R2, GHR, PGC-1 $\beta$ , NF $\kappa$ B, MyD88 and FGF7. This also suggests that dysregulation of miR expression under stress may participate in dysregulation of markers of macrophage phenotype.

We studied the effect of ameliorating miR levels on wound healing and the potential application of miR inhibitor as therapeutic treatment in stress-impaired wound healing. Inhibition of miR-98 by LNA-98 and its impact on miR-98 target GHR and GH signaling pathway were studied. GH signaling pathway is involved in bacterial clearance, inflammation and tissue growth. Treatment of stress group with LNA-98 significantly improved/restored levels of miR-98, and gene expression of GHR and genes induced by GH pathway (SCD1, Bcl-2, Myc, SOCS2). LNA-98 treatment enhanced wound closure and improved bacteria clearance under stress, which provides a novel and exciting therapeutic approach to ameliorate stress-impaired wound healing.

This work provides strong evidence that stress dysregulates expression of genes involved in the Inflammatory phase of wound healing, in part via miRs, and alters neutrophil and macrophage recruitment and functions leading to impaired bacterial

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clearance and wound healing. In addition, to modulate miR levels constitute a potential therapeutic treatment of stress-impaired wound healing.

#### **INTRODUCTION**

# 1. Wound healing:

When a tissue is damaged as a result of a surgical procedure or an accident, a complex series of cellular and molecular events occur at the wound site to prevent bacterial infection, to repair the damaged tissue and to restore its function. Wound healing requires the timely orchestration and efficient completion of four overlapping phases, namely hemostasis, inflammation, proliferation and resolution/remodeling. These events are interconnected, and rely on the successful execution of anterior events, thus dysregulation of one phase will have a domino effect on downstream events.. This interconnection highlights the importance of the early phases of wound healing, particularly inflammatory.

Whereas healing wound usually results in the recovery of the tissue structure and function with the formation of a scar, abnormal wound healing may lead to hypertrophic scarring, infection of the wound, or transition toward a state of chronic wound. The poor esthetic of hypertrophic scars may have psychological impact on the patient depending on the severity and location. Infections occur in 2-20% of surgery patients, depending on the site of surgery (Samson, 2004). Another negative outcome is the infection. Infection not only prolongs the discomfort of the patient but also increases the cost of the treatment by \$17,708 per patient and leads to extended hospital stays averaging approximately 10.2 days (Whitehouse et al., 2002; Zoutman et al., 1998). Finally, a wound can evolve into a chronic wound. In the United States alone, chronic wounds affect 6.5 million patients (Crovetti et al., 2004; Singer and Clark, 1999) with an overall cost of \$20 billion (AHRQ, 2001).

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Given the large number of surgical procedures performed every year, the number of victims of natural disasters and the need for wound care of veterans (Sen et al., 2009), a deeper understanding of normal and impaired wound healing is crucial to ameliorate therapeutic treatment of wounds.

#### a. Hemostasis:

Hemostasis starts immediately after tissue damage. Activated platelets aggregate. In addition, fibrinogen helps form a fibrin clot to stop local hemorrhage from the rupture blood vessels. This clot acts as a temporary matrix for growth factors to bind to and a support for infiltrating cells (Nurden et al., 2008). Activated platelets themselves secrete growth factors such as TGF $\beta$ , and promote angiogenesis, inflammation, and migration of keratinocytes and fibroblasts. In addition, growth factors and interleukins in the serum also contribute to the initiation of downstream wound healing phases.

### b. Inflammation:

The inflammatory phase starts minutes after injury and continues for approximately 48h. During this phase, dead tissues are removed, and bacteria present in the wound are cleared to protect from infection. Finally, the inflammatory phase promotes the proliferation phase.

After injury, endogenous products released from damaged cells and microbial products are recognized by resident tissue cells as activated. Activated cells produce interferons, pro-inflammatory cytokines (TNF $\alpha$ , IL-1, IL-6, IL-12) and chemokines (IL-8 and MIP-2), which induce surrounding cells to secrete more chemokines and adhesion molecules to recruit inflammatory cells to the site, including neutrophils and macrophages. Neutrophils and macrophages are cells from the innate immune system responsible for removing debris and clearing bacteria at the wound site as well as secreting mediators regulating wound healing. After injury, neutrophils and macrophages leave the peripheral blood to reach the wound. The active recruitment of neutrophils and

then macrophages from nearby vessels is orchestrated by signals from the resident cells and serum, and by foreign epitopes such as the lipopolysaccharides (LPS) of invading microorganisms (Eming et al., 2007). Together, these signals trigger local endothelial cell `activation' and thus, expression of selectins, which are adhesion molecules on the surface of endothelial cells. Selectins control the rolling and subsequent tethering of leukocytes to the vessel wall and subsequent crossing of the endothelial barrier (Yukami et al., 2007). This is enhanced by vessel dilation and an increase in vascular permeability that is triggered by inflammation-associated nitric oxide (NO), mast cellderived histamine, tissue plasminogen activator and other factors (Eming et al., 2007). Adhesion molecules on the phagocyte cell surface (e.g. CD11b) facilitate migration of recruited cells toward a gradient of chemoattractant leading to the inflammatory site. Neutrophils arrive first, following a trail of chemokines KC and MIP-2 in mice and IL-8 in humans (Engelhardt et al., 1998; Kernacki et al., 2000; Wetzler et al., 2000). Macrophages attracted by MIP-1 $\alpha$  and MCP-1, reach the wound shortly after (DiPietro et al., 1998; Maus et al., 2001). On site, neutrophils and macrophages clear bacteria primarily via phagocytosis. Cytokine, chemokine and the recognition of bacteria in the wound influence neutrophil/macrophage activation, which also affects macrophage phenotype and function altering their phagocytic abilities or pro-inflammatory response. This allows macrophages to adapt to the environment as healing progresses.

The elimination of bacteria in the wound is necessary for healing to follow its normal course. Failure to do so results in a prolonged pro-inflammatory response and the damage of bystander cells. Macrophages and neutrophils are responsible for removing bacteria. Neutrophils not only participate in bacterial clearance via phagocytosis, oxidative burst also helps kill bacteria via the formation of superoxide and reactive oxygen intermediates (ROIs). As a drastic resort neutrophil release the toxic contents of their granules while oxidative burst occurs simultaneously; proteases stored in theses granules have potent antimicrobial properties and serve to combat a diverse array of pathogens (Dovi et al., 2004; Nathan, 2006). Unfortunately, these highly potent agents do not discriminate between bacteria and host targets, thus damaging tissues. Macrophages also participate in bacterial clearance via phagocytosis and oxidative burst. Another important role for macrophages is to remove apoptotic neutrophils to limit collateral damages. Thus, macrophages accelerate the regression of the inflammatory response via the elimination of neutrophils, allowing progression to later phase. The inflammatory phase also promotes the following phases of wound healing, such as the proliferation phase, with neutrophils expressing several pro-inflammatory cytokines to activate local fibroblasts and keratinocytes.

Previous studies have shown that the appropriate recruitment and functions of neutrophils and macrophages are crucial for efficient removal of microbial agents (Bullard et al., 1996; Savill, 1997) and any factor tampering their recruitment and/or functions would potentially impair bacterial clearance.

c. Proliferation phase:

Proliferation, migration and contraction characterize the proliferation phase (2-10 days post-wounding) which results in the closure of the wound and replenishment of lost tissues. Proliferation and migration of keratinocytes from the wound margin result in reepithelialization of the epidermis (Martin, 1997). New blood vessels are synthesized to restore the vascular network (angiogenesis). In the dermis, the granulation tissue formed by fibroblasts replaces the fibrin clot (Hinz, 2007). Myofibroblasts, which are contractile cells derived from fibroblasts, bring the edges of a wound together and contribute to the synthesis, organization and alignment of collagen fibers (Hinz, 2007) of the mature scar. Growth factors secreted in the tissue regulate the cellular and molecular events of the proliferation phase. Fibroblast growth factor 7 (FGF7 or KGF2) stimulates fibroblast and keratinocyte proliferation. Growth hormone GH, when bound to its receptor GHR on cell surface such as neutrophils, macrophages and fibroblasts, triggers a signaling cascade that induces gene expression and tissue growth.

Another important factor is TGF $\beta$ . TGF $\beta$  is produced by a variety of different cells, including platelets, macrophages, fibroblasts and keratinocytes. In the proliferative phase, activated macrophages produce TGF $\beta$ , which plays an integral role in angiogenesis, by stimulating keratinocytes. These accelerate the rate of epithelialization and produce vascular endothelial growth factor (VEGF). The latter stimulates endothelial precursors to form a primitive vascular network, which is then remodelled to produce the complex capillary system that is essential for wound healing. TGF $\beta$  also induces wound fibroblasts to differentiate into myofibroblasts, which are essential for wound contraction. TGF $\beta$  stimulates the production of extracellular matrix components, and promotes collagen formation. The loss of TGF $\beta$  signaling in the dermis results in reduced collagen deposition and remodeling associated with a reduced extent of wound contraction (Martinez-Ferrer et al., 2010).

#### d. <u>Remodeling phase:</u>

The remodeling phase (weeks to months) is essential to restore the functions of the injured tissue. During this phase, proliferation and migration of keratinocyte come to an end. The newly formed blood vessels are refined and mature to form a functional network (Adams and Alitalo, 2007). The rough extracellular matrix deposited early in repair is remodeled to restore normal architecture. This remodeling is a delicate balance of collagen synthesis, bundling and degradation (for example, by matrix metalloproteinases). Myofibroblasts undergo apoptosis (Hinz, 2007). During the resolution/remodeling phase regression of a number of cell types occurs. Remnants of

the inflammatory response are cleared out. Neutrophils are cleared from the wound site, at least in part by apoptosis and phagocytosis by macrophages (Haslett, 1992). Some neutrophils and macrophages return to the vasculature. Macrophages are deactivated by anti-inflammatory cytokines, glucocorticosteroids, cell-cell contact or phagocytosis (Ma et al., 2003). Other strategies to help resolve inflammation include sequestration of pro-inflammatory chemokines and production of endogenous anti-inflammatory molecules such as resolvins (Schwab et al., 2007).

After normal completion of wound healing in adults, the repaired tissue does not exactly compare to its pre-wound state; a scar remains where the cellular organization of newly formed tissue differs and lacks epidermal appendages such as hair follicles and sebaceous glands. In addition, dysregulation of the remodeling phase affects the morphology of the scar. Defective arrest of the proliferative phase and/or incorrect resolution of the inflammatory phase can result in hyperproliferation, and/or prolonged inflammation which would ultimately induce fibrosis and exessive scar formation with the potential discomfort and esthetic challenges associated with it. From hemostasis to the remodeling phase, the normal time required to heal a wound varies depending on the size and location of the injury. The successful completion of each phase and the timely transition to the next stage of tissue repair occurs through the regulation of gene expression. Clearly, changes in gene expression play an essential role in regulating these processes. For example,  $TGF\beta$ , TLR4 or GH signaling pathways, when activated, induce transcription of genes involved in the regulation of wound healing.

# e. Growth Hormone Signaling Pathway:

The Growth Hormone (GH) signaling pathway is one of the many pathways participating in wound healing. Secreted GH binds to its receptor, growth hormone

receptor (GHR), and triggers a signaling cascade inducing gene expression. These molecules stimulate the proliferation and/or differentiation of a range of cells or tissues including fibroblasts which proliferate and produce collagen production in the skin (Tavakkol et al., 1992). Activation of GH signaling pathway via Jak2/STAT5 induces the transcription of genes important for cell cycle progression, cell growth, cytokine and growth factor-receptor signaling components (such as c-Myc, IGF-1, EGFR), and cell survival (Bcl-2). Previous work showed that excessive GH in human leads to overgrowth of various organs including skin (Melmed, 1990), whereas GH deficiency or GHR loss (Laron's syndrome) results in short stature and other phenotypic disturbances (Maheshwari et al., 1998; Rosenfeld et al., 1994). In addition, elevation of serum GH led to enhanced wound healing and accelerated the healing of skin graft donor sites in burn patients (Herndon et al., 1995). This serum elevation emphasizes the role of GH signaling pathway in tissue growth and repair, which characterizes the proliferation phase. Interestingly, the GH signaling pathway is also involved in the inflammatory phase of healing; the pathway affects cytokine production, primes neutrophils and stimulates superoxide production and phagocytosis (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). Thus the GH signaling pathway participates in an inflammatory response and proliferative phase of tissue repair, and the regulation of this pathway is sensitive to multiple factors including the stress hormone glucocorticoid, age, sex hormones, and nutrition (Jux et al., 1998).

#### f. Factors affecting wound healing:

As reviewed by Guo and DiPietro (2010), a plethora of factors can alter the adequate succession of events leading to complete healing. These factors can be divided in two categories: local and systemic factors. Local factors include oxygenation of the wound, infection, the presence of a foreign body and venous sufficiency. Age and gender are

amongst systemic factors influencing wound healing. Engeland et al. reported that aging slowed healing of oral mucosa in subjects between 50 to 88 years old as compared to subjects 18 to 35 years old (2006). The same study also showed delayed healing in females versus males. Diseases (diabetes, fibrosis), obesity and immunocompromised conditions, including cancer and AIDS, also impair healing. Chronic wounds are often associated with diseases such as diabetes or obesity and affect 6.5 million patients (Crovetti et al., 2004; Singer and Clark, 1999) in the United States alone. Behavior related factors such as nutrition, alcohol consumption and smoking can be detrimental to healing. Fitzgerald et al. reported that acute ethanol consumption before injury altered early dermal inflammatory responses including neutrophil activity and production of MIP-2, KC, and IL-1 $\beta$  in mice (2007). Last but not least, stress has been shown to delay wound healing. These factors can act at the transcriptional level to activate or repress gene expression, including glucocorticoid-glucocorticoid receptor complexes, which directly bind to DNA, or can act at the post-transcriptional level by the regulation of mRNA stability and its translation. Finally some factors can modulate a cellular response via proteins, by activating, inhibiting or sequestering their target protein.

Wound healing is a complex series of events closely intertwined, tightly regulated, where "the beginning [the inflammatory phase] programs the end" (Serhan and Savill, 2005). The inflammatory phase sets the stage for downstream phases and macrophages are hallmarks of inflammation. Modulating the inflammatory phase via the regulation of macrophage activation would potentially affect downstream events and wound healing overall.

### 2. Macrophage Activation:

Macrophages are the hallmark of inflammatoy phase and they also participate in later phases of wound healing. Macrophages kill pathogens present at the wound site, and remove necrotic tissue and apoptotic cells; they promote and help resolve inflammation, contribute to cell proliferation, differentiation, migration and participate in angiogenesis (Mirza et al., 2009). In addition, macrophages promote matrix remodeling. Several studies have analyzed skin wound healing during macrophage depletion (Mirza et al., 2009). Mice depleted before injury show impaired re-epithelialization, granulation tissue formation, angiogenesis, wound cytokine production and wound contraction. The multifunction of macrophages renders them key partners of the innate immune response during wound healing.

Far from being a homogeneous population, macrophages display a wide range of phenotypes and physiological properties depending on the signal(s) inducing their activation (recognition of pathogen, cytokine and chemokines) (Gordon & Taylor, 2005; Martinez et al., 2006).

### a. Populations of activated macrophages:

Activation signals induce distinct gene expression profiles as macrophages acquire targeted functions according to local stimuli (Martinez et al., 2006). Activated macrophages can be classified into three groups depending on the signal(s) triggering their activation: Classically Activated Macrophages (CAM), Wound Healing Macrophages (WHM) and regulatory macrophages (Edwards et al., 2006; Mosser and Edwards, 2008). Each sub-population exhibits specific markers and functions.

Classically activated macrophages (CAM) are induced by cytokines and the recognition of microbial patterns via Toll-like receptors (TLR) (Padgett et al., 1998b). These CXCL10 expressing macrophages secrete pro-inflammatory cytokines (TNFα, IL-

 $1\beta$ , IL-6 or IL-12) and efficiently kill pathogens by phagocytosis and production of NO and ROS (Martinez et al., 2008). CAM promote inflammation, pathogen killing and debris removal. They are vital components of host defense, but their activation must be tightly controlled because the cytokines and mediators they produce can lead to host-tissue damage.

Wound healing macrophages (WHM) arise in response to interleukin-4 (IL-4) and are less proficient than CAM to clear bacteria. However, WHM secrete components of the extracellular matrix and express numerous markers of tissues remodeling which are important during proliferation and remodeling phases of wound healing (Resistin Like Molecule Alpha (RELM $\alpha$ ), Insulin Growth Factor 1 (IGF-1)). WHM also mediate the winding down of inflammatory response (Rodero and Khosrotehrani, 2010).

Regulatory macrophages are generated in response to various stimuli, including TLR and immune complexes, prostaglandins and glucocorticoids. These regulatory cells need two stimuli to induce their anti-inflammatory activity. Regulatory macrophages promote the resolution of inflammation, and express CCL1 and IL-10 a potent anti-inflammatory cytokine (Sironi et al., 2006). Unlike wound healing macrophages, regulatory macrophages do not contribute to the production of the extracellular matrix. Macrophage activation phenotype is not static; macrophages retain their plasticity and are responsive to their environment, which allows them to adapt their phenotype and expression profile to progress with the wound (Daley et al., 2010; Rodero and Khosrotehrani, 2010). Dysregulation of macrophage phenotypes has been shown to contribute to impaired healing (Mirza and Koh, 2011).

b. <u>Macrophage activation during wound healing:</u>

Analysis of the temporal profiles of macrophage activation markers suggests that both classically activated and alternatively activated macrophages are present early during wound healing, whereas wound healing and regulatory macrophages are predominant later in repair (Deonarine et al., 2007).

Recent work on aseptic wounds using an implantable sponge model described wound macrophages as cells expressing both classical activation markers, such as TNF $\alpha$ , and markers of non-classical activation such as mannose receptor (Daley et al., 2010). One day post-wounding (D1) macrophages produced more TNF $\alpha$  and IL6 and less TGF $\beta$  as compared to day 7 macrophages suggesting a transition from an inflammatory to a regulatory or tissue remodeling state. However, it is important to consider that neither IL-4 nor IL-13 were detectable in this model. Impaired wound healing often exhibits a defect in the progression from the inflammation to tissue formation stage. Loots et al reported increased infiltration of chronic and diabetic wounds by wound-associated macrophages compared to control wounds (Loots et al.). Similarly, in diabetic db/db mice, wounds are characterized by a prolonged inflammatory response and the persistence of the wound healing macrophages (Wetzler et al., 2000).

In summary, the environmental conditions influence the phenotype of the macrophages and their contribution during wound healing. This plasticity allows macrophages to participate in homeostatic processes, such as tissue remodeling as well as in host defense (Stout and Suttles, 2004). However, each of these types of activation can have potentially negative consequences if not appropriately regulated. Moreover, the studies presented above sustain the hypothesis that modulating the environment of macrophages recruited at the wound site might affect their phenotype and function and contribute to improve impaired wound healing. Because of their plasticity and the central position of macrophages during wound healing, they appear as ideal targets for clinical interventions in impaired wound healing.

Macrophage activation of a specific population, like other events in wound healing, is accomplished through the regulation of gene expression. Gene regulation can

occur at the transcriptional or post-transcriptional levels. Interestingly, microRNAs (miRs) have emerged as powerful post-transcriptional regulators.

### 3. microRNAS:

MicroRNAs (miRs) are small endogenous non-coding RNAs (approximately 22 nt) derived from the intergenic regions or introns regions. Mature miRNAs are produced from long primary transcripts that contain the pre-miRNA, through a series of maturation steps. Most miRNAs are transcribed by RNA Polymerase II as long primary miRNA transcripts (pri-miRNA) with 5' caps and 3'poly(A) tails. The pri-miRNAs are processed into corresponding pre-miRNA stem-loops of ~60 nucleotides in length by the nuclear RNase III enzyme Drosha and its partner DGCR8 (DiGeorge syndrome critical region gene 8). The pre-miRNAs are actively transported to the cytoplasm by Exportin-5 and are cleaved into ~22-nucleotide duplexes by the cytoplasmic RNase III enzyme Dicer. The resulting double-stranded miRNA duplexes are processed further with one of the strands ("the guided strand") being selectively incorporated into the RNA-induced silencing complex (RISC) while the other strand (referred to as miRNA\*) may be degraded.

MiRs bind to the complementary sequence in the 3'UTR of target mRNA(s) and can prevent protein synthesis by triggering the degradation of target mRNAs (Lim et al., 2005) and/or inhibiting their translation (Jackson and Standart, 2007; Pillai et al., 2007). A major determinant of this intermolecular interaction is the "seed", a short 6-8nt stretch at the 5'end of the miR.

Each miR can regulate multiple genes and multiple miRs can target the same transcript, which makes the miR regulation network extremely intricate. MiRs constitute potentially widespread regulatory mechanisms with key roles in diverse regulatory pathways, including immune regulation, cell proliferation, cell migration and apoptosis (Asirvatham et al., 2008; Sonkoly et al., 2008). These events are part of the healing process, which suggests that miRs participate in gene regulation during wound healing.

MiR expression is tissue and cell specific. MiR-146, absent in keratinocytes and dermal fibroblasts, is expressed in immune cells in the skin, whereas miR-125b and miR-21 are expressed in both structural and inflammatory cells (Liu et al., 2009; Sand et al., 2009; Zavadil et al., 2007). These miRs have been of particular interest for investigations associated with inflammatory and immune responses. For example, miR-21, miR-146 and miR-155 were induced by pro-inflammatory stimuli as IL-1 $\beta$ , and TLRs (Sheedy and O'Neill, 2008). In addition, recent studies have shed light on miRs involved in later events of wound healing such as angiogenesis. Two research teams explored the role of Dicer and found the enzyme is a key player during angiogenesis *in vivo* and *in vitro* (Kuehbacher et al., 2008; Suarez et al., 2007). Yet the role miRs in the regulation of genes involved in early phases of wound healing such as inflammation and proliferation largely remains unexplored.

The effects of miRs regulating the expression of multiple genes involved in key physiological events are reminiscent of the broad effects of stress observed on gene regulation. Similarly to miRs, stress dysregulates the expression of a plethora of genes in a systemic manner, including genes involved in wound healing healing such as IL-1 $\beta$  (Hayashi et al., 2004; Padgett et al., 1998a).

### 4. Stress:

Multiple factors can derail the course of successful wound healing, and studies in humans and animals have shown that stress impairs wound healing. Stress can be defined as the perception by the brain of a potential or actual threat (stressor) that requires immediate changes in behavior and physiology to survive (Dhabhar and McEwen, 1997). Stress triggers a neuroendocrine response by activating the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis.

The ANS provides the most immediate response to stressor exposure through its sympathetic (including the sympatho-adreno-medullary axis-SAM) and parasympathetic arms, which induces rapid alterations in physiological states through neural innervation of end organs. ANS is characterized by the quick fight-or-flight response via the sympathetic nervous system, which is short-lived due to parasympathetic counterbalancing effects. Stimulation of SAM increases circulating levels of adrenaline (primarily from the adrenal medulla) and noradrenaline (primarily from sympathetic nerves), heart rate and force of contraction, peripheral vasoconstriction, and energy mobilization and immune response preparing the body to react to threat. Stress can also modulate the parasympathetic response mediated by acethylcholine, which often have opposite effects.

Activation of the hypothalamic-pituitary-adrenocortical (HPA) axis results in elevations in circulating glucocorticoids. Peak plasma glucocorticoid levels occur tens of minutes after the initiation of stress. The HPA endocrine response to stress is slow compared to SAM actions but it induces longer-lasting changes. For the HPA axis, stressor exposure activates neurons in the paraventricular nucleus of the hypothalamus that secrete releasing hormones, such as corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), into the portal circulation of the median eminence. These releasing hormones act on the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH), which in turn acts on the inner adrenal cortex to initiate the synthesis and release of glucocorticoid hormones (GCs) influencing the immune system, digestion, emotions, and energy storage.

Interestingly, glucocorticoids potentiate numerous sympathetically mediated effects, such as peripheral vasoconstriction. Moreover, the adrenal cortex is directly innervated by the sympathetic nervous system, which can regulate corticosteroid release. Both systems, HPA and SAM, participate in the cross-talk between the brain and the immune system during stress response via the regulation of gene expression directly or indirectly.

The duration and intensity of the stressor promotes different physiological responses for the body to adapt. Whereas short intense (acute) stress can potentially have positive effects, long-term (chronic) stress is physiologically detrimental and impacts the immune system.

# a. Stress and Immunity:

Stress has been shown to have detrimental effects on the innate and adaptive immune system. The sympathetic nervous system (SNS), and the parasympathetic nervous system generally inhibit inflammation at a regional level, through innervation of immune organs. Activation of the SNS that occurs following stressful stimuli is associated with decreased NK-cell activity (Benschop et al., 1993). Furthermore, large release of noradrenaline can be immunosuppressive (Chelmicka-Schorr et al., 1988). In vitro, noradrenaline mediates its suppressive effects on monocytes by inhibiting the production of pro-inflammatory cytokines, including TNF, IL-1, IL-6 and IL-12. It also upregulates the production of anti-inflammatory cytokines, such as IL-10, from these cells (Maestroni and Mazzola, 2003; van der Poll et al., 1994). Interestingly, depletion of noradrenaline has been shown to reduce the resistance to some bacterial infections (Straub et al., 2000).

In stress-induced parasympathetic response, activation of cholinergic receptors on macrophages inhibits NFkB signaling, thereby inhibiting pro-inflammatory cytokine production (Wang et al., 2003). In addition to the autonomic nervous system, the HPA axis provides an important physiological feedback loop of inflammation through the antiinflammatory effects of glucocorticoids. Glucocorticoids act on immune cells both directly via binding of the GC to its receptor, GR, and repression of gene expression, and particularly the suppression of inflammation. They inhibit the production of proinflammatory cytokines, such as IL-1 and TNF $\alpha$ , while promoting the production of antiinflammatory cytokines, such as IL-10, by macrophages and dendritic cells. They also promote apoptosis of macrophages, dendritic cells and T-cells, leading to inhibition of immune responses. Previous reports have shown that stress modulates neutrophil and macrophage recruitment, chemokine gene expression as well as the cell surface expression of adhesion molecules (Curry et al., 2010; Filep et al., 1997; Heasman et al., 2003; Mizobe et al., 1997; Viswanathan and Dhabhar, 2005; Zhang et al., 1998) In addition, stress was reported to alter neutrophils and macrophages anti-microbial functions (Ehrchen et al., 2007; Khanfer et al., 2010; Palermo-Neto et al., 2003). Given the broad anti-inflammatory effects of stress mentioned above, its impact on the inflammatory phase of wound healing and of downstream events is ineluctable.

b. Stress and Wound Healing:

A previous study on the healing of caregivers for Alzheimer's patients, whose responsibilities lead to a state of chronic stress, showed that caregivers took 24% longer to heal dermal wounds on their arms compared to their age-matched controls (Kiecolt-Glaser et al., 1995). Similarly, dental students healed mucosal wounds 40% slower during examination compared to their healing rate during summer break (Marucha et al., 1998). Stress up-regulates GCs and reduces pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  levels at the wound site and decreases the production of chemoattractants to initiate inflammation. Gosain et al. showed that the norepinephrine modulates the inflammation and proliferative phases of wound healing (2006). In addition, restraint stress impairs early wound healing in mice via alpha-adrenergic, but not beta-adrenergic

receptors (Eijkelkamp et al., 2007). Stress suppresses cell differentiation and proliferation, promotes the production of anti-inflammatory agents and negatively impacts angiogenesis. Finally, stress leads to impaired matrix deposition. Thus, stress dysregulates each and every stage of wound healing.

In the murine model of stress by restraint, Padgett et al showed a delay in wound closure of 27% (Padgett et al., 1998a). Similar to human studies, the restraint-stress mice model exhibits lower gene expression of IL-1 $\beta$  and FGF7 one day post-wounding, and higher expression of IL-1 $\alpha$  and IL-1 $\beta$  at five days (Mercado et al., 2002). In addition, restraint stress increased susceptibility to opportunistic infection. Rojas et al (2002), reported that even though bacteria levels were equal at the time of wounding and 6 hours post-wounding, under stress, bacterial clearance was impaired during wound healing leading to an increased bacteria load in the wound as early as D1 and up to 3 log higher in stressed group at D5. Blocking the HPA axis by treatment with GC antagonist RU486 resulted in partially ameliorating stress-impaired wound healing. Treatment with RU486 restored IL-1 $\beta$  gene expression one day post-wounding but had only a partial effect on the bacterial clearance (Mercado et al., 2002; Rojas et al., 2002).

As SNS might also participate in bacterial clearance (Maestroni and Mazzola, 2003), SNS-related stress response was blocked using antagonist either alone or in conjunction with blockade of HPA axis. Under these conditions, the inflammatory response and bacterial clearance were partially restored. These studies suggest that in addition to the induction of HPA and SNS axis, stress might have an effect on a broad regulatory system contributing to impair wound healing; miRs regulation could be such a system.

# c. Stress and miRs:

Stress is known to have adverse physiological effects that contribute to cardiovascular disease, impaired immune function, inflammatory diseases, and impaired neural function

and behavior (Grippo and Johnson, 2009; Sapolsky *et al.*, 2000). Similarly, miRs have been shown to regulate a broad spectrum of physiological events, and miRs were reported to play a role in cellular responses to stress.

Rinaldi et al. reported a significant increase in the expression levels of miR-9, miR-26a/b and let-7a in the frontal cortex after acute stress while only minor changes were observed after repeated restraint (Rinaldi et al., 2010). Another study from Mele's team observed that miR-132 level is upregulated in hippocampus during chronic immobilization stress, but not in acute stress (Rinaldi et al., 2010). Interestingly, miR-132 not only contributes to the cholinergic stress reaction in the brain, but miR-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase in the periphery (Shaked et al., 2009). In peripheral blood, Gidron's human study showed that brief academic stress alters expression of miR-21 and let-7b (2010). Reciprocally, miRs can modulate glucocorticoids and the glucocorticoid receptor (GR) function. MiR-18 and miR-124a downregulate the GR protein in the paraventricular nucleus in Fischer 344 (F344) (Vreugdenhil et al., 2009). Yet the effect of stress on miR expression during wound healing remains unexplored.

If recent work has shown that stress can influence miRs expression (the mechanism by which still remains unexplored), reciprocally microRNAs can modulate glucocorticoids and the glucocorticoid receptor (GR) function (Rainer *et al.*, 2009). Indeed, miR-18a downregulates GR protein in the paraventricular nucleus in Fischer 344 rats (Uchida *et al.*, 2008). Vreugdenhil *et al.*, found that miR-18 and miR-124a reduce GR-mediated events and decrease GR protein levels (2009). Studies investigating the effect of stress on miRs and their downstream targets are sparse and how stress affects miR expression and miR regulation of downstream target genes involved in wound healing remains to be explored.

#### 5. Purpose of the Present Study:

Wound healing is a complex series of events that rely on the successful completion of the previous phase, which means that the completion of the inflammatory phase is crucial. The goal of this study is to further investigate how stress regulates inflammation during wound healing to impair bacterial clearance.

As previously stated, macrophages are hallmarks of inflammation, and their primary function is to clear bacteria and prevent infection of the wound in conjunction with neutrophils. Macrophage function is determined by activation signals that trigger gene expression of specific markers. Stress has been shown to differentially regulate a broad spectrum of physiological events including immune functions and healing. Stress can alter these events through its affects on gene expression either at the transcriptional level or through posttranscriptional regulation. MiRs constitute a potentially widespread post-transcriptional regulatory mechanism with key roles in diverse regulatory pathways, including pathways involved in wound healing.

Overarching Hypothesis: stress dysregulates expression of genes involved in the Inflammatory phase of wound healing, in part via miRs, and alters neutrophil and macrophage recruitment and functions leading to impaired bacterial clearance and wound healing.

Specific Hypothesis Chapter 1: restraint stress alters neutrophil and macrophage recruitment and/or functions during wound healing, therefore impairing bacterial clearance.

Specific Aim Chapter 1: to determine the effect of stress on neutrophils and macrophages recruitment and/or functions during wound healing.

Specific Hypothesis Chapter 2: stress affects gene expression during the inflammatory response in part by altering expression of miRs that globally regulate inflammation during would healing.

Specific Aim Chapter 2: to identify miRs differentially regulated by stress during wound healing and assess their downstream targets.

Specific Hypothesis Chapter 3: to modulate miRs differentially regulated under stress to restore their expression reestablishes gene expression of their target and ameliorates wound healing.

Specific Aim Chapter 3: to determine the effect of ameliorating miR levels on wound healing and the potential application of miR inhibitor as therapeutic treatment in stress-impaired wound healing.

### CHAPTER 1

# RESTRAINT STRESS ALTERS NEUTROPHILS AND MACROPHAGES PHENOTYPES DURING WOUND HEALING

# 1. Introduction

Wound healing requires the timely orchestration and efficient execution of three major overlapping phases: inflammation, proliferation and resolution/remodeling. These phases prevent bacterial infection, repair the damaged tissue and restore tissue function.

Unfortunately many factors, including stress, can hinder a successful outcome. Stress delays wound healing and impairs bacterial clearance. Whether the wound is caused by an accident or a surgical procedure, inefficient removal of bacteria at the site of an injury elevates the risk for opportunistic infection. Infection can potentially prolong discomfort to the patient, increase the cost of wound treatment and extend the hospital stay. Neutrophils and macrophages are innate immune system cells responsible for bacterial clearance at the wound. Previous studies have shown that the appropriate recruitment and functions of these cells are crucial for efficient removal of microbial agents (Bullard et al., 1996; Savill, 1997).

After injury, neutrophils and macrophages leave the peripheral blood to reach the wound. Adhesion molecules on the cell surface (e.g. CD11b) facilitate their migration toward a gradient of chemoattractant leading to the inflammatory site. Neutrophils arrive following a trail of chemokines KC and MIP-2 (Engelhardt et al., 1998; Kernacki et al., 2000; Wetzler et al., 2000). Macrophages recruited by MIP-1 $\alpha$  and MCP-1, reach the wound shortly after (DiPietro et al., 1998; Maus et al., 2001). At the wound site,

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neutrophils and macrophages clear bacteria using oxidative burst and phagocytosis. The phenotype and function of the macrophages vary depending on how they are activated. Both pathogen recognition as well as the cytokine and chemokines in the environment shape macrophage activation. Activated macrophages can be classified in three groups: classically activated macrophages (CAM), wound-healing macrophages (WHM) and regulatory macrophages (Edwards et al., 2006; Mosser and Edwards, 2008). Each subpopulation exhibits specific markers and functions. Classically activated macrophages (CAM) are induced by recognizion of microbial patterns via Toll-like receptors (TLR) (Padgett et al., 1998) and cytokines. These pro-inflammatory macrophages express CXCL10 and efficiently kill pathogens (Martinez et al., 2008). Wound-healing macrophages (WHM) arise in response to interleukin-4 (IL-4) and are less proficient than CAM to clear bacteria. However, WHM secrete components of the extracellular matrix and express numerous markers of tissue-remodeling which are important during the proliferation and remodeling phases of wound healing (Resistin Like Molecule Alpha (RELM $\alpha$ ), Insulin Growth Factor 1 (IGF-1)). WHM also mediate the diminishing of the inflammatory response (Rodero and Khosrotehrani, 2010). Finally, regulatory macrophages show anti-inflammatory properties, which help the resolution of inflammation. These cells express CCL1 and IL-10, a potent anti-inflammatory cytokines (Sironi et al., 2006). The macrophage phenotype is not static; macrophages retain their plasticity and are responsive to their environment, allowing them to adapt their phenotype and expression profile to progress with the wound (Daley et al., 2010; Rodero and Khosrotehrani, 2010).

Given the crucial role of neutrophils and macrophages in the removal of bacteria, any factor diminishing their recruitment and functioning could impair bacterial clearance. Previous reports have shown that stress modulates neutrophil recruitment, macrophage recruitment, chemokine gene expression and the cell surface expression of adhesion molecules (Curry et al., 2010; Filep et al., 1997; Heasman et al., 2003; Mizobe et al., 1997; Viswanathan and Dhabhar, 2005; Zhang et al., 1998) In addition, stress was reported to alter neutrophil and macrophage microbicidal functions (Ehrchen et al., 2007; Khanfer et al., 2010; Palermo-Neto et al., 2003). Previous studies in a murine model of cutaneous healing showed that restraint stress increased susceptibility to opportunistic infection. Rojas et al (2002), reported that even though bacteria levels were equal at the time of wounding and 6 hours post-wounding, under stress, bacterial clearance was impaired leading to an increased bacterial load in the wound as early as day 1 and up to 3 log higher day 5 post-wounding.

We hypothesized that restraint stress alters neutrophil and macrophage recruitment and/or functions during wound healing, thereby impairing bacterial clearance. The effect of restraint stress on neutrophil and macrophage recruitment and chemokine gene expression during wound healing was investigated. Anti-microbial functions of neutrophils and macrophages were assessed. The expression of cell surface markers on macrophages that are involved in cell adhesion and bacteria recognition was examined. Finally, we explored how restraint stress affected the sub-populations of activated macrophages participating in wound healing.

### 2. Material and Methods

**Animals.** Eight-week old female SKH-1 mice obtained from Charles Rivers, Inc. (Wilmington, MA) were allowed to acclimate for 7-10 days. Animals were handled according to a protocol approved by the Institutional Animal Care and Use Committee.

**Wounding and Tissue harvest.** Mice were anesthetized with an i.p. injection of 250µL of ketamine-xylazine-saline solution (ratio 4:1:35) consisting of ketamine 100mg/Kg and xylazine 5mg/Kg. The dorsal skin was cleaned with alcohol pads and two

full-thickness wounds were created on their backs using a sterile 3.5mm biopsy punch (Miltex Inc.). Wounds were harvested with a 6mm biopsy punch (Miltex Inc.) on anesthetized mice before euthanasia.

**Restraint Stress.** Mice were stressed by restraint in well-ventilated 50mL conical tubes for 12 hours/cycle for three cycles prior to wounding, and five additional cycles after wounding as previously described by Gajendrareddy et al, (2005). Since animals in the tubes did not have access to food and water, control mice were deprived of food and water during the same 12 hours for 8 days, but were allowed to roam free.

**Myeloperoxidase (MPO) Assay.** As described by Zhou et al (1996), harvested wounds were homogenized in 1mL of 50mM sodium phosphate buffer, pH 6.0, with 0.5% HTAB (Sigma). Homogenates were centrifuged at 12,000g for 20 min and underwent 3 cycles of freeze/thaw for MPO extraction. Supernatants were mixed 1:15 with 80mM sodium phosphate buffer pH 5.4, containing 16mM TMB (Sigma) previously dissolved in dimethylformamide (Sigma). Reactions were started by adding 0.03% oxygen peroxide, incubated for 2min at 37°C, and stopped by adding 200mM sodium phosphate buffer pH 3.0. Absorbance was measured at 650nm for each sample. Units of MPO per wound were determined by regression analysis using a standard curve determined with commercial MPO (Sigma), with concentrations of 0.5-0.0025 units/mL (Schierwagen et al., 1990).

**Macrophage quantification.** Formalin-fixed wounds were sectioned at  $5\mu$ m and incubated overnight at 4°C with rat-anti-mouse CD11b/CD18 antibody at 1:800 (Pharmingen) (Ho and Springer, 1983). Anti-rat Vectostain ABC kit was used following the manufacturer's protocol to allow for detection. The tissues were counterstained with hematoxylin. A blinded observer counted the number of CD11b/CD18 positive and total cells in three different fields (magnification x40) for each section. The average

percentage of CD11b/CD18 + cells was determined and expressed as mean ±SEM as previously described (Engelhardt et al., 1998).

mRNA analysis. Harvested wounds were stored in RNAlater® (Sigma) at 4°C and later homogenized in TRIzol<sup>®</sup> (Invitrogen) using a Tissue-Tearor (Cole-Parmer). Total RNA was extracted and reverse transcription performed using SuperScript<sup>™</sup> First Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR primers and probes were purchased from Applied Biosystems TagMan®Gene Expression Assays for mouse MIP-1α (#4331182-Mm00441259 g1) MIP-2 (#4331182-Mm00436450 m1), MCP-1 (#4331182- Mm00441242\_m1), KC (#4331182-Mm04207460\_m1), CXCL10 (#4331182-Mm00445235 m1), CCL5 (#4331182-Mm01302427 m1), CCL22 (#4331182-Mm00436439 m1), RELMα (#4331182-Mm00445109 m1), IGF-1 (#4331182-Mm00439560 m1), CCL1 (#4331182-Mm00441236 m1), IL10 (#4331182-

Mm00439614\_m1) and CD68 (#4331182-Mm03047340\_m1). Amplification of target cDNA was accomplished using the ABI Prism 7000 Sequence Detection System.

**Phagocytic activity and superoxide production.** Blood was collected by cardiac puncture and aliquoted 0.5mL in 5mL tubes containing final concentration of 10 units/mL of heparin (Elkins-Sinn, Inc.). Phagotest <sup>®</sup> was used according to the manufacturer's protocol to quantify the phagocytic activity of monocytes and granulocytes. This protocol was modified to load neutrophils and macrophages with the reagent to measure oxidative burst by adding dihydroethidium (DHE) (Biotium, Inc.) at 2.5µg/mL and incubating for 30min at 37°C on a shaker. Live YFP-*E.coli* (kind gift from Dr. Ye from the College of Medicine, UIC) was added to test the phagocytic activity. Fluorescence was quenched by adding Trypan blue pH 7.0 at 4mg/mL for one minute to each tube before analysis with Beckman Coulter Cyan II.

**Cell surface markers analysis.** Blood was collected as previously described. The appropriate amounts of conjugated antibodies were added to the tubes (F4/80-APC, CD284-PE, CD11b-FITC (eBioscience), CD206-Alexa Fluor®488 (BioLegend); CD68-Alexa Fluor®700 (ABD)) following the manufacturer's protocol and incubated on ice for 30min. The samples were washed with 1mL PBS, and centrifuged for 2min at 2000rpm. Each pellet was resuspended in 0.5mL of 4% paraformaldehyde and analyzed using Beckman Coulter CyAn II.

Immunohistochemistry and Laser Capture Microdissection. Harvested wounds were embedded in HistoPrep<sup>™</sup>(Fisher Scientific) and stored at -80°C. Frozen sections were cut at 6µm thickness and mounted on MembraneSlides PEN-Membrane 2,0µm (MicroDissect GmbH). The Trogan et al (2002) staining protocol was followed for immunochemistry staining of macrophages using rabbit anti-mouse CD68 antibody (Abbiotec) and against rabbit Vectastain Elite ABC kit (Vector Laboratories, Inc.). The macrophages were microdissection using a Leica LMD7000 Laser Microdissection System. Cells harvested by laser dissection were processed for qRT-PCR using RNAqueous®-MicroKit (Ambion Inc).

**Statistical Analysis.** Statistics were performed using SPSS. Differences between groups over time were assessed using repeated-measurement ANOVA. Univariate ANOVA analysis was performed to compare between 2 groups at a single time point. Data represented as mean  $\pm$  SEM from 3 or more experiments. Statistical significance was determined at  $p \le 0.05$  (\*), and *p*-values approaching significance (0.05<*p*<0.1) were represented as # (ANOVA).
#### 3. Results

### a. Neutrophils accumulate in the wound site under restraint stress.

To assess the effects of stress on neutrophil recruitment, myeloperoxidase (MPO), a marker of neutrophil infiltration, was quantified by a colorimetric assay in unwounded tissue and in excisional wounds at 6h, day 1, day 3, day 5 and day 7 post-wounding. Low levels of MPO were detected in unwounded skin (Day 0) from control and stressed mice (Fig.1A). In control mice, peak MPO levels were found at day 1 (D1) after wounding, with similar levels in stressed mice. At day 3, stressed mice had significantly increased levels of MPO, while MPO in control started to decline ( $p \le 0.05$ ). At day 5 (D5) post-wounding, MPO levels were elevated 4 fold ( $p \le 0.05$ ) in stressed mice as compared to control mice. By day 7, MPO levels had returned to near control levels in both control and stressed mice.

b. Gene expression of neutrophil-attracting chemokines increases during stressimpaired wound healing.

To determine whether altered kinetics of neutrophil recruitment was due to chemokine expression, MIP-2 and KC mRNA levels were measured by qRT-PCR in unwounded tissue, and wounds excised at day 1 and day 5 post-wounding in both control and stressed groups.

MIP-2 expression increased during the peak of neutrophil recruitment (D1) in controls, and went back to basal level after 5 days (Fig.1B). MIP-2 gene expression was significantly higher in stressed groups as compared to control groups at D5. KC expression level did not significantly vary over time in control groups during wound healing (Fig.1C). Similar to MIP-2, KC gene expression increased in stressed mice, approaching significance at D5.



Figure 1. Neutrophil accumulation and gene expression of chemokines MIP-2 and KC.

(A) (In manuscript but part of Gina Rojas' thesis 2001) Excised wounds of 6h, D1 to D7 and control skin were subjected to myeloperoxidase (MPO) extraction. MPO was quantitated by a colorimetric assay and expressed as units of MPO/gram of wound tissue. Data represent mean  $\pm$  SEM from 2 polled experiments. At each time-point, n=7-12 mice/group, except for D7 and control skin (n=3-5). \* p≤0.05 compared to control (ANOVA). (B-C) Unwounded skin and wounds were harvested at D1 and D5 days. The gene expressions of MIP-2 (B) and KC (C) were measured and compared to GAPDH using qRT-PCR. Data represent mean  $\pm$  SEM from 4 experiments. At each time point n=20 mice/group. \* *p*≤0.05 compared to control, # 0.05<*p*<0.1 (ANOVA).



**Figure 2**. <u>Macrophage recruitment and gene expression of chemokines MCP-1 and MIP-1a</u>. (A) (In manuscript but part of Gina Rojas' thesis 2001). Wounds excised from D1 D7 were sectioned and immunostained with an antibody against CD11b/CD18. Circles represent CD11b/CD18+ cells (**O** for control, for **o** stressed group. Squares represent Average total cells/field ( $\Box$  for control, and **e** for stressed group). Data represent mean number of cells and mean percentage of CD11b/CD18+ cells per counting field (±SEM) from 2 polled experiments. At each time point n=6-7 mice/group, except for D7 (n=3). Unwounded skin and wounds were harvested at D1 and D5 days. The gene expressions of MCP-1 (B) and MIP-1a (C) were measured and compared to GAPDH using qRT-PCR. Data represent mean ± SEM from 4 experiments. At each time point n=20 mice/group. No significant group differences were found.

c. Restraint stress does not affect macrophage recruitment or the expression of macrophage chemokines at the wound site.

To assess the effects of restraint stress on macrophage recruitment, wounds excised at day 1 through day 7 post-wounding were prepared for immunostaining with monoclonal antibody against murine CD11b/CD18, a marker of activated macrophages. CD11b/CD18 positive (CD11b/CD18+) and total cells were counted, and the average percentage of CD11b/CD18 + cells was determined. Both control and stressed mice had similar macrophage wound infiltration (Fig.2A). The expression profiles of macrophage chemokines MCP-1 and MIP-1 $\alpha$  were examined by qRT-PCR (Fig.2B-C). MCP-1 mRNA level did not significantly vary over time in either control or stressed groups. MIP-1 $\alpha$  relative gene expression increased at D1 compared to unwounded tissue and D5 tissues in control; nevertheless, restraint stress did not affect the expression of MCP-1 and MIP-1 $\alpha$ .

d. Restraint stress decreases phagocytic activity but does not affect oxidative burst during wound healing.

Phagocytosis and oxidative burst are crucial functions of phagocytic cells for killing pathogens. Phagocytosis activity was assessed by measuring the mean fluorescence intensity emitted by ingested YFP-*E.coli* incubated with whole blood harvested one, three and five days post-wounding (Fig.3). Interestingly, the mean fluorescence intensity was significantly reduced by 18-30% in stressed mice at all time points as compared to control, suggesting that fewer bacteria were phagocytozed during stress-impaired wound healing. Superoxide production by phagocytic cells was the second key bactericidal function tested.



**Figure 3.** <u>Phagocytic activity and superoxide production of phagocytic cells.</u> To measure oxidative burst, DHE ( $2.5\mu$ g/mL) was added to the heparinized whole blood harvested at D1 and D5. Live YFP-*E.coli* were added to test the phagocytic activity. Mean fluorescence intensity for YFP and DHE were measured. Data represent mean ± SEM from 2-3 experiments. At each time point n=10-15 mice/group. \* *p*≤0.05 compared to control (ANOVA).



Figure 4. Cell surface expression of F4/80+ cells one day post-wounding.

Collected blood was incubated with fluorochrome-conjugated antibodies against F4/80, CD11b, CD284 (TLR4), CD206, CD68 and CD62L. F4/80+ cells were gated and the mean cell surface expression for Cd11b, CD284, CD206 and CD68 was measured using Beckman Coulter CyAn II. Data represent mean $\pm$  SEM from 4 experiments of pooled blood from n=5 mice per condition per time point.\* *p*≤0.05 compared to control (ANOVA). Overlays of the curves of distribution CD11b (B) and CD284 (C) mean cell fluorescence intensity are shown for control (strips) and stressed groups (solid grey) at D1.

Upon stimulation with *E.coli*, the oxidative burst from phagocytic cells was measured as the mean fluorescence intensity from the release of DHE product. The mean fluorescence intensity did not vary between control and stressed groups at one, three and five days post-wounding.

# e. Cell surface expression of CD11b and TLR4 is down regulated in stressed group.

The cell surface expression of monocyte/macrophage markers, adhesion molecules and TLR4 was measured by flow cytometry at D1 and D5 post-wounding. F4/80+ monocytes were gated. The mean cell fluorescence for cell surface macrophage marker CD68 did not significantly change under restraint stress (Fig.4A), and nor did CD62L, a marker involved in leukocyte rolling and homing (data not shown). Interestingly, the cell surface expression of CD11b, which regulates migration and participates in cell adhesion to bacteria, was lowered by 43 % at D1 in stressed animals (p=0.002) as compared to control. Figure 4B represents the shift in the curve of the mean cell fluorescence intensity for CD11b between control and stressed groups observed at D1. TLR4 (CD284), which recognizes Gram (-) bacteria and triggers pro-inflammatory response, was expressed 35% less at D1 in stressed group as compared to control (p=0.018) (Fig.4A). Figure C shows the shift of distribution curves of mean cell fluorescence of TLR4 for stressed and control groups at D1. Cell surface expression of mannose receptor (CD206), which is a marker of alternatively activated macrophages, was not affected by stress.

# <u>f. Gene expression of CAM and WHM markers and the regulatory macrophage</u> marker CCL1 are reduced in stressed groups, whereas IL10 gene level increases.

To investigate the effect of stress on the phenotype of activated macrophages present at the wound site, the gene expression of markers of classically activated macrophages (CXCL10, CCL5 and TLR4), wound healing macrophages (CCL22, RELM $\alpha$  and IGF-1) and regulatory macrophages (CCL1 and IL10) were measured by qRT-PCR in unwounded tissue and wounds from D1 and D5. Both CXCL10 and CCL5 mRNA levels were down regulated in unwounded tissue and D1 wounds for CCL5, and D1 only for CXCL10 (Fig.5). TLR4 gene expression was not differentially regulated under stress (data not shown).

CCL22 and IGF-1 gene expression, which are markers of wound healing macrophages, were significantly lower in stressed at D1 and D5 post-wounding with a 6 fold decrease for CCL22, and a 3 fold decrease at both D1 and D5 for IGF-1 respectively (Fig.6). Interestingly, mRNA levels of RELM $\alpha$  decreased 6 fold in unwounded tissue in stressed groups as compared to the controls ( $p \leq 0.05$ ). The cytokines IL4 and IL13, reported to induce wound-healing macrophage phenotype, were not detected by qRT-PCR in the whole wound (data not shown).

Regulatory macrophages markers CCL1 mRNA level increased five days postwounding in the controls; whereas under restraint stress, its gene expression was 8 fold and 12 fold lower at D1 and D5 (Fig.7). IL10 gene expression increased over time in stressed mice, and at D5 the mRNA level of anti-inflammatory cytokine was significantly higher in stressed than control ( $p \le 0.05$ ).

To further confirm the previous data from whole wounds, macrophages were harvested from wound sections of control and stressed mice at D5 using laser capture microdissection, which allows for single cell dissection. The gene expression of IGF-1 was measured and t expressed as a ratio against expression of the macrophage marker CD68 (Fig.8). As expected, IGF-1 gene expression from dissected macrophages reflected previous results from the whole wound; the ratio of IGF-1 mRNA- CD68 mRNA level was higher in macrophages from control as compared to stressed animals.



**Figure 5.** <u>Markers of classically activated macrophage.</u> Unwounded skin and wounds were harvested at 1 and 5 days post-wounding. The gene expression of CXCL10 (A), CCL5 (B) were measured and compared to the housekeeping gene GAPDH using qRT-PCR. Data represent mean  $\pm$  SEM from 4 experiments. At each time point n=20 mice/group. \* *p*≤0.05 compared to control (ANOVA).



**Figure 6.** <u>Markers of wound-healing macrophage.</u> Unwounded skin and wounds were harvested at 1 and 5 days post-wounding. The gene expressions of CCL22 (A), RELM $\alpha$  (B), IGF-1 (C) were measured and compared to the housekeeping gene GAPDH using qRT-PCR. Data represent mean  $\pm$  SEM from 4 experiments. At each time point n=20 mice/group. \* *p*<0.05 compared to control (ANOVA).



**Figure 7.** <u>Markers of regulatory macrophage.</u> Unwounded skin and wounds were harvested at 1 and 5 days post-wounding. The gene expression of CCL1 (A) and IL10 (B) were measured and compared to the housekeeping gene GAPDH using qRT-PCR. Data represent mean  $\pm$  SEM from 4 experiments. At each time point n=20 mice/group. \*  $p \le 0.05$  compared to control (ANOVA).



**Figure 8.** <u>Gene expression of IGF-1 in macrophages isolated by LCM</u>. Tissue sections of wounds from control and stressed mice at D5 were stained against CD68. Macrophages were microdissected using LCM and qRT-PCR was performed. Data represent mean  $\pm$  SEM from approximately 4000 cells/sample.

# 4. Discussion

Restraint stress had been shown to increase susceptibility to opportunistic infection. This study investigated the effect of stress on neutrophils and macrophages responsible for bacterial clearance. Impaired bacterial clearance observed as early as one day post-wounding under stress (Rojas et al., 2002) cannot be attributed to a reduced neutrophil recruitment. Restraint stress did not alter the number of neutrophils during the early stage of the wound healing (day 1 post-wounding) when neutrophil presence is at its peak in the control group. However, stress did lead to increased neutrophil accumulation later during the healing process, at days 3 and 5. It is well established that neutrophil presence mirrors bacterial number, so the increase of bacterial number under stress suggests that the continued recruitment of neutrophils is driven by bacteria. Neutrophil numbers followed the timeline of bacteria, including the reduction after the stress is removed at day 5 and the numbers of neutrophils returning back to normal at day 7. MIP-2 and KC gene expression were increased at day 5, which is consistent with prolonged neutrophil infiltration. Similarly Sakamoto et la (1996) found increased mRNA expression of the rat homologue of KC in the brain after immobilization stress. These results are in contrast to the Rovai study (1998), which observed insensitivity of MIP-2 and KC to regulation by the stress hormone glucocorticoid. Liu & Cousin (1999) previously demonstrated that glucocorticoids prolonged neutrophil viability for 12 to 48h in vitro. The excessive infiltration and/or longer retention of neutrophils at the wound might further damage the tissue with the release of oxidants and hydrolytic enzymes from activated neutrophils. In addition, Zheng et al, reported that ingestion of apoptotic neutrophils actively suppresses stimulation of macrophages (2004), which may alter macrophage response. The mechanism(s) resulting in neutrophil accumulation (increased recruitment and/or longer life-span) should be investigated. Our data on

neutrophil presence in the wound further emphasized the crucial role of macrophages in bacterial clearance.

Macrophage recruitment and the gene expression of chemokines MIP-1 $\alpha$  and MCP-1 were not significantly affected by restraint stress during wound healing. The strong presence of neutrophils at a later time during which macrophage population in the tissue is unchanged might overwhelm the macrophages in charge of removing apoptotic neutrophils. This effect might lead to additional tissue damage and would impair the resolution of inflammation. Interestingly, studies on the effects of stress on phagocytic cell accumulation have yielded conflicting results. Bilbo et al reported that after 2 hours of restraint, increased numbers of neutrophils and monocytes accumulated in a surgical sponge implanted in a mouse (2002), whereas Zhang's team reported that restraint suppressed the migration of neutrophils and macrophages into the peritoneal cavities after IP inoculation of *L. monocytogenes* (1998). These differences between studies may come from the type of stress, namely acute stress (Bilbo et al., 2002) versus chronic stress, as well as the presence of a large number of bacteria in Zhang's model of L. *monocytogenes* infection. The present study measured the recruitment of macrophages in a naturalistic model, wound healing in a model that represents a more persistent stressor.

Even though stress did not affect macrophage presence in the wounded tissue, how stress may differentially regulate macrophage phenotype and function in the blood and the wound during healing was investigated. Our results showed that stress diminished the phagocytic ability of the inflammatory cell population in the blood during wound healing; yet restraint stress did not alter the cells' capacity for oxidative burst. Impairment of phagocytic function by stress was similarly observed in the Palermo-Neto study (2003) during which mice were exposed to the response delivered by other mice receiving inescapable footshock. Kang et al found that examination stress increases superoxide production (1997). Acute psychological stress was reported to increase phagocytic ability and reduce superoxide production of neutrophils in humans (Khanfer et al., 2010), suggesting that the type of stress experienced has different effects on phagocytic abilities. Our current study did not distinguish between the contribution from neutrophil and monocyte/macrophage phagocytosis, which will be investigated later. Harvesting phagocytic cells directly from wounds could provide information about phagocytic and oxidative burst abilities but the isolation process may activate the cells. Oxygen, required for oxidative burst, is less available at the site of injury due to the disruption of the vascular system and to the stress-induced activation of the sympathetic nervous system and vasoconstriction. Decreasing oxygen delivery could potentially impair bacteria clearance, yet preliminary studies do not indicate that supplementing oxygen systemically decreases bacterial load.

In the blood. CD11b cell surface expression circulating of monocytes/macrophages was down-regulated at day 1 post-wounding under stress, whereas CD68 and CD62L levels did not vary. CD11b is involved in leukocytes' rolling, homing and bacteria recognition. Even though low CD11b expression may have slowed down cell trafficking, macrophage recruitment was not altered. Stress also decreased TLR4 (CD284) cell surface expression during inflammation. Similarly, Du et al reported that stress hormones such as corticosterone and epinephrine induced down-regulation of TLR4 in macrophages (2010) in vitro. CD11b and TLR4 decreased expression could potentially impede bacteria recognition and binding by macrophages.

TLR4 binding to LPS triggers activation of classically activated macrophages, which are highly efficient at bacterial killing and which promote a pro-inflammatory response. In tissue, TLR4 gene expression was unchanged, however, the mRNA levels of other markers of classically activated macrophages, such as CXCL10 and CCL5, were decreased in the stressed group. Our data is in agreement with the study of Ehrchen et al (2007) that showed that treatment of human monocytes with glucocorticoids for 2 hours led to the down-regulation of CXCL10 and CCL5 *in vitro*.

Expression of the markers for wound-associated macrophages CCL22 and IGF-1 declined under stress at day 1 and day 5 post-wounding. Dissection of macrophages from wounds of control and stressed mice at day 5 confirmed IGF-1 down-regulation. IGF-1 induces keratinocyte and fibroblast proliferation and migration (Lee et al., 2010). Over-expression of IGF-1 in mouse keratinocytes increased their proliferation and migration, which improved wound healing (Semenova et al., 2008). This suggests that an IGF-1-decreased expression under stress would have detrimental effects on wound closure and tissue repair. In addition, lower RELM $\alpha$  expression was observed in the stressed group before wounding occurred, suggesting that restraint stress also alters the deposition of extracellular matrix. It is worth pointing out that even though IL4 /IL13 were reported to be necessary for the development of the wound-healing macrophage phenotype (Mosser and Edwards, 2008), IL4 and IL13 could not be detected in the wound in either the control or the stressed groups in this study. This observation supports similar findings by Daley (2010) and Bryan (2005) groups.

Stress also dysregulated gene expression of markers of regulatory macrophages, which promote the resolution of inflammation. The mRNA level of CCL1 decreased at D1 and D5 in the whole wounds of the stressed group, whereas gene expression of IL10 increased as previously shown by Curtin and Sesti-Costa's groups. (Curtin et al., 2009; Sesti-Costa et al., 2012).

During normal tissue repair, the macrophage phenotype evolves from a proinflammatory phenotype of CAM to a less-inflammatory/anti-inflammatory phenotype that is characteristic of WHM and regulatory macrophages. In diabetic db/db mice, the proinflammatory phenotype persisted through ten days post-injury (Mirza and Koh, 2011). This group reported that dysregulation of macrophage phenotypes contributes to impaired healing in a diabetic mouse model. Interestingly, our study shows that stress did not promote a shift of the macrophage population, but did dysregulate the gene expression of markers of all three of the macrophage populations present during wound healing without a significant decrease in macrophage numbers. The mechanism behind this general dampening of macrophage phenotype and function is unclear. Macrophage dysregulation by stress, in addition to the dampening of macrophage function resulting from excessive neutrophil phagocytosis, would contribute to impaired bacterial clearance.

Studying the expression of markers linked to macrophage activation in the whole wound enabled the determination of how stress affects the macrophage environment, and influences the phenotype. The LCM study focused on gene expression of isolated macrophages. Our work highlights the need to explore multiple markers of macrophage activation in order to understand the complexity of macrophage phenotypes involved in wound healing.

Altogether, our findings suggest that stress-induced impaired bacterial clearance is in part due to stress altering phagocytic abilities of neutrophils and macrophages. In addition, stress impacts macrophage activation by inducing the overall dysregulation of macrophage response during tissue repair. A better understanding of the mechanisms behind normal transition of macrophage phenotype during wound healing would provide crucial information to resolve stress-induced changes in macrophage phenotype with the goal to dysregulate their activation in order to ameliorate bacterial clearance and wound healing. To modulate macrophage phenotype could provide a new therapeutic approach in the clinical treatment of wound.

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# CHAPTER 2

# RESTRAINT STRESS DYSREGULATES MIR EXPRESSION DURING WOUND HEALING

# 1. Introduction

To prevent bacterial infection, repair the damaged tissue and restore tissue function, wound healing constitutes a complex series of events that requires the timely orchestration and efficient completion of three overlapping phases: inflammation, proliferation and resolution/remodeling. As the different phases of wound healing overlap and rely on the successful execution of anterior events, any factor altering early healing will also affect later phases. As the first phase, inflammation has a particularly crucial role.

Soon after injury, neutrophils infiltrate the wound, followed by macrophages. At the wound site, neutrophils and macrophages kill pathogens via phagocytosis and oxidative burst, releasing pro-inflammatory cytokines and growth factors. These factors initiate the proliferation phase. Proliferation and migration of keratinocytes and fibroblasts contribute to re-epithelialization, participate in the formation of granulation tissue and the contraction of the wound to restore integrity and functions of the damaged tissue.

The accurate timing of events occurring during wound healing is accomplished through gene regulation at the transcriptional and post-transcriptional levels. MicroRNAs (miRs) have emerged as powerful post-transcriptional regulators. MiRs are small endogenous non-coding RNAs (approximately 22 nt) derived from the intergenic regions or introns regions. MiRs bind to a complementary sequence in the 3'UTR of target

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mRNA(s) and can prevent protein synthesis by triggering the degradation of target mRNAs (Lim et al., 2005) and/or inhibiting their translation (Jackson and Standart, 2007; Pillai, 2007). Each miR can regulate multiple genes, and multiple miRs can target the same transcript. This interplay makes the miR regulation network extremely intricate. MiRs potentially constitute regulatory mechanisms with key roles across a widespread and diverse range of regulatory pathways, including immune regulation, cell proliferation, cell migration and apoptosis (Asirvatham et al., 2008; Sonkoly et al., 2008). These events are part of the healing process, which suggests that miRs participate in gene regulation during wound healing. For example, miR-21, miR-146 and miR-155 are induced by pro-inflammatory stimuli that are important in wound healing, such as IL-1 $\beta$ , and TLRs (Sheedy and O'Neill, 2008). MiR expression is tissue and cell specific. MiR-146, absent in keratinocytes and dermal fibroblasts, is expressed in immune cells in the skin, whereas miR-125b and miR-21 are expressed in both structural and inflammatory cells (Liu et al., 2009; Sand et al., 2009; Zavadil et al., 2007).

Similar to miRs, stress has been shown to differentially regulate a broad spectrum of physiological events including immune functions and healing. Stress has been shown to delay wound healing, to decrease and delay inflammation, and to impair bacterial clearance, thereby elevating the risk for opportunistic infection (Padgett et al., 1998; Rojas et al., 2002). It has also been shown to alter expression of genes involved in wound healing such as IL1 $\beta$  (Hayashi et al., 2004; Padgett et al., 1998) and could also dysregulate miRs during healing. In fact, Rinaldi et al. reported a significant increase in the expression levels of miR-9, miR-26a/b and let-7a in the frontal cortex after acute stress while only minor changes were observed after repeated restraint (Rinaldi et al., 2010). In the peripheral blood, Gidron's human study showed that brief academic stress alters expression of miR-21 and let-7b (2010). Reciprocally, miRs can modulate glucocorticoids and glucocorticoid receptor (GR) function. MiR-18 and miR-124a

downregulate GR protein in the paraventricular nucleus in Fischer 344 (F344) (Vreugdenhil et al., 2009). Yet the effect of stress on miR expression during wound healing remains unexplored.

Thus, we hypothesize that stress affects gene expression during the inflammatory response in part by altering expression of miRs that globally regulate inflammation during would healing.

Using microarray technology, the expression profile of approximately 700 miRs was analyzed to identify miRs differentially regulated by stress during wound healing. Nine miRs were selected for further study. The effect of stress on the expression of these miRs and on the gene expression of potential targets of these miRs involved in wound healing was investigated using qRT-PCR.

# 2. Material and Methods

**Animals.** Previously described p.23

Wounding and Tissue Harvest. Previously described p.23

**MicroArray Analysis of miRs.** Tissue samples harvested at the day of wounding, as well as 1 and 5 days post-wounding were processed to isolate RNA, which was sent to LC Sciences, LLC (Houston, TX) (n=3 mice per time point). MicroRNAs were labeled with Cy3 and Cy5 and analyzed using chip MRA-1002 (LC Sciences, LLC Houston, TX). ANOVA and t-tests were performed to analysis the data.

**miR Target Prediction.** TargetScan release 5.0 was used to predict potential binding sites for miRNA miR-21, miR-132, miR-146b and miR-155 on the 3'UTR region of murine genes (Lewis et al., 2003).

**mRNA and miR Analysis.** Harvested wounds were stored RNAlater® (Sigma-Aldrich Corp. St. Louis, MO) at 4°C and later homogenized in TRIzol<sup>®</sup> (Invitrogen Grand Island, NY) using a Tissue-Tearor (Cole-Parmer, Vernon Hills, IL). Total RNA was extracted and reverse transcription using SuperScript<sup>™</sup> First Strand Synthesis System for RT-PCR (Invitrogen Grand Island, NY). Reverse transcription of the isolated microRNA was performed using TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) using 100ng of total RNA for each RT reaction.

Real-time PCR primers and probes were purchased from Applied Biosystems TaqMan®Gene Expression Assays for CXCL10 (#4331182-Mm99999072\_m1), TGFβR2 (#4331182-Mm00436977 m1), PDCD4 (#4331182- Mm01266062 m1), GHR (#4331182-Mm00439093 m1), PGC-1 (#4331182-Mm01208835 m1), NFκB (#4331182-Mm00476361 m1), TLR4 (#4331182-Mm00445273 m1), IRAK1 (#4331182-Mm01193538 m1), MyD88 (#4331182-Mm00440338 m1) and FGF7 (#4331182-Mm00433291 m1). Primers and probes were also purchased from TagMan®MicroRNA Assays for miR-21 (# 4427975-000397), let-7b (# 4427975-002619), miR-27b (# 4427975-000409), miR-30d (# 4427975-000420), miR-98 (# 4427975-000577), miR-125b (# 4427975-000449), miR-132 (# 4427975-000457), miR-146b (# 4427975-001097), miR-155 (# 4427975-002571). Amplification of target cDNA was accomplished using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Mouse GAPDH (#4331182- Mm99999915\_g1) and miR endogeneous control snoRNA202 (Applied Biosystems, Carlsbad, CA, # 4427975-001232) were used as controls for mRNA and for miR RT-PCR respectively.

**Statistical Analysis.** Statistical analyses were performed using SPSS version 19. Differences over time were assessed using repeated-measurement ANOVA. Data represented as mean  $\pm$  SEM from 5 experiments. Statistical significance was determined at  $p \le 0.05$  (a or \*), and *p*-values approaching significance (0.05<p<0.1) were represented as # (ANOVA).

# 3. Results

# a. Microarray Profiling of miRs during stress-impaired wound healing.

Microarray analysis showed that wounding induced the differential expression of 167 miRs by a magnitude of at least two fold in the control group. Interestingly, 230 miRs were differentially regulated in the stress group as compared to the control at all time points. Amongst these 230 miRs, 10% showed a differential basal level expression in unwounded tissue under stress as compared to control. Of these, 3% had a higher expression, and 7% showed a lower basal expression (Fig.9). Most (62%) of miRs that were differentially regulated during wound healing under stress were significantly up- or down-regulated during the inflammatory phase. Finally, during the proliferation phase, 28% of miRs (including miR-146b and let-7b) were significantly increased or decreased in stressed group.

#### b. MiRs and downstream target genes.

Nine miRs were selected for validation and further analysis based on the magnitude of the differential expression and/or their potential involvement in the inflammatory phase of healing. To investigate the effect of stress on miR expression during wound healing, miR levels were measured at different time points in the control and stressed groups using qRT-PCR. Furthermore, bioinformatic software, TargetScan, was used to predict potential mRNA targeted by selected miRs to investigate how miR dysregulation by stress affects downstream mRNA expression during wound healing. Potential targets involved in wound healing were selected based on their aggregate Pct value (high aggregate Pct would correlate with higher probability of binding) and/or previous studies reporting a direct binding between the potential target and the miR of interest.



**Figure 9.** <u>Distribution of miRs differentially regulated during wound healing under stress.</u> Number of miRs differentially expressed with increased (Up) or decreased (Down) by at least 2 fold under stress in unwounded tissue (D0), inflammatory (D1) or proliferation phase (D5) during wound healing.

**miR-21.** In controls, miR-21 expression significantly increased in wounds at D5 as compared to the level in unwounded tissue (p=0.016). Under stress a similar augmentation of miR-21 was observed, but at an earlier time point (D1) (Fig.10). CXCL10 was predicted to be a miR-21 target using TargetScan (Aggregate Pct=0.4),. MiR-21 was showed to directly target TGF $\beta$ R2 and PDCD4(Kim et al., 2009; Chen et al., 2008) therefore higher miR-21 level would induce a decreased expression of these genes.

In the control, CXCL10 gene expression increased at D1, while the CXCL10 mRNA level did not significantly vary under stress. While miR-21 expression increased during the inflammatory phase under stress, CXCL10 gene expression did not increase which led to a 5 fold greater mRNA level in the control as compared to the stress group. Even though the TGF $\beta$ R2 mRNA basal level was 6 fold higher in the stressed group (*p*=0.046) compared to control, TGF $\beta$ R2 expression was lower by 2 fold in wounds at D1 (*p*=0.05) and by 3 fold at D5 (*p*=0.047) as predicted, compared to control. PDCD4 gene expression did not significantly vary under stress during wound healing (data not shown).

**miR-98.** MiR-98 levels decreased at D1 in the control group (p=0.002) as compared to the unwounded tissue. Interestingly, miR-98 expression pattern changed drastically under stress; stress enhanced expression of miR-98 during the inflammatory phase of healing (D1) by 38 fold (p=0.023) as compared to control (Fig.11). MiR-98 was predicted to bind to and inhibit gene expression of the growth hormone receptor (GHR) and PPARgamma coactivator 1 beta (PGC-1 $\beta$ ) with Aggregate Pct of 0.87 and at least 0.99 respectively. GHR and PGC-1 $\beta$  gene expression were up-regulated in the control at D5 as compared to unwounded tissue. But GHR expression under stress decreased at D1 as compared to control, and the drastic up-regulation of GHR gene expression observed in the control at D5 was not as great under stress.



**Figure 10.** Expression of miR-21 and downstream targets CXCL10 and TGF $\beta$ R2. MiR-21 relative expression and relative gene expression of CXCL10 and TGF $\beta$ R2 were measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one (D1) and five (D5) days post-wounding. Data represent mean ± SEM from 6 experiments. At each time point *n*=30 mice/group. (a) *p*≤0.05 compared to unwounded tissue (D0) in control (ANOVA). \* *p*≤0.05 stressed group compared to control at the same time point (ANOVA).



**Figure 11**. Expression profile of miR-98 and targets GHR and PGC-1 $\beta$ . MiR-98 relative expression and relative gene expression of GHR and PGC-1 $\beta$  were measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one (D1) and five (D5) days post-wounding. Data represent mean  $\pm$  SEM from 6 experiments. At each time point *n*=30 mice/group. (a) *p*≤0.05 compared to unwounded tissue (D0) in control (ANOVA). \* *p*≤0.05 and # 0.05<*p*<0.1 in stressed group compared to control at the same time point (ANOVA).

Two fold lower expression of GHR was observed at D1 (p=0.002) and D5 (p=0.046) in the stress group as compared to control.

PGC-1 $\beta$  gene expression was down-regulated by 2 fold at D1 (*p*=0.006) under stress as compared to control, and the increase of PGC-1 $\beta$  gene expression observed at D5 in the control was of smaller amplitude under stress (*p*=0.066) (Fig.11).

**miR-132.** Similarly to miR-98, miR-132 levels decreased at D1 in the control group (p=0.028) compared to unwounded tissue. Under stress, miR-132 level was enhanced by 3 fold at D1 (p=0.001) as compared to the control (Fig.4).

The pro-inflammatory transcription factor NF $\kappa$ B is a potential target of miR-132 (Aggregate Pct=0.34). MiR-132 up-regulation at D1 in the stressed group correlated with the decrease of NF $\kappa$ B mRNA at D1 by 2 fold (*p*=0.002) and also at D5 by 3 fold (*p*=0.027) in the stressed group as compared to controls (Fig.12).

**miR-155.** In the control group, miR-155 levels increased during the inflammatory and proliferation phases. Under stress, miR-155 expression drastically increased by 4 (p=0.043) at D1 in the stress group when compared to the control. Interestingly, miR-155 expression during the inflammatory phase in the stressed group was comparable to its level during proliferation in the control (Fig13). MiR-155 was predicted to bind to 3'UTR mRNA region of fibroblast growth factor 7 (FGF7) with an aggregate Pct of 0.85. In addition, miR-155 was previously showed to bind to MyD88 (Tang et al., 2010). As expected, gene expression of MyD88 was down-regulated by 3 fold (p=0.043) at D1 in stressed group compared to control. FGF7 mRNA level as well was significantly lower by more than 2 fold in stressed group in unwounded tissue at D1 and D5.

**Let-7b and miR-146b.** Of the nine miRs selected in this study, only two miRs were down-regulated during stress-impaired wound healing (Fig.14). In controls, miR-146b expression increased during the proliferation phase (D5). Interestingly, stress caused a 4 fold lower miR-146b level at D5 as compared to the control (p=0.05).



**Figure 12.** Effect of stress on level of miR-132 and NF $\kappa$ B mRNA MiR-132 relative expression and relative mRNA levels of NF $\kappa$ B were measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one (D1) and five (D5) days post-wounding. Data represent mean ± SEM from 6 experiments. At each time point *n*=30 mice/group. (a) *p*≤0.05 compared to unwounded tissue (D0) in control (ANOVA). \* *p*≤0.05 in stressed group compared to control at the same time point (ANOVA).



**Figure 13.** Expression of miR-155, its targets MyD88 and FGF7. MiR-155 relative expression and relative mRNA levels of targets MyD88 and FGF7 were measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one (D1) and five (D5) days post-wounding. Data represent mean  $\pm$  SEM from 6 experiments. At each time point *n*=30 mice/group. (a) *p*≤0.05 compared to unwounded tissue (D0) in control (ANOVA). \* *p*≤0.05 in stressed group compared to control at the same time point (ANOVA).



**Figure 14.** Decreased level of miR-146b and Let-7b in stress-impaired wound healing. Relative expression of miR-146b and let-7b were measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one (D1) and five (D5) days post-wounding. Data represent mean  $\pm$  SEM from 6 experiments. At each time point *n*=30 mice/group. (a) *p*≤0.05 compared to unwounded tissue (D0) in control (ANOVA). \* *p*≤0.05 in stressed group compared to control at the same time point (ANOVA).

Similarly, under stress let-7b expression was reduced 2.5 fold during the proliferation phase (p=0.033) as compared to the control. MiR-146b binds the mRNA of IRAK1 (Zilahi et al., 2012) and let-7 family was predicted to bind mRNA of TLR4. Nevertheless, IRAK1 and TLR4 gene expression were not differentially regulated under stress during wound healing (data not shown).

**miR-27b**, **miR-125b**, **miR-30d**. Even though miR-27b and miR-125b expression increased at D1 and D1 and D5 respectively, and miR-30d expression decreased at D5, stress did not affect their expression (data not shown).

# 4. Discussion

Profiling analysis of miRs showed that most stress-induced changes in miR expression occurred during the inflammatory phase, which would be expected given that delayed wound closure was observed under stress as early as one day post-injury (Padgett et al., 1998). MiRs selected for further analyses were involved in the inflammatory response and bacterial clearance by neutrophils and macrophages.

Macrophages participate in pathogen removal and their function is modulated by activation signals present in macrophage environment. Classically activated macrophages expressing CXCL10, bear potent phagocytic properties and are proinflammatory, whereas alternatively activated macrophages (wound-healing and regulatory macrophages) are less efficient in bacterial killing, contribute to tissue remodeling and are anti-inflammatory. While both macrophage populations are present during wound healing, classically activated macrophages are most important during early inflammation, whereas alternatively activated macrophages help resolve the inflammatory phase and the transition to proliferation phase. In this study, downregulation of the classically activated macrophage marker, CXCL10, correlated with miR- 21 increased expression under stress, which would potentially reduce the proinflammatory response during wound healing under stress. The effect of stress on miR-21 expression has yielded conflicting results depending on the duration of GC exposure and model used in studies. Whereas Moschos et al. (2007) reported that pre-treatment with dexamethasone for 1 hour had no effect on miR-21 expression in mouse lung following exposure to lipopolysaccharide (LPS), Gidron's human study shows that brief academic stress decreased expression of miR-21 in the blood (2010). Nevertheless, Ehrchen et al.(2007) showed that treatment of human monocytes with glucocorticoids for 2 hours led to down-regulation of CXCL10. Whether stress exerts an effect on CXCL10 directly or indirectly via miR-21 regulation should be determined. MiRs may also participate in the regulation of the alternatively activated macrophage phenotype.

The increased levels of MiR-98 at D1 and D5 under stress correlated with the PGC-1 $\beta$  mRNA decrease at these time points. Based on the study by Vats et al (Vats et al.), which showed that transgenic expression of PGC-1 $\beta$  primed macrophages for M2 phenotype and strongly inhibited pro-inflammatory cytokine production, miR-98 inhibition of PGC-1 $\beta$  gene expression would potentially prolong inflammation. Interestingly, miR-155 was recently reported to target IL13R1 $\alpha$ , the receptor for typical pro-M2 cytokine interleukin 13 (IL-13) (Martinez-Nunez et al., 2011). How dysregulation of miRs expression by stress affects macrophage activation and functions remains to be investigated.

Recognition of bacteria present in the wound by neutrophils and macrophages activates the TLR4 signaling pathway, triggering gene expression of pro-inflammatory molecules. Let-7b, miR-146b, miR-155 and miR-132 target the TLR4 signaling pathway, namely, TLR4, IRAK1, MyD88 and NFkB. In our study, miR-155 levels increased at D1 under stress, and as predicted, MyD88 gene expression decreased. Du et al (2010) found that exposure to corticosterone did not affect MyD88 mRNA levels in rat macrophages, which suggests that MyD88 differential expression results from miR-155 regulation. Interestingly, Tang et al (2010) reported that miR-155 decreased MyD88 expression at the protein level but not the mRNA level in human gastric and embryonic cell lines subject to *H.pylori* infection. The mechanism by which miR-155 acts to inhibit gene expression (translational repression or mRNA degradation) may depend on the tissue and cell type.

NFkB regulates the transcription of pro-inflammatory genes downstream of the TLR4 signaling pathway, and is a predicted target of miR-132. MiR-132 level increased at D1 under stress and NF $\kappa$ B mRNA diminished at D1 and 5, which could decrease transcription of pro-inflammatory genes. Mele's team reported that miR-132 level was up-regulated in the hippocampus during chronic immobilization stress, but not under acute stress (Rinaldi et al., 2010), which is in agreement with our results in skin. Almawi et al (2002) showed that GC negatively regulated activation and functions of NF $\kappa$ B, but did not alter its expression. The differential regulation of NFκB gene expression observed in this study could result from miR-132 regulation. Additional experiments investigating miR-132 binding on NF $\kappa$ B mRNA, would reveal if NF $\kappa$ B down-regulation was a direct consequence of miR-132 action. Shaked et al. (2009) provided evidence that miR-132 targets acetylcholinesterase in peripheral macrophages, inducing an increase in the neurotransmitter, acetylcholine, and mediates an anti-inflammatory effect. The anti-inflammatory role of miR-132 in the neuroimmune interface during wound healing under stress, resulting from NF $\kappa$ B and/or acetylcholinesterase gene regulation, requires further investigation.

Rojas et al (2002) reported that bacterial clearance is impaired under stress during wound healing leading to an increased number of bacteria present at the wound site as early as D1 and with up to 3 log augmentation at D5. Urbich et al. (2008) observed that LPS stimulates miR-21, miR-146b and miR-155 expression, whereas miR-98 expression decreased in macrophages following LPS exposure (Liu et al, 2011). The increased number of bacteria in the wound in stressed groups could explain the augmentation of miR-21 and miR-155, but not the increase of miR-98 observed in our study. An increased miR-98 level would predict a lower gene expression of the miR-98 target, GHR. GHR promotes bacterial clearance: GHR bound to its ligand GH induces GH signaling pathway, which primes neutrophils, stimulates superoxide production and phagocytosis (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). Interestingly, Dejkhamron et al (2007) observed that in a cell culture model of bacterial sepsis, LPS suppressed expression of GHR, via activation of TLR4 signaling pathway. Whether GHR decreased expression occurs via miR-98 regulation or TLR4 pathway should be investigated, nevertheless GHR lower expression may contribute to impaired bacterial clearance during wound healing under stress.

Given the global effect of miRs on multiple target genes, miRs selected in this study not only regulated genes in inflammatory phase, but also genes involved in the proliferation phase of wound healing. Cell proliferation, cell migration and wound contraction characterize the proliferation phase to replenish lost tissues, close the wound and form a scar. Growth factors secreted in the tissue, such as FGF7, regulate cellular and molecular events of the proliferation phase. FGF7 stimulates re-epithelialization and is a target of miR-155. mRNA levels of FGF7 decreased under stress, potentially altering fibroblast proliferation. FGF7 lower gene expression during stress impaired wound healing was reported by Mercado et al (2002), but whereas a reduction of FGF7 gene expression was measured at D1 using competitive PCR, in this study diminished FGF7 mRNA levels were detected by qRT-PCR at all time points. The strength of gene expression results from the difference between the production of mRNA and their

specific degradation by miRs. This effect should be magnified at the protein level, which should be investigated.

GH signaling pathway previously mentioned for its role in inflammation also participates in tissue growth. Dysregulation of miR-98 expression in conjunction with PGC-1 $\beta$  and GHR mRNA down-regulation under stress would potentially impair keratinocyte differentiation and tissue repair. TGF $\beta$  activates the TGF $\beta$  signaling pathway via binding to TGF $\beta$ R2, to replenish the extracellular matrix and scar formation. Recently, Yu et al (2011) observed that down-regulation of miR-21 enhanced luciferase-TGF  $\beta$  R2-3′ UTR activity confirming TGF  $\beta$  R2 as one of the direct targets of miR-21. MiR-21 high expression under stress correlated with the down-regulation of TGF $\beta$ R2 gene expression in our study, which would diminish the effects of TGF $\beta$  signaling pathway. Based on Martinez-Ferrer study (2010), which reported that the loss of TGF $\beta$  signaling in the dermis resulted in reduced collagen deposition and remodeling associated with reduced wound contraction, our data suggests that miR-21 higher expression could induce a potential delay in re-epithelialization.

Interestingly, two miRs in this study (let-7b and miR-146b) differentially regulated under stress during wound healing did not affect mRNA level of downstream targets selected for the study. This absence of differential expression could be explained by the fact that miRs expression is triggered after gene expression is induced; therefore, if transcription of a specific mRNA is not active then those mRNAs cannot be regulated by miRs. Alternatively, as multiple miRs can target the same transcript, the effect on a specific target mRNA would be the summation of effects from individual miRs. Finally, miR silencing of these particular genes might be solely through translational repression, which would be reflected in protein levels. As previously mentioned, miRs can prevent protein synthesis by triggering the degradation of target mRNAs (Lim et al., 2005) and/or inhibiting their translation (Jackson and Standart, 2007; Pillai et al., 2007). Therefore the effect of miR dysregulation under stress on mRNA levels of targets will likely be amplified at the protein level. Finally, gene expression of additional predicted targets of let-7b and miR-146b should be studied.

This study provides further evidence that stress alters miR expression and affects mRNA levels of miR targets in the inflammatory and proliferation phases of wound healing, yet the direct or indirect mechanism behind this regulation remains to be explored. This work constitutes, to our knowledge, the first descriptive study focusing on the effect of stress on miR expression and miR target gene expression during cutaneous wound healing. The global effect of miRs on genes involved in both inflammatory and proliferative phases of healing highlight the potential use of miRs modulators for therapy to ameliorate stress-impaired wound healing.
#### CHAPTER 3

### INHIBITION OF MIR-98 AMELIORATES STRESS-IMPAIRED WOUND HEALING

#### 1. Introduction

Wound healing constitutes a complex series of events that require the timely orchestration and efficient completion of three major phases, namely inflammation, proliferation and resolution/remodeling. These events help prevent bacterial infection, repair the damaged tissue and restore function of the damaged tissue. As the different phases of wound healing overlap and rely on the successful execution of anterior events, any factor altering early healing will also affect later phases, which emphasizes the crucial role of inflammation.

Previous studies have shown that stress delays wound healing. Stress decreases and delays inflammation and impairs bacterial clearance thereby elevating the risk for opportunistic infection (Padgett et al., 1998; Rojas et al., 2002). We previously showed that neutrophil recruitment is altered under stress and that neutrophil and macrophage phagocytic abilities are compromised in the blood. During healing, stress dysregulated the gene expression of markers of macrophage activation, which are associated with macrophage function, all of which would contribute to impaired bacterial clearance.

Stress-induced regulation of gene expression can occur at the transcriptional level or post-transcriptional level. Indeed our group and others reported that stress dysregulated miRs, which are potent post-trancriptional regulators (Gidron et al., 2010; manuscript in preparation). MiRs are small endogenous non-coding RNAs (approximately 22 nt) that bind to complementary sequence in the 3'UTR of target mRNA(s) and can prevent protein synthesis by triggering the degradation of target mRNAs (Lim et al., 2005) and/or

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inhibiting their translation (Jackson and Standart, 2007; Pillai et al., 2007). Interestingly, one miR can regulate multiple genes and multiple miRs can target the same transcript.

A previous study showed that miR-98 level was significantly increased by more than 30 fold during the inflammatory phase in stressed mice as compared to controls. At the same time, the gene expression of miR-98 target growth hormone receptor (GHR) was decreased three fold.

Binding of the growth hormone (GH) to its receptor GHR triggers GH signaling pathway, which is involved in skin homeostasis and immune response. The GH signaling pathway induces the expression of proteins important for cell cycle progression and cellular growth, cytokine and growth factor-receptor signaling component, and cell survival. The GH signaling pathway affects cytokine production, primes neutrophils and stimulates superoxide production and phagocytosis during inflammation (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). In addition, this signaling cascade regulates fibroblast proliferation, collagen production and overall tissue growth. Thus, the GH signaling pathway participates in both the inflammatory response and proliferative phases of wound healing. Interestingly, systemic GH treatment has been shown to significantly improve the wound-healing rate in burn patients (Herndon et al., 1995). Animal models showed that GH therapy improves healing time and increases collagen deposition, granulation tissue, and the tensile strength of skin (Ghofrani et al., 1999; Jorgensen and Andreassen, 1987). Nevertheless, side effects associated with GH treatment, including hypoglycemia, electrolyte imbalance, and edema, limit the use of GH treatment and emphasizes the need for new treatment approach (Kemp and Frindik, 2011; Pierre et al., 1997).

We hypothesized that inhibition of miR-98 during stress-impaired wound healing restores miR-98 levels and the GH signaling pathway, and ameliorates wound closure and bacterial clearance. Inhibition of miR-98 by LNA-98 was first assessed *in vivo* during

stress-impaired wound healing. Given the role of the GH signaling pathway in tissue growth and bacterial clearance, the relative wound closure and bacteria load in wounds of an LNA-98-treated group were investigated. Finally, the effects of LNA-98 treatment on the gene expression of GHR and genes induced by GH signaling pathway was examined.

# 2. Material and Methods

Animals. (Previously described)

#### Wounding and Tissue harvest. (Previously described)

**Treatment with miR-98 inhibitor LNA-98.** One day before wounding, mice were injected subcutaneously with 50uL of miRCURY LNA <sup>TM</sup> microRNA inhibitor for miR-98 (LNA-98) or negative control A (LNA-scramble) (Exiqon) with Lipofectamine (Invitrogen) dissolved in saline solution with a final concentration of 1 $\mu$ M. The day of wounding, another 10 $\mu$ L of solution of LNA-98 inhibitor or LNA-scramble were applied to the wounds.

**Measurement of wound area.** Each wound was photographed daily form the day of wound placement (D0) until 5 days post-wounding (D5). A standard-sized dot was placed next to the wound while the picture was taken. The area of each wound was measured using Adobe and compared to the area of a standard-sized dot. This ratio was compared to the ratio at D0 to determine the ratio of wound closure.

**Bacteria count in the wound.** Mice were anesthetized as previously described, and wounds were harvested 5 days post-wounding using a 6.0mm punch biopsy. Each wound was homogenized in 1mL sterile PBS on ice, using a Tissue Tearor Homogenizer (Cole-Parmer, Vernon Hills, IL). Serial dilutions (1:10) of the homogenate were plated in

duplicate on brain-heart-infusion agar plates. After a 24h incubation at 37°C, colonies formed on the plates were counted to determine the colony forming units (CFU) per wound.

mRNA and miR analysis. Harvested wounds were processed as previously described. Real-time PCR primers and probes were purchased from Applied Biosystems TaqMan®Gene Expression Assays for GHR (#4331182- Mm00439093\_m1), SOCS2 (#4331182- Mm00850544\_g1), SCD1 (#4331182- Mm01197142\_m1), Bcl-2 (#4331182- Mm00477631\_m1), Myc (#4331182-Mm00487804\_m1). TaqMan®MicroRNA Assays were purchased for miR-98 (# 4427975-000577). Amplification of the target cDNA was accomplished using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Mouse GAPDH (#4331182- Mm99999915\_g1) and microRNA endogeneous control snoRNA202 (Applied Biosystems, Carlsbad, CA, # 4427975-001232) were used as controls for mRNA and for miR RT-PCR respectively.

**Statistical Analysis.** (Previously described) Statistics were performed using SPSS. Differences over time were assessed using repeated-measurement ANOVA. Data was represented as mean  $\pm$  SEM from 5 experiments. Statistical significance was determined at (a) *p*≤0.05 in the stressed group as compared to control at the same time point, \* *p*≤0.05 in the stressed group treated with LNA-98 as compared to LNA-scramble treatment at the same time point. *p*-values approaching significance (0.05<p<0.1) were represented as #a (ANOVA).

#### 3. <u>Results</u>

#### a. LNA-98 treatment decreases miR-98 level in the wound.

To assess miR-98 inhibition by LNA-98, miR-98 levels were measured by qRT-PCR in unwounded tissue and wounds at D1 in control and stressed groups, as well as stressed

groups treated with LNA-98 or LNA-scramble. As previously observed, miR-98 level was significantly greater (more than 20 fold) in the stressed group as compared to the control at D1 (*p*=0.002) (Fig.15), As expected, the stressed group treated with miR-98 inhibitor LNA-98 showed significantly decreased miR-98 levels compared to the group treated with LNA-scramble. To further test the specificity of miR-98 inhibition via LNA-98, miR-132 levels were measured. A previous study reported that miR-132 expression increased at D1 under stress, similar to miR-98 (manuscript in preparation). As expected, LNA-98 and or LNA-scramble did not affect miR-132 levels (data not shown).

# b. LNA-98 treatment ameliorates wound closure during stress.

MiR-98 targets GHR, which triggers the GH signaling pathway involved in two important wound healing events: bacterial clearance and re-epithelialization,. To assess the effect of treatment with LNA-98 on wound closure we determined the relative wound area by measuring the wound each day in all groups and comparing it to the original wound size. As previously reported, the relative wound area in the stressed group without treatment was on average 35% greater than the control (p<0.05) (Fig.16). No significant difference was observed in wound closure between LNA-scramble and untreated stressed groups. Under stress, treatment with LNA-98 significantly increased wound closure by 10% as compared to untreated stressed group. Moreover, LNA-98 treatment led to a significantly greater wound closure than the LNA-scramble treatment at D2 to D5 post-wounding (p<0.05) (Fig.16). Surprisingly, treatment with LNA-scramble seemed to have a non-specific effect as the wound closure was improved in that group as compared to untreated stressed group.



**Figure 15.** <u>LNA-98 treatment decreases miR-98 level in the wound.</u> MiR-98 relative expression was measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one day post-wounding (D1) in control (FWD), stressed (RST) and stressed groups treated with LNA-98 (RST-LNA98) or LNA-scramble (RST-LNAscr). Data represent mean  $\pm$  SEM from 5 experiments. At each time point n=25 mice/group. (a) *p*≤0.05 in stressed group (RST) compared to control (FWD) and (\*) *p*≤0.05 in the stressed group treated with LNA-98 (RST-LNA98) as compared to LNA-scramble (RST-LNAscr) treatment at the same time point (ANOVA).



**Figure 16.** <u>LNA-98 treatment ameliorates wound closure during stress.</u> Each wound was photographed daily. A standard-sized dot was placed next to the wound while the picture was taken. The area of each wound was measured and compared to the area of the standard-sized dot. This ratio was compared to the ratio at D0 to determine the relative wound area. Data represent mean  $\pm$  SEM from 3 experiments. At each time point n=15 mice/group. (a) *p*≤0.05 stressed group (RST) compared to control (FWD) and (\*) *p*≤0.05 in stressed group treated with LNA-98 (RST-LNA98) compared to LNA-scramble (RST-LNAscr) treatment at the same time point (ANOVA).

# <u>Effect of LNA-98 treatment on stress-impaired bacterial clearance during wound</u> healing.

The GH signaling pathway participates in bacterial clearance, and under stress this pathway is altered. Therefore, improvement of GH signaling pathway during stress via LNA-98 treatment may improve bacterial clearance. As expected, our data showed an 8 fold decrease (p=0.004) in bacterial load at D5 in the LNA-98 group as compared to the LNA-scramble group (Table 1).

#### d. mRNA level of GHR, a miR-98 target, increases after LNA-98 treatment.

MiR-98 targets GHR and, therefore, an increase in miR-98 level would potentially correlate with a decrease in the GHR mRNA level. Similarly, inhibition of miR-98 via LNA-98 would alleviate the miR-98-induced silencing of GHR, and help restore its gene expression. To investigate the effect of miR-98 inhibition on its target, GHR mRNA levels were measured by qRT-PCR in each group. Without treatment, GHR mRNA basal levels in control and stressed group were comparable (Fig.17). One day post-wounding GHR gene expression decreased 2 fold in the stressed group (p=0.045). As predicted, LNA-98 treatment induced an increase in GHR mRNA levels of at least 2 fold at D0 (p=0.028) and more than 3 fold at D1 (p=0.048) as compared to the group treated with LNA-scramble.

# e. <u>LNA-98 treatment modulates expression of genes induced by the GH signaling</u> <u>pathway.</u>

Binding of GH to GHR triggers the activation of GH signaling pathway. The following signaling cascade induces transcription of genes, such as SCD1. Therefore, as GHR expression is restored via inhibition of miR-98, gene expression of downstream genes would also be re-established.

#### TABLE II

# LNA-98 TREATMENT IMPROVES BACTERIAL CLEARANCE IN STRESS-IMPAIRED WOUND HEALING

	Mean CFU/wound
RST-LNA98	14.9E+05 (± 8.97E+05)*
RST-LNAscr	128.1E+05 (±63.4E+05)

Wounds were harvested from stressed groups treated with LNA-98 or LNS-scramble at D5, and homogenized. Serial dilutions (1:10) of the homogenate were plated. After a 24h incubation at 37°C, colonies formed on the plates were counted to determine the colony forming units (CFU) per wound. Data from 2 experiments, n=15 mice/group.



#### Figure 17. mRNA level of GHR, a target of miR-98, after LNA-98 treatment.

GHR relative gene expression was measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one day post-wounding (D1) in control (FWD), stressed (RST) and stressed groups treated with LNA-98 (RST-LNA98) or LNA-scramble (RST-LNA-scr). Data represent mean  $\pm$  SEM from 5 experiments. At each time point n=25 mice/group. (a) *p*≤0.05 in stressed group (RST) compared to the control (FWD) and (\*) *p*≤0.05 in stressed groups treated with LNA-98 (RST-LNA98) as compared to LNA-scramble (RST-LNAscr) treatment at the same time point (ANOVA).



#### Figure 18. Expression of genes induced by GH pathway under LNA-98 treatment.

Relative gene expression of SCD1, Bcl-2, Myc and SOCS2, was measured using qRT-PCR in unwounded tissue (D0) and wounds harvested one day post-wounding (D1) in control (FWD), stressed (RST) and stressed groups treated with LNA-98 (RST-LNA98) or LNA-scramble (RST-LNA-scr). Data represent mean  $\pm$  SEM from 5 experiments. At each time point n=25 mice/group. (a) *p*≤0.05 and (#a) 0.05<*p*<0.1 in stressed group (RST) as compared to the control (FWD) and (\*) *p*≤0.05 in stressed group treated with LNA-98 (RST-LNA98) compared to LNA-scramble (RST-LNAscr) treatment at the same time point (ANOVA).

To determine the effect of LNA-98 treatment on genes downstream of GH signaling pathway, mRNA levels of SCD1, Bcl-2, Myc and SOSC2 were measured by qRT-PCR. \_Stearoyl CoA desaturase-1 (SCD1) is an enzyme involved in the formation of monounsaturated fatty acids, which contributes to the barrier function of the skin. In untreated groups, SCD1 mRNA level was significantly higher (3 fold) in the controls in unwounded tissue and wounds at D1 as compared to stress group (Fig.18). Similar to the control group, the stressed group treated with LNA-98 showed higher expression at D0 by more than 3 fold (p=0.047) as compared to the LNA-scramble treated group. At D1, SCD1 mRNA levels in both treated groups were comparable.

The gene expression of the anti-apoptotic, Bcl-2, is induced by the GH signaling pathway. Bcl-2 mRNA levels in the control were significantly higher (2 fold) in unwounded tissue and D1 wounds (p=0.046 and p=0.049 respectively) as compared to the stressed group (Fig.18). Interestingly, Bcl-2 mRNA levels in the LNA-98 treated stressed group mirrored gene expression in the control, whereas Bcl-2 gene expression in the LNA-scramble group paralleled the untreated stressed group at each time point. (p=0.023 at D0 and p=0.04 at D1).

Activation of GH signaling induces the expression of Myc, which drives cell proliferation and cell growth. In unwounded tissue, Myc mRNA level was greater in the control than in the stressed group by 2 fold (p=0.049). As predicted, Myc gene expression was also higher in the LNA-98 treated stress group by 2 fold (p=0.05), as compared to the LNA-scramble group. No significant difference in Myc mRNA level was measured within untreated groups, or within pre-treated groups at D1.

Finally, SOCS2 gene expression was lower at D0 (p=0.073) in the control as compared to the untreated stressed group. Similarly, the stressed group treated with LNA-98 showed diminished mRNA level of SOCS2 as compared to the group treated

with LNA-scramble (*p*=0.01). No significant difference in SOCS2 mRNA level was observed at later time point.

# 4. Discussion

Rojas et al (2002) reported that under stress, the bacterial load increased at the wound site as early as D1 and with a 3 log augmentation at D5 as compared to the control. Liu's study (2011) reported that miR-98 expression decreased in macrophage following LPS exposure. Based on Liu's work, miR-98 would be expected to decrease in the stressed group in correlation to the increased bacterial load. Yet our results showed that miR-98 level in the wound increased under stress. This suggests that miR-98 expression is regulated by a stress-induced mechanism other than LPS stimulation and/or that the mechanism for LPS mediated induction of miR98 was blocked. The mechanism by which stress regulates miR-98 expression should be investigated.

Because the miR-98 level was greatly increased one day post-wounding under stress, pre-treatment of the stressed group with miR-98 inhibitor LNA-98 before surgery would inhibit the induction of this peak, and have a greater effect. As expected, LNA-98 treatment specifically inhibited miR-98 expression in the wound tissue of stressed animals. In our study, the LNA-98 solution was injected subcutaneously a day before surgery where the wound would be placed; therefore, it is not surprising that the miR-98 level was decreased in the unwounded tissue and at D1. The inhibition of miR-98 by LNA-98 was specific. MiR-132 levels, which follow the same pattern as miR-98 (manuscript in preparation), were not affected by LNA-98 treatment (data not shown). Nevertheless, LNA treatment had a non-specific effect on miR-98 level as observed in the LNA-scramble group. This effect might result from the presence of lipofectAMINE, which can be cytotoxic (Madry et al., 2001). Indeed, the use of lower LNA concentration (1/10) did not abrogate the non-specific effect (data not shown).

Various *in vivo* and *in vitro* studies have demonstrated that cationic liposomes cause oxidative stress leading to the generation of reactive oxygen species (Boon et al., 2011) that is associated with its cytotoxicity (Dokka et al., 2000; Kongkaneramit et al., 2008). LipofectAMINE was reported to induce a dose-dependent increase in ROS generation after pulmonary administration that induced apoptosis in mice (Dokka et al., 2000). Interestingly, both LNA treated groups overall showed lower miR-98 levels.

Modified LNA with a complete phosphorothioate backbone have been shown to be more stable with low cytoxicity and can be used without a carrier, which would allow the removal of lipofectAMINE (Boon et al., 2011; Garchow et al., 2011). Nevertheless, if a carrier is necessary for the cellular uptake of the miR-98 inhibitor, nanoparticles should be tested. Indeed nanoparticle technology, including polyLysine, and gold nanoparticles, show promising results in miR-related delivery *in vitro* and *in vivo* (Crew et al., 2012; Jin et al., 2012; Liu et al., 2011a).

Upon GH binding, GHR triggers the GH signaling pathway. The GH signaling cascade promotes bacterial clearance by priming neutrophils, stimulating superoxide production and phagocytosis (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). GHR is a target of miR-98; therefore, increased miR-98 level would predict a lower GHR gene expression than was observed at D1 in this study. Interestingly, Dejkhamron et al. (2007) reported that in a cell culture model of bacterial sepsis, LPS suppressed expression of GHR, via activation of TLR4 signaling pathway. Based on the Dejkhamron study, and given that the bacteria load is increased under stress, GHR gene expression would be expected to decrease in the stressed group as compared to the control. Indeed, in this study, GHR mRNA was diminished under stress. Interestingly, LNA-98 treatment of the stressed group restored GHR gene expression at D1 to the

level observed in control, but treatment with LNA-scramble did not. This result suggests that GHR gene expression is at least in part regulated via miR-98.

Decreased expression of GHR under stress would affect the activation of the GH signaling pathway, and increased GHR mRNA level in the stressed group treated with LNA-98 would help restore normal activation of GH pathway and the expression of downstream genes such as MYC, SCD1, Bcl-2, SOCS2. These genes are important for cell cycle progression, cellular growth, cell survival, and cytokine response. The stressed group showed significantly lower basal gene expression of SCD1, Bcl-2 and MYC as compared to the control. In addition, SCD1 and Bcl-2 mRNA levels were still decreased at D1 under stress. After LNA-98 treatment, SCD1, Bcl-2 and MYC gene expression in the stressed group were restored and comparable to the control. Bcl-2 mRNA level in the LNA-98 treated group was also equal to the control at D1.

SCD1 is an enzyme involved in the formation of mono-unsaturated fatty acids, which contributes to cell proliferation, cell survival, and helps maintain the barrier function of the skin. In recent years, functional relevance of SCD1 in cell proliferation and survival has emerged (Igal, 2010). Cell proliferation requires fatty acids synthesis and alterations of fatty acid synthesis induces apoptosis. Inhibition of SCD1 activity blocks cell cycle progression and induces programmed cell death in lung cancer cells (Hess et al., 2010). Studies performed in oncogene-transformed and cancer cells reported that ablation of SCD1 notably increases the rate of apoptosis (Scaglia and Igal, 2008), implying that SCD1 is an important factor for cell survival. Diminished gene expression of SCD1 at D0 and D1 in the untreated stressed group may promote a pro-apoptotic state in the cells. Based on Georgel et al (2005), mutation of SCD1 gene were found to cause skin abnormalities, to compromise the immune function of the skin and to reduce the ability of the mice to fight against bacterial infection. Thus, lower SCD1 mRNA level might alter the immune response to clear bacteria during inflammation.

LNA-98 treatment inhibited the decrease of SCD1 mRNA level in unwounded tissue. Interestingly, SCD1 mRNA is itself a predicted target of miR-98; thus, regulation of SCD1 expression may result from direct (miR-98 inhibition) or indirect effects (via GH pathway) of LNA-98 treatment.

Gene expression of Bcl-2 promotes cell survival. The balance between cell survival and apoptosis is key to guarantee the maintenance of homeostasis in the skin and the control of the inflammatory response during tissue repair. Interestingly, the stress hormone glucocorticoid GC promotes apoptosis. In the stressed group, the Bcl-2 gene expression was diminished in unwounded skin and wounds at D1 compared to control. Bcl-2 mRNA levels in LNA-scramble group were comparable to the levels observed in untreated stressed group, whereas LNA-98 treatment restored Bcl-2 gene expression levels to control levels in both unwounded tissue and wounds at D1. The stress-induced decrease of Bcl-2 gene expression would potentially create an imbalance towards a pro-apoptotic state, though LNA-98 treatment inhibited this effect. Interestingly, Wang et al (2011) reported that Let-7/miR98 directly regulated Fas and Fas-mediated apoptosis. Wang's work in addition to our study emphasizes the involvement of miR-98 in cell fate.

MYC is a well-studied transcription factor involved in cell proliferation and cell growth. Similarly to SCD1 and Bcl-2, basal gene expression of MYC was decreased under stress as compared to the control and restored by LNA-98 treatment in unwounded tissue. Lower gene expression of MYC under stress may impair the tissue propensity for cell proliferation needed for skin repair, which would be restored by LNA-98 treatment. In addition to its role in proliferation, Hemann et al (2005) demonstrated that MYC can also trigger apoptosis by neutralizing Bcl-2, which implies that MYC contributes to the determination of cell fate during wound healing. It is worth mentioning that Sampson et al reported that Let-7a, another member of Let-7/miR-98 family, down-

regulated MYC (2007). LNA-98 treatment restored MYC mRNA levels, yet whether the regulation occurred solely via restoring GH signaling pathway or not remains to be determined.

Finally, SOCS2 mRNA level increased in the stressed group and LNA-treatment decreased SOCS2 gene expression to reach a level comparable to the control group. SOCS2 has a pivotal and indispensable role as a negative feedback inhibitor of GH signaling and is necessary to prevent excessive growth. In our study, SOCS2 gene expression followed an opposite trend as compared to GHR mRNA levels in both control and stressed groups. SOCS2 might also be involved in the regulation of the immune response upon infection. Indeed, SOCS2-/- mice have decreased microbial proliferation, abnormal leukocyte infiltration, an uncontrolled production of proinflammatory cytokines, and an elevated mortality upon infection (Metcalf et al., 2000). It could be inferred that increased gene expression of SOCS2 would potentially correlate with a greater bacterial load.

In agreement with on Tollet-Egnell's work (1999), which showed that glucocorticoids enhanced SOCS2 expression in primary hepatocytes, the basal SOCS2 mRNA level was enhanced in the stressed group as compared to the control. Treatment with LNA-98 restored SOCS2 gene expression, suggesting that SOCS2 gene expression is regulated at least in part by miR-98.

Our results show that LNA-98 treatment helped restore the expression of GHinduced genes involved in cell proliferation, cell survival and cytokine response that were dysregulated by stress via miR-98. As previously mentioned, the GH signaling pathway affects cytokine production, primes neutrophils and stimulates superoxide production and phagocytosis during inflammation (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). It is well-established that stress alters cytokine production. More recently, our team reported that even though superoxide production in the blood was not affected by stress, the phagocytic abilities of neutrophils and macrophages were altered. Additionally, the gene expression of macrophage activation markers characteristic of their functions were down-regulated during stress-impaired wound healing (manuscript in preparation). It could be speculated that restoration of GH signaling pathway, via miR-98 inhibition, would improve the phagocytic abilities of neutrophils and macrophages at the wound site, thus improving bacterial clearance and overall wound healing under stress.

Altogether our study demonstrates that miR-98 inhibition, via LNA-98, helped restore GH signaling pathway and re-establish expression of genes involved in cell survival and cell proliferation. Thus, promoting tissue growth and bacterial clearance and ultimately ameliorates wound healing under stress. LNA-98 treatment provides a novel and exciting therapeutic approach, which could be used clinically to prepare the tissue of patients at risks before surgery in order to boost tissue repair. Given that miR-98 has multiple targets, it is possible that other pathways involved in wound healing could be affected by miR-98 inhibition and have contributed to the improvements in wound healing we observed. The effect of miR-98 inhibition on such targets should be further investigated, as it would broaden the potential applications of LNA-98 as a therapeutic. In addition, LNA-98 treatment could potentially replace GH local treatment, which has dangerous side effects, offering an alternative treatment approach.

#### **GENERAL DISCUSSION**

Wound healing is a complex series of events that rely on the successful completion of the previous phase, which means the completion of the inflammatory phase is crucial. We hypothesized that stress dysregulates expression of genes involved in the Inflammatory phase of wound healing, in part via miRs, and alters neutrophil and macrophage recruitment and functions leading to impaired bacterial clearance and wound healing.

In the first study presented here we showed that stress-induced impaired bacterial clearance was in part due to stress altering phagocytic abilities of neutrophils and macrophages. In addition, stress impacts macrophage activation by inducing the overall dysregulation of macrophage response during tissue repair via differential regulation of activation. The indication of their differential regulation is the change in the expression of markers for different sub-population Indeed the phagocytic abilities of neutrophils and macrophages in the blood was decreased. The contribution from neutrophil versus monocyte/macrophage phagocytosis should be investigated.

In our model, stress did not affect the number of macrophages recruited in the wound.

Interestingly, studies on the effects of stress on phagocytic cell accumulation have yielded conflicting results. Bilbo et al reported that after 2 hours of restraint, increased numbers of neutrophils and monocytes accumulated in a surgical sponge implanted in a mouse (2002), whereas Zhang's team reported that restraint suppressed the migration of neutrophils and macrophages into the peritoneal cavity after IP inoculation of *L. monocytogenes* (1998). These differences between studies may come from the type of stress, namely acute stress (Bilbo et al., 2002) versus chronic stress, as well as the presence of a large number of bacteria in Zhang's model of *L. monocytogenes* infection.

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The present study measured the recruitment of macrophages in a naturalistic model, wound healing, in a model that represents a more persistent stressor.

We showed that stress did not promote a shift of the macrophage population, but did dysregulate the gene expression of markers of all three of the macrophage populations present during wound healing without a significant decrease in macrophage numbers. It is worth pointing out that even though IL4 /IL13 were reported to be necessary for the development of the wound-healing macrophage phenotype (Mosser and Edwards, 2008), IL4 and IL13 could not be detected in the wound in either the control or the stressed groups in this study. This observation supports similar findings by Daley (2010) and Bryan (2005) groups.

Most importantly, a better understanding of the mechanisms behind normal transition of macrophage phenotype during wound healing would provide important information to resolve stress-induced changes in macrophage phenotype with the goal to dysregulate their activation to ameliorate bacterial clearance and wound healing.

The second study focused on the effects of stress affects on expression of miRs and downstream targets during the inflammatory phase of tissue repair. Microarray analysis helped identify 230 miRs differentially regulated during stress-impaired wound healing. Profiling analysis of miRs showed that most stress-induced changes in miR expression occurred during the inflammatory phase.

Nine miRs were selected as well as their predicted mRNA gene targets for further analysis. They were selected upon their potential roles in inflammatory and proliferation phases of wound healing. MiR-21, miR-98, miR-132, miR-155 were upregulated during inflammation in wounds of stressed mice, which as expected correlated with decreased mRNA levels of their target genes CXCL10, TGFβR2, GHR, PGC-1β, NFκB and MyD88 and FGF7. MiR-146b and let-7b were down-regulated during the proliferation phase under stress, yet, the gene expression of their targets TLR4 and IRAK1, respective, was not significantly changed. Finally, miR-27b, miR-125b and miR-30d, which were differentially expressed during wound healing, were not affected by stress. This study provided strong evidence that stress alters miR expression and affects mRNA levels of miR targets in inflammatory and proliferation phases of wound healing, yet the direct or indirect mechanism behind this regulation remains to be explored.

We observed an inverse correlation between miR-21 and miR-132 levels and the expression levels of their potential targets as expected, yet there is no direct evidence of binding of these miRs to the 3'UTR region of their mRNA targets to date. Additional experiments should confirm the link between these miR and their targets.

Three miRs targeted genes involved in macrophage activation phenotype, suggesting that miRs may participate in the regulation of macrophage function: CXCL10, a marker of classically activated macrophage is a potential target of miR-21; miR-98 targets PGC-1 $\beta$  which primes macrophages for anti-inflammatory phenotype (2006); and miR-155 was recently reported to target IL13R1 $\alpha$ , the receptor for macrophage switch IL-13 (Martinez-Nunez et al., 2011). This suggests that dysregulation of miR expression under stress may participate in dysregulation of macrophage phenotype. Because multiple miRs seem to contribute to the regulation of macrophage phenotype, the modulation of multiple miRs might be needed to improve the macrophage response under stress.

Thus, the modulation of gene expression of macrophage activation markers via regulation of miRs may help restore the functions of activated macrophage in the wound, and should be further investigated.

It is worth mentioning that two miRs in this study (let-7b and miR-146b) differentially regulated under stress during wound healing, did not affect mRNA level of downstream targets selected for the study. This absence of differential expression could be explained by the fact that miR expression is triggered after gene expression is induced, therefore if

transcription of a specific mRNA is not active then those mRNAs cannot be regulated by miRs. Alternatively, as multiple miRs can target the same transcript, the effect on a specific target mRNA would be the summation of effects from individual miRs. Finally, miR silencing of these particular genes might be solely through translational repression, which would be reflected in protein levels. As previously mentioned, miRs can prevent protein synthesis by triggering the degradation of target mRNAs (Lim et al., 2005) and/or inhibiting their translation (Jackson and Standart, 2007; Pillai et al., 2007). Therefore, the effect of miR dysregulation under stress on mRNA levels of targets will likely be amplified at the protein level, and should be tested. Finally, the gene expression of additional predicted targets of let-7b and miR-146b should be studied.

In the first two studies we showed that stress altered neutrophil and macrophage response via gene regulation and that the expression of miRs, which are post-transcriptional regulators, was dysregulated under stress. Based on these findings we decided to determine the effect of ameliorating miR levels on wound healing and the potential application of miR inhibitor as therapeutic treatment in stress-impaired wound healing. The third study demonstrated that inhibition of miR-98, via LNA-98, helped restore the mRNA levels of its target, GHR, and its GH signaling pathway. This could also ameliorate the expression of genes involved in cell survival and cell proliferation thus, promote tissue growth, bacterial clearance and ultimately ameliorate wound healing impaired by stress.

Even though miR-98 expression was previously reported to be regulated by LPS exposure, stress regulates miR-98 expression, not bacteria in our model. The mechanism by which stress regulates miR-98 expression remains to be investigated . Indeed little is know about how miR-98 expression is regulated. Bhat-Nakshatri et al (2009) reported that estradiol induced the up-regulation of miR-98 in breast cancer cells. MiRs being non-coding RNAs derived from the intergenic regions or introns regions,

their expression relies on expression of genes in close proximity. MiR-98 sequence lies on Chromosome X, close to the gene for  $17\beta$ - hydroxysteroid dehydrogenase 10 (HSD17B10). This versatile enzyme functions in fatty acid oxidation, amino acid degradation and the metabolism of sex steroid hormones (including estradiol synthesis) and neuroactive steroids (GABAa) and thus has important roles in mammalian development and aging (He and Yang, 2006). HSD17B10 catalyses the conversion of 17beta-OH (-hydroxy)/17-oxo groups of steroids, and is essential in mammalian hormone physiology. It also is believed to be involved in glucocorticoid metabolism (Shafqat et al., 2003). Whether the regulation of miR-98 is linked to the regulation of HSD17B10 should be investigated.

LNA treated groups showed overall lower miR-98 levels. This off-target effect might result from the presence of lipofectAMINE. Various *in vivo* and *in vitro* studies have demonstrated that cationic liposomes cause oxidative stress leading to the generation of reactive oxygen species that is associated with its cytotoxicity (Dokka et al., 2000; Kongkaneramit et al., 2008).

Modified LNA with a complete phosphorothioate backbone have been shown to be more stable with low cytoxicity and can be used without a carrier, which would allow the removal of lipofectAMINE (Boon et al., 2011) (Garchow et al., 2011). Never the less, if a carrier is necessary for the cellular uptake of miR-98 inhibitor nanoparticles should be tested. Indeed nanoparticle technology, including polyLysine, and gold nanoparticles, show promising results in miR-related delivery *in vitro* and *in vivo* (Crew et al., 2012; Jin et al., 2012; Liu et al., 2011).

LNA-98 solution was first injected as a pre-treatment before the wound was placed, and we observed changes in miR-98 levels and gene expression of targets before wounding and one day post-wounding. Use of modified LNA with a longer half-life or repeated administration of LNA-98 should be tested to prolong the effect at the gene level and possibly enhance the ameliorations we observed in wound closure and bacterial clearance.

MiR-98 inhibition restored expression of genes induced by GH signaling pathway, such as SCD1 and MYC. Interestingly, SCD1 is also a predicted target of miR-98, and MYC was reported to be targeted by another member of Let-7/miR-98 family, let-7a.

As previously mentioned, GH signaling pathway affects cytokine production, primes neutrophils and stimulates superoxide production and phagocytosis during inflammation (Manfredi et al., 1994; Peterson et al., 2000; Wiedermann et al., 1991). Even though superoxide production in the blood was not affected by stress, the phagocytic abilities of neutrophils and macrophages were altered. It could be speculated that restoration of GH signaling pathway, via miR-98 inhibition, would improve phagocytic abilities of neutrophils and macrophages and contribute to improve bacterial clearance. This should be determined in a future study.

MiR-98 has multiple targets potentially involved in wound healing, such as PGC-1 $\beta$ . These targets would likely be affected by miR-98 inhibition and may have contributed to the improvements we observed. The effect of miR-98 inhibition on such targets should be further investigated, as it would broaden the potential applications of LNA-98 as therapeutic.

As previously mentioned two major axis, HPA and SNS, contribute to the stress response. .Eijkelkamp et al. (Eijkelkamp et al.) reported that blockade of SNS alphaadrenergic receptors by phentolamine attenuated impairments in wound closure ins stressed mice. The improvement in wound closure was observed during the inflammatory phase but unfortunately was not sustained past D3. In addition, Rojas et al. (2002a) showed that blockade of HPA axis via glucocorticoid receptor antagonist RU486 reduced opportunistic bacteria by nearly 1 log at D3 in the wounds of stressed mice (Rojas et al., 2002b). The blockade of both pathways together did not enhance these effects. The partial amelioration of wound healing under stress by blockade of these axis suggests that another regulatory mechanism was induced by stress. We hypothesized that miR were involved in the stress response. Our study showed that inhibition of miR-98 via LNA-98 treatment significantly improved wound closure from D2 to D5 and ameliorated bacterial clearance by 8 fold at D5 in our stress model, and confirmed our hypothesis. The effect of the blockade of SNS and/or HPA axis on miR-98 expression would provide additional information to solve how stress-induced miR-98 differential expression.

LNA-98 treatment provides a novel and exciting therapeutic approach, which could be used clinically to prepare the tissue of patients at risks before surgery. Most studies conducted on miRs have focused on miRs as diagnostic tools and few studies have investigated miRNA therapeutics. Never the less a Phase I clinical trial is active on targeting miR-122 for therapy of HCV-infection (Isis Pharmaceuticals).

In addition, LNA-98 treatment could potentially replace GH local treatment, which has dangerous side effects, therefore, offering an alternative treatment approach.

Altogether, this work demonstrates that stress dysregulates expression of genes involved in the Inflammatory phase of wound healing, in part via miRs, and alters neutrophil and macrophage recruitment and functions leading to impaired bacterial clearance and wound healing. This series of study constitutes to our knowledge the first descriptive study focusing on the effect of stress on miR expression and miR target gene expression during cutaneous wound healing. The global effect of miRs on genes involved in both inflammatory and proliferative phases of healing highlight the potential use of miRs modulators for clinical application to ameliorate stress-impaired wound healing.

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# VITA

## **EDUCATION**

- **2002** Maitrise de Biochimie, Université de Nice Sophia-Antipolis, France
- **2000** Licence (B.Sc) de Biochimie, Université de Nice Sophia-Antipolis, France
- **1998 Diplôme Universitaire de Technologie, Biologie and Biochimie Appliquées**, Institut Universitaire de Technologie, Toulon, France

## **PUBLICATIONS**

Characterization of Marburg virus glycoprotein in viral entry. Manicassamy B., Wang J., Rumschlag E., Tymen S., Volchkova V., Volchkov V., Rong L. Virology. 2007; 358(1): 79–88.

## AWARDS

03/10/2011 First Place Graduate Student Basic Science, UIC College of Dentistry Award

### **CONFERENCES & SYMPOSIA ATTENDED**

Gordon Research Conference "Tissue Repair and Regeneration", June 2009- Attendee PNIRS Conference, December 2009- Attendee Wound Healing Symposium, April 2010- Attendee PNIRS Conference, June 2011- **Presenter** PNIRS Local Conference Chicago, December 2011-**Presenter** *IADR/AADR Annual Meeting, March 2012-Presenter Wound Healing Society, April 2012-Presenter* 

### **POSTERS PRESENTATIONS**

College of Dentistry Clinic & Research 2010 UIC Student Forum June 2010 College of Dentistry Clinic & Research 2011 *College of Dentistry Clinic & Research 2012- Upcoming* 

## **TEACHING**

Teaching Assistant Microbiology Lab M2 Fall 05, Fall 06

### **PROFESSIONAL MEMBERSHIPS:**

Wound Healing Society 2009-present PNIRS 2009-present AADR 2011-present