Function of Microfibril Associated Protein 5 (MFAP5)

in Wound Healing

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THESIS

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DEDICATION

I dedicate this work first to God, my ultimate strength when I feel weak and my refuge when it gets hard, to you I owe it all. I pray that I live a life that pleases you.

To my dad, my role model who might have left us too early but definitely did not leave our hearts at all. You set the bar high, I know I have a high standard to reach to walk along your steps. To my mom, your affection and warm prayers got me here. Your constant guidance navigated my way and allowed me to take the right decision at the right time. You both always pushed me to be the best version of myself and I hope I make you proud.

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LIST OF ABBREVIATIONS

α-SMA	Alpha Smooth Muscle Actin
αvß3	Alpha-v-Beta 3
ECM	Extracellular Matrix
ACTA2	Actin Alpha Cardiac/Skeletal Muscle 2
ANOVA	Analysis of Variance
CAM	Cellular Adhesion Molecules
COL	Collagen gene
COL1A1	Collagen Type I alpha 1
COL3A1	Collagen type III alpha 1
COL4A4	Collagen type IV alpha 4
COL5A1	Collagen type V alpha 1
COL6A1	Collagen type VI alpha 1
COL7A1	Collagen type VII alpha 1
COL11A1	Collagen type XI alpha 1
COL14A1	Collagen type XIV alpha 1
COL16A1	Collagen type XVI alpha 1
COL17A1	Collagen type XVII alpha 1
CTGF	Connective Tissue Growth Factor
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
FGF2	Fibroblast growth factor 2

LIST OF ABBREVIATIONS

G	Green
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-1ß	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IPA	Ingenuity Pathway Analysis
KGF	Keratinocyte Growth Factor
LOX	Lysyl oxidase
LPP	Lipoma-Preferred Partner
PMN	Polymorphonuclear Leukocytes
MFAP5	Microfibril Associated Protein 5
MAGP2	Microfibril Associated Glycoprotein 2
MMP	Matrix Metalloproteinase
MMP1	Matrix metalloproteinase 1 (also known as interstitial collagenase)
MMP2	Matrix metalloproteinase 2 (also known as gelatinase A)
MMP3	Matrix metalloproteinase 3 (also known as stromelysin 1)
MMP7	Matrix metalloproteinase 7 (also known as matrilysin 1)
MMP9	Matrix metalloproteinase 9 (also known as gelatinase B)

LIST OF ABBREVIATIONS

MMP10	Matrix metalloproteinase 10 (also known as stromelysin 2)
MMP11	Matrix metalloproteinase 11 (also known as stromelysin 3)
NS	Normal Skin
PDGF	Platelet Derived Growth Factor
PBS	Phosphate-buffered saline
PS	Picrosirius
R	Red
RGD	Arg-Gly-Asp
rMFAP5	Recombinant Microfibril-Associated Protein 5
T	Trichrome
TGF-β	Transforming Growth Factor-beta
TNF-α	Tissue Necrosis Factor-alpha
TGFB1	Transforming growth factor beta 1
TGFB3	Transforming growth factor beta 3
VEGF	Vascular Endothelial Growth Factor

SUMMARY

Tissue injury leads to extensive extracellular matrix (ECM) changes throughout the wound healing process. MFAP5 is a 25 kD serine and threonine rich small microfibril-associated protein, involved in the regulation of major ECM pathways and microfibril function. Interestingly, the role MFAP5 plays in the wound healing process is currently unknown. This study was undertaken to identify the genes that are most differentially expressed between skin and oral mucosa as related to wound healing and fibrosis. Previously available human gene array data from scar-forming skin wounds and minimally scarring oral wounds was utilized to investigate the differential expression of genes that are closely related to wound healing and fibrosis in these alternate healing phenotypes. This analysis led to the identification of MFAP5 as a factor that was highly expressed in skin but not oral mucosal wounds, and thus a candidate profibrotic mediator in healing wounds. To directly examine the role of MFAP5 in wound healing, a murine model of full-thickness excisional skin wounds was employed, and the effect of MFAP5 neutralization on healing was assessed. Mice were randomly assigned to three wound treatment groups: phosphate-buffered saline (PBS), immunoglobulin G (IgG), and anti-MFAP5 antibodies. Histologic wound samples were stained with Masson's Trichrome and Picrosirius stains, and AxioVision software was used to quantify collagen deposition and the ratio of mature/immature collagen, respectively. Data was analyzed by 2-way ANOVA and multiple t-test. It was found that in humans, MFAP5 expression was significantly higher in skin versus oral wounds at baseline and throughout the course of wound healing. Furthermore, in the murine model, antibody neutralization of MFAP5 *in vivo* led to decreased collagen deposition with lower mature collagen and significantly higher immature collagen when compared to the control

groups. These results suggest that MFAP5 promotes collagen deposition during wound healing in skin *in vivo*. Therefore, the production of MFAP5 may have significant implications for scar formation in skin and other fibrotic conditions.

I. INTRODUCTION

A. Introduction:

Wound healing refers to the body's natural process of repairing damaged tissue following an injury. It involves a complex series of coordinated physiological processes working to close and restore the wounded area. This typically involves four overlapping stages: hemostasis (the cessation of bleeding), inflammation, proliferation (the growth and movement of new cells and tissue), and remodeling (the maturation and strengthening of the new tissue). The specific course and duration of wound healing can differ depending on various factors such as the nature and severity of the injury, as well as individual variables like age and general health. Healing is a multifaceted process that involves coordinated interactions between multiple biological and immunological systems like blood vessels, platelets, white blood cells, growth factors, extracellular matrix proteins, and different types of cells, such as fibroblasts, keratinocytes, and endothelial cells. In the first phase, hemostasis, a blood clot is formed and platelets aggregate to stop the bleeding. In the second phase, inflammation, immune cells and white blood cells clear the wound of debris and pathogens. The third phase, proliferation, involves the formation of new blood vessels and cells to replace the damaged tissue. Finally, in the remodeling phase, the new tissue is strengthened and restructured to restore normal function. This is the typical process that occurs when all the components function properly and carry out their respective roles effectively.

The extracellular matrix (ECM) plays a crucial role in the wound healing process by providing structure, organization, and signals to cells and tissues. It serves as a guide for cell migration, proliferation, apoptosis, differentiation, and adhesion to direct morphogenesis and

cellular metabolism. The ECM is composed of complex compounds, with collagen comprising a large part of it. Collagen is a key factor in the wound healing process, providing strength and structure to the wound. However, aberrant wound healing may result in numerous pathological outcomes, one of which is fibrosis.

Fibrosis is a pathological outcome of wound healing that results in excess connective tissue depositing in organs or tissue, eventually replacing normal tissue parenchyma and forming a thickened scar tissue. Many studies have tried to identify the key factors in the occurrence of fibrosis. Prior studies have suggested that some components contribute to an inadequate healing process, such as fibroblasts, MMPs, CTGF, MFAP5. Additionally, studies have suggested that mechanical forces could contribute to fibrosis, as more tension can lead cells to produce aberrant ECM, making the tissue as a whole more rigid. Some components are more expressed in the skin than in the oral mucosa resulting in slower wound healing in skin with increased scar formation as compared to oral mucosa. Researchers have used this fact to compare both types of tissues when it comes to fibrosis formation. It has been shown that MMP7 and MMP9 are overexpressed in skin, which is linked to delayed wound healing. The overexpression of MMP2 and MMP3 as seen in oral wounds play an important role in keratinocyte migration, which may explain the overall accelerated and scarless oral wound healing in oral mucosa compared to skin. In skin wounds, as well as internal wounds, the tendency to heal through fibrosis/scarring instead of regeneration, places a significant strain on public health. Studies have investigated the role of different components in the formation of fibrosis. Our study focuses on the function of MFAP5 and its role in scar formation.

B. Objectives

The objectives of this study are to 1) investigate which are the most differentially expressed genes between skin and mucosa, 2) identify the genes that are closely related to wound healing and fibrosis, 3) determine if the modulation of the identified genes alters wound collagen deposition.

C. Hypotheses

We hypothesize that certain genes that are differentially expressed in skin versus mucosa are more related to fibrosis, and that MFAP5 may be a candidate factor that contributes to more fibrotic outcomes in skin wound healing.

II. BACKGROUND

A. Wound healing and the factors affecting its process

Following tissue injury, the wound goes through a complex multifactorial process influenced by the interaction of different cells and signaling molecules resulting in healing. This intricate process is critical to the survival of the organism. Numerous studies have described dermal wound healing and focus on the biological aspects of this process, which involves coagulation, inflammation, angiogenesis, collagen synthesis, epithelialization, wound contraction, and remodeling (Diegelmann and Evans, 2004). Despite the continuous nature of the healing process, the division into distinct phases helps provide a better comprehension of the process.

The phases of wound healing have similar characteristics in various tissues throughout the body (Richardson et al., 2004), but have been best studied in skin. There are four classic phases that harmoniously develop after injury; these include hemostasis, inflammation, proliferation, and remodeling (Figure 1) (Gosain and DiPietro, 2004). The wound healing process has been described by the activation of leukocytes, dendritic cells, lymphocytes and the associated production of growth factors and inflammatory cytokines that sequentially stimulates stem cell proliferation (Karin et al., 2016; Zheng et al., 2020).

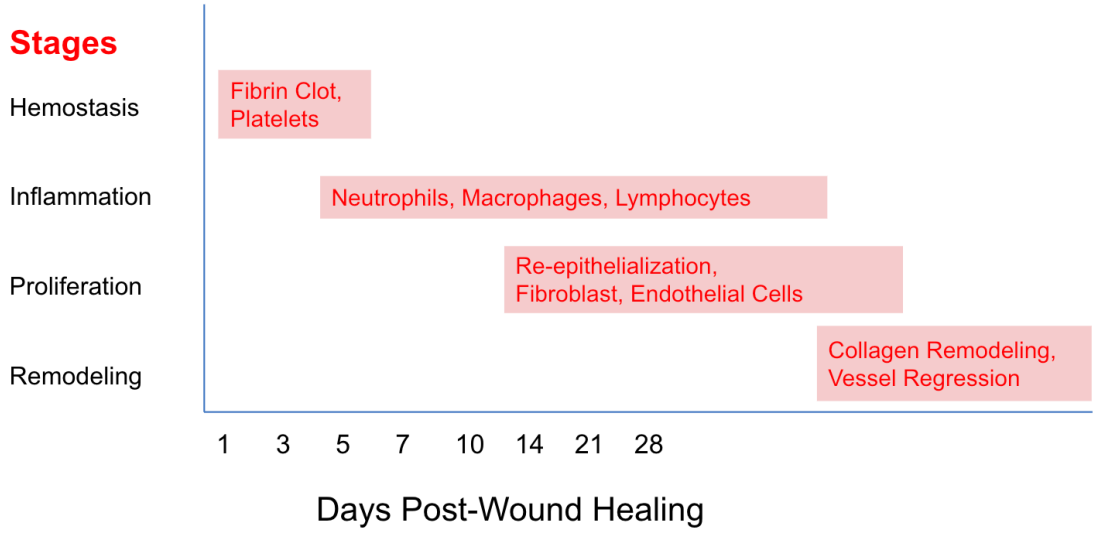


Figure 1: Stages of wound healing. Immediately after injury, hemostasis occurs with an initial fibrin clot forming due to platelet activation. After hemostasis, there is the inflammatory phase, which is when immune cells migrate to the site of tissue injury. Overlapping the inflammatory phase, the proliferation phase occurs, during which the epithelium is reformed, and granulation tissue is deposited to fill the wound gap. Tissue forming cells like fibroblasts, keratinocytes, and endothelial cells characterize this phase. As the proliferation phase resolves, the remodeling phase begins. Remodeling is the longest phase and is marked by capillary regression, wound contracture and collagen maturation.

Hemostasis begins directly after the skin is damaged and is marked by vasoconstriction and blood clot formation. The exposure of collagen triggers both the intrinsic and extrinsic blood

clotting pathways signaling platelet recruitment to the site of injury. The fibrin clot is formed as a result of platelet aggregation and the release of different mediators and adhesive proteins (DiPietro, 2016). The release of these mediators enhances cells' function and facilitates their communication in the healing process. Additionally, these mediators initiate chemotactic signals to recruit inflammatory cells to the injured site.

Polymorphonuclear leukocytes (neutrophils, PMN) are among the first immune responders to the wounded site. Their presence within the blood clot magnifies the leukocyte response through their release of chemotactic mediators which attract other cells involved in the inflammatory phase. The infiltration of leukocytes into the wound site as a response to the chemotactic function of these mediators is the main characteristic of the inflammatory phase. Therefore, the role of inflammatory mediators in controlling the healing process is critical to the inflammation stage of wound healing. Several pro-inflammatory cytokines are released to coordinate the immune response and promote tissue repair. Some of the key pro-inflammatory cytokines involved in this phase include IL-1, IL-6, IL-8, and IFN-γ. These cytokines typically regulate the growth, activation, differentiation, and migration of immune cells to areas of infection to control and eliminate pathogens (Turner et al., 2014). In tandem, the level of cellular adhesion molecules (CAMs) increases and facilitates inflammatory cell migration (Singer and Clark, 1999). Among the immune cells in wounds, macrophages play a critical role both in inflammation and in stimulating the later proliferative phase. During the inflammatory phase, macrophages release proinflammatory mediators to attract leukocytes and activate endothelium. During the proliferation phase, macrophages produce growth factors that can attract fibroblasts, stimulate ECM formation, and promote angiogenesis (Koh and DiPietro, 2011). Mast cells also

play an important role in dermal wound healing, as they release an array of proinflammatory cytokines leading to progressive inflammatory and vascular changes. Their numbers surge as tissue repair proceeds (Wilgus and Wulff, 2014).

During the proliferation phase, the temporary fibrin scaffold created during the hemostasis phase gets replaced by granulation tissue, which has an elevated number of fibroblasts, granulocytes, macrophages, blood vessels, and collagen. This tissue provides a provisional structure to support the restoration and return of function to the damaged skin (Schultz and Wysocki, 2009). Fibroblasts actively synthesize collagen in this phase as a response to the cytokines and growth factors such as PDGF, TGF-β, KGF, VEGF, and FGF2 that are released by platelets and actively produced by fibroblasts, epithelial cells, and inflammatory cells, particularly macrophages (Hinz et al., 2007; Schultz and Wysocki, 2009). The recruitment and proliferation of keratinocytes allows for re-epithelialization and eventual reformation of the epithelial barrier (DiPietro, 2016). Integrins are involved in re-epithelialization and granulation tissue formation during wound healing through their function in cell adhesion and signaling. Integrins are cell surface-associated dimeric glycoproteins that function as cell-to-ECM adhesion receptors. Hence, the synergy between integrin and growth factor receptors is probably a key factor in the regulation of cell proliferation (Koivisto et al., 2014).

Following granulation tissue formation and re-epithelialization, a lengthy remodeling phase begins. Mechanical stress and cytokines such as TGF-β encourage fibroblasts to differentiate into myofibroblasts, which produce α-smooth muscle actin (α-SMA) resulting in wound contraction (Hinz et al., 2007). During this phase, the quickly formed collagen III in the

ECM is replaced by stronger but more slowly-deposited collagen I. In the meantime, overgrown blood vessels begin to regress by apoptosis, resulting in a final vascular density similar to that of normal skin (DiPietro, 2016). Matrix metalloproteinases (MMPs) have a significant impact on the wound healing process in the proliferation and remodeling phases, with the continuous changes in the expression and activity of MMPs (Cui et al., 2017). MMPs also play a role in reducing scarring and fibrosis by either directly breaking down the ECM or indirectly influencing the cellular behavior involved in proteolysis (Giannandrea and Parks, 2014). Each stage of wound healing is crucial for a successful outcome, and adequate progress through each stage is vital.

B. The role of ECM and its importance in wound healing

The ECM is an acellular scaffold that is made up of structural proteins such as collagens, laminins, elastins, and fibronectins that offer flexibility and strength to the dermis. Proteoglycans, and hyaluronan in the ECM can sequester growth factors and water in the surrounding space through their high ability to bind water. Glycoproteins like integrins function to control cell attachment and communication between cells and the ECM. The ECM provides structure, organization, and direction to cells and tissues. In addition, ECM serves as a guide for cell migration, proliferation, apoptosis, differentiation, and adhesion directing morphogenesis and cellular metabolism, regulating cell behavior and performance through direct binding with integrins and other cell surface receptors (Dominguez-Bendala et al., 2012). The ECM also serves as a storage area for growth factors and controls their availability (Schultz and Wysocki, 2009). Studies have shown that imbalances in the ECM can lead to the development of fibrosis, a condition in which excessive ECM accumulation leads to tissue dysfunction.

Collagen is a distinct, three-stranded protein molecule that makes up the majority of the ECM in the skin. The ECM in skin is composed of collagen along with other substances such as fibronectin, elastin, glycosaminoglycans, proteoglycans, laminin, and cellular components (Hopkinson, 1992 a, b; Berry et al., 1998; Enoch and Leaper, 2005). Collagen is primarily produced by fibroblasts and there are currently 21 identified variations of this protein. Of these, 6 are found in the skin, with type I being the most prevalent, accounting for 70% of the collagen in the skin, while type III makes up 10%. There are also small amounts of collagen types IV, V, VI, and VII in the skin (Uitto et al., 1989; Hay, 1991). Collagen is a key factor in increasing the strength of a wound. As the wound healing process progresses, collagen is deposited and remodeled which leads to an increase in the tensile strength of the wound. This strength increases to 20% of normal skin strength by three weeks after injury and continues to increase until it reaches 70% (Desmouliere et al., 1995). The process of healing in tissues is different depending on the type of tissue involved. While epithelial structures can heal through regrowth, connective tissues cannot and instead relies primarily on repair through the formation of scar tissue made of collagen (Berry et al., 1998). The scar tissue is primarily made of type I collagen. Although the collagen architecture in scars is not completely normal, the ECM nevertheless functions to restore tissue integrity, strength, and generally normal function.

C. Skin wound healing and fibrosis

The typical wound healing process in adult mammals, including humans, involves repair rather than the regeneration of the injured tissue, and thus results in the formation of a scar, which is an abnormal tissue with altered ECM structure. In skin, the fibrotic scar is mainly

composed of fibroblasts and disordered collagen fibers in the ECM (Xue and Jackson, 2015). These scars occur partly due to inflammation, which has been shown to foster less than ideal wound healing (Jeschke et al., 2011). Amongst the inflammatory cytokines, TGF-β promotes the transformation of fibroblasts into myofibroblasts to aid in wound healing. Myofibroblasts, the cells responsible for the contractile activity leading to fibrosis, are contractive fibroblasts that contain α-SMA. α-SMA containing myofibroblasts are considered a marker of fibrotic diseases such as liver and renal fibrosis. Prolonged activation of TGF-β signaling sends a signal to myofibroblasts to keep producing ECM, leading to the formation of pathological scarring (Sarrazy et al., 2011). MMPs can have either a suppressing or promoting effect on fibrosis. Some MMPs can decrease fibrosis, while others can enhance it (Giannandrea and Parks, 2014). Initially, one might think that proteins that can break down the matrix, such as MMPs, would be under-expressed in fibrosis or, if present, could help clear the excessive matrix. This is true for some MMPs, which have anti-fibrotic properties (MMP1, MMP2, MMP3, and MMP10), while others can actually contribute to fibrosis (MMP7, MMP9, MMP11) (Giannandrea and Parks, 2014). The development of fibrosis has also been shown to be influenced by many other factors, such as connective tissue growth factor (CTGF), and microfibrillar-associated protein 5 (MFAP5). CTGF contributes to fibrosis by regulating the proliferation of fibroblasts and production of the extracellular matrix. Elevated levels of CTGF are present in many fibrotic diseases, including liver and pulmonary fibrosis (Kalluri et al., 2016). Another component of the ECM is MFAP5; it is a protein that helps keep the fibers of the extracellular matrix organized and stable. It has been observed to increase in fibrotic diseases such as liver and lung fibrosis and may play a role in the development and spread of fibrosis by controlling the formation of fibrotic ECM (Zhu et al., 2021; Broekelmann et al., 2020; Chen et al., 2020; Tabib et al., 2021).

D. The role of wound tension in fibrosis and scar formation

Cutaneous scarring, which is a common skin disorder, is increased in areas of tissue tension, as fibroblasts have a mechanobiologic response to strain. Numerous studies in recent years have indicated that mechanical forces may play a significant role in the development of pathological scarring (Ogawa et al., 2011; Ogawa et al., 2016). The level of tension in our skin varies depending on the part of the body and is influenced by factors such as movement and age (Rosińczuk et al., 2016). When there is more tension, the ECM that is produced is abnormal and increased in stiffness. This increased stiffness can be caused by various factors, including increased forces from cell contraction, the deposition of ECM, or the presence of specific cell types that contribute to the skin's mechanical environment (Rosińczuk et al., 2016; Pawlaczyk et al., 2013). Tension was found to act as a mechanical stimulation inducing inflammatory immune response, which if prolonged and of a chronic nature, may result in more scarring compared to less scarring with less tension. These findings were based on a comparison between stretched and non-stretched wounds in a murine model (Harn et al., 2019).

Compared to adults, fetal wound healing is characterized by faster healing of the outer layer of skin, quicker migration of cells that produce connective tissue, and faster deposition of the matrix that supports cell growth. Moreover, fetal wounds created within the first two trimesters heal scarlessly with a complete restoration of normal tissue structure. It has been suggested that the physical characteristics of the ECM in fetal skin may play a role in the absence of scarring during the healing process (Lorenz et al., 1993; Satish et al., 2010). When examined, the skin of fetal mice was found to have low levels of natural tension and it did not

develop significant scarring during healing. However, when the same level of tension found in human skin was applied to the fetal mouse skin, it developed fibrosis similar to hypertrophic scar formation (Aarabi et al., 2007). The non-scarring outcome of fetal skin has also been shown to be related to the difference in the level of inflammatory cells in fetus skin versus human skin wounds. Whereas the fetal wounds contain few or no inflammatory cells, adult skin wounds have a significant presence of inflammatory cells and expression of proinflammatory cytokines (Coolen et al., 2010; Krummel et al., 1987). In addition, the fetal skin has high levels of hyaluronic acid, (Longaker et al., 1991; Mast et al., 1993), higher ratio of type III to type I collagen (Larson et al., 2010), fewer and less mature mast cells (Larson et al., 2010), and lower expression of TGF-β1 and TGF-β2 (Lo et al., 2012; Hantash et al., 2008).

E. Comparison between oral and skin wound healing

Despite the similarity that skin and oral mucosa share in morphology and function, the comparison of the differences in the healing process are critical to understand the superiority of mucosal healing over skin. Both tissues proceed through the classic stages of wound healing. However, the timeline and duration of these phases varies from mucosa to skin. Mucosa heals faster with minimal scar formation with rare occurrence of scars in the oral cavity. There are different factors that positively impact mucosal wound healing, including thicker epithelium with more cell layers and higher proliferation rate in the basal lamina resulting in higher proliferation rate, less inflammatory infiltrate with lower levels of inflammatory cytokines (e.g. IL-1α, IL-1ß, TNF-α), more highly regulated angiogenic response, presence of moist environment leading to faster re-epithelialization, angiogenesis, and maturation of the wound bed, the existence of saliva which contains an abundance of peptides, proteins, and histatins, including growth factors

stimulating wound healing (Gibbs et al., 2000; Szpaderska et al., 2003; Schrementi et al., 2008; Mak et al., 2009). The presence of a wet environment with salivary flow promotes re-epithelization, angiogenesis, and maturation of the wound bed (Dyson et al., 1992; Junker et al., 2013; Svensjo et al., 2000; Vogt et al., 1995). Studies on de-salivated mice showed a decrease in healing compared to sham-operated ones (Bodner et al., 1991; Bodner et al., 1993 a, b). Another factor that promotes healing without scarring is the decreased ratio of TGF-ß1/TGB-ß3 that is suggested to predict scar formation in mucosal wounds (Schrementi et al., 2008). The presence of TGF-β, which is mainly secreted by immune cells such as T-cells and macrophages has some effect on the process of healing through its effect on fibroblast proliferation. TGF-β1 is associated with scar formation while TGF-β3 is more associated with scarless fetal wound healing (Penn et al., 2012). Studies in murine models have demonstrated that higher levels of TGF-β1, but lower or similar levels of TGF-β3 are found in skin compared to mucosal wounds (Schrementi, 2008; Pastar et al., 2014). As mentioned earlier, MMP production has also been linked to fibrosis, and differences in MMP levels have been described in oral mucosal versus skin wounds. More specifically, the expression of MMP1, MMP2, MMP3, and MMP10 was higher in oral compared to skin fibroblast whereas, MMP7, MMP9, and MMP11 were more highly expressed by skin fibroblasts (Mah et al., 2014; Stephens et al., 2001; Chinnathambi et al., 2005). Studies have shown that the overexpression of MMP7 and MMP9 is linked with delayed wound healing, while MMP2 and MMP3 play an important role in keratinocyte migration which may therefore explicate the overall accelerated and scarless oral wound healing in oral mucosa compared to skin (Caley et al., 2015; Krishnaswamy et al., 2017; Letra et al., 2013; Reiss et al., 2010). Although pathogenic microbes colonizing wounds can significantly slow down the healing process, both in mucosal and skin wounds, research has shown that a healthy oral biofilm

can lead to a higher production of antimicrobial peptides and improved protective properties in lab-grown human gingival tissue (Shang et al., 2018; Laheji et al., 2013).

Recent studies that have investigated the genomic response to injury in skin and mucosa have demonstrated dramatic differences in the gene expression patterns of oral and skin wounds. (Chen et al., 2010; Leonardo et al., 2022; Iglesias-Bartolome et al., 2018). These studies have shown that compared to skin wounds, the genomic response of oral wounds is reduced in intensity. They have also identified multiple differentially expressed genes that are present both at baseline and in wounded tissues. For example, the transcriptionally regulators SOX2 (sex-determining region Y-box 2) and PITX1 (paired-like homeodomain have been shown to be upregulated in oral mucosa versus skin, a feature that likely partially explains the differential healing in the two tissues.

F. The effect of pathological scar formation in wound healing on public health

The healing process through fibrosis/scaring, instead of regeneration, places a significant strain on public health. Pathological scars in humans include hypertrophic scars, keloids, and contracture scars (Figure 2). The exact cost of illnesses caused by pathological scars in terms of economic expenses is challenging to determine, but it is estimated to be in the tens of billions of dollars (Mathieu et al., 2006; Menke et al., 2007). Therefore, the transformation of fibrotic healing into a regenerative one where original tissues are restored is paramount to relieve the burden and improve human health and quality of life (Jeschke et al., 2011).

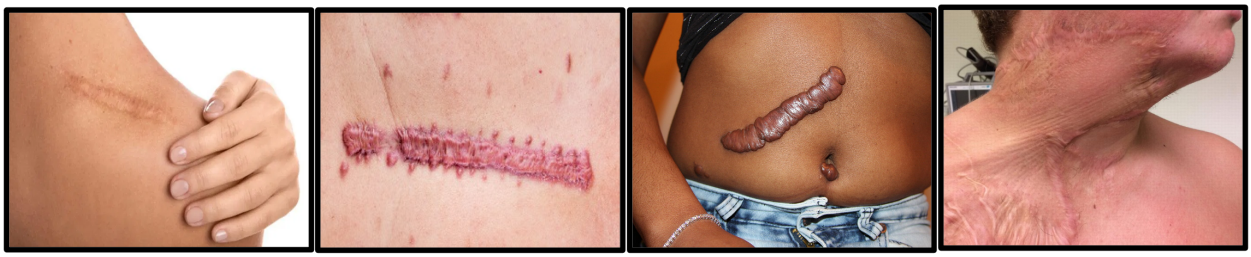


Figure 2. Different types of scars. In adult skin, the repair of the dermis nearly always results in a scar with abnormal ECM architecture and less tensile strength than normal skin. Fibrous skin scars are a major clinical problem with outcomes ranging from mild to hypertrophic scars, keloids, and painful contractures. Pathologic skin scarring or fibrosis often leads to aesthetic consequences, functional deficits, and adverse psychological effects.

F. Identification of the most differentially expressed genes related to wound healing and collagen deposition when comparing skin to oral mucosa wounds

A large number of genes become differentially regulated in response to wound injury, with an estimated 1/3 of the genome exhibiting significantly differential expression levels in skin wounds (Chen et al., 2010). Therefore, identification of the genes, across the genome, that are

closely related to the wound healing process, and those that might be important mediators of scar formation, is of importance to human healing outcomes.

In this study we utilized existing micro-array gene expression data from of skin and oral wounds. Our strategy was to compare broad gene expression profiles in skin and oral wounds, with an aim to identify pro-fibrotic genes that were highly expressed in skin, but not oral mucosal wounds. This analysis resulted in the identification of MFAP5 as a candidate pro-scar forming gene for further study.

III. MATERIALS AND METHODS

A. Human oral and skin wound microarray

The genomic dataset for human oral and skin wounds was previously obtained under IRB approval as described in Leonardo et al. 2022 article (Leonardo et al., 2022).

Using a microarray gene expression data of tissue samples from humans, we compared the expression levels of each microarray dataset of the 30 genes that were identified to be most closely related to essential roles in wound healing in the skin and oral to identify possible profibrotic genes mucosa. These two tissue types have distinct regenerative and scarring phenotypes. The candidate genes included These genes were grouped into families according to their structure and functions: MMPs (MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, MMP12, MMP13, MMP19), TIMPs (TIMP1, TIMP2, TIMP3), collagens (COL1A1, COL3A1, COL4A4, COL5A1, COL6A1, COL7A1, COL11A1, COL14A1, COL16A1, COL17A1), growth factors (TGFß1, TGFß3, CTGF, FGF2), MFAPs (MFAP2 and MFAP5), other relevant genes (ACTA2 and LOX). Through the analysis of these gene expression data, MFAP5 was identified as the most highly differentially expressed gene among those we examined in skin wounds as compared to oral wounds.

B. Animal study

To investigate if neutralizing MFAP5 in skin wounds would affect collagen deposition or scar formation, a mouse excisional wound model was used. Eight- to 10-week-old female C57/BL/6j mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups of five in a temperature-controlled vivarium (22 to 24°C) on a 14-h:12-h light-

dark cycle and had free access to food and water. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). The shaved dorsal skin was cleaned with 70% isopropyl alcohol. Four 5mm full-thickness excisional skin wounds were made using a punch-biopsy instrument (Acu Punch, Acuderm Inc, Fort Lauderdale, FL). Mice were randomly assigned to one of three treatment groups: phosphate buffered saline (PBS), normal mouse IgG isotype control (ThermoFisher Scientific), or an anti-MFAP5 monoclonal antibody (clone 130A) (Yeung et al., 2019). Each wound was topically treated with 40 µl of PBS, 1.5 µg of mouse IgG control or 1.5 µg of anti-MFAP5 monoclonal antibody in 40 µL PBS immediately after injury. The wounds were treated through subcutaneous injections under each wound with the same amount of antibodies administered topically on days 3, 6, 9, 12, and 15 post-wounding. On days 7, 14, and 21 post-wounding, the wound tissues were harvested. The excised skin removed during wounding was used as the uninjured or normal skin (NS) sample (day 0). Samples were fixed in 10% neutral buffered formalin solution for 24 hours. Eight µm paraffin-embedded sections were used for Masson's trichrome and Picrosirius red staining as described below. All animal procedures performed were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois Chicago.

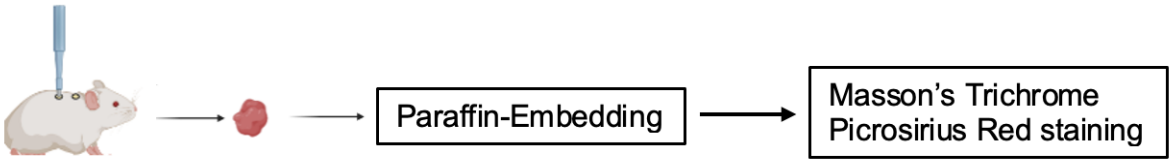


Figure 3: Schematic diagram depicting workflow following mouse wound tissue collection in an excisional dorsal skin wound healing model. Mice were wounded with a 5-mm punch biopsy and treated with either PBS, IgG, or anti-MFAP5 antibodies. Mouse wounds were collected throughout wound healing and then stored in 10% neutral buffered formalin solution, paraffin embedded and sectioned. Histological slides were stained with Masson's Trichrome and Picrosirius Red staining to assess collagen deposition and maturity.

a. Masson's trichrome staining and image analysis

After fixation of samples in formalin and embedding them in paraffin, 5µm sample sections were stained using Masson's trichrome staining as previously described (Zhao et al., 2016) to visualize the blue collagen content. Slides were evaluated under a microscope (Carl Zeiss Microscopy); photomicrographs were taken at 20 magnification and analyzed in imageJ software using standardized color thresholds to identify and quantify collagen deposition. Collagen content in the wound bed was calculated as follows: (blue area)/(total area of wound bed) X 100. One image per sample was taken and analyzed.

b. Picrosirius red staining and image analysis

After fixation of samples in formalin and embedding them in paraffin, 5µm sample sections were stained using picrosirius red staining as previously described (Zhao et al., 2016) to visualize the immature (collagen III) and mature (collagen I) collagen content. Slides were evaluated under a polarized microscope (Carl Zeiss Microscopy); photomicrographs were taken at 20 magnification and analyzed in imageJ software using standardized color thresholds to identify and quantify areas of mature (red-orange) and immature (green-yellow) collagen. The percentage of the pixel area of green immature collagen/total pixel area and the percentage of the pixel area of red-orange mature collagen/total pixel area in the wound bed were calculated. The percentage of immature collagen or mature collagen in relation to total collagen was calculated as follows: green pixel area or red-orange pixel area/total pixels of red-orange area+green area x100. one to three images per sample were taken and analyzed.

C. Statistical analyses

Data from the human gene array picrosirius red staining data from the animal study were analyzed by two-way ANOVA followed by post-hoc Benferonni test. Trichrome staining data from the animal study was analyzed by multiple t-test (GraphPad Software, San Diego, CA). N numbers for the human gene array and the animal studies are listed in Table 1 below. Statistical significance marked when p values were less than 0.05.

Table 1: N numbers for human gene array and animal studies

Human					
Hours post-wounding	0	6	24	72	168
Oral samples (n)	17	6	12	10	6
Skin samples (n)	13	7	10	9	6
Mouse					
Days post-wounding	7	14	21		
Samples (n) per group	5	5	5		

IV. RESULTS

A. Gene array analysis

Previous work in our lab investigated the changes in gene regulation in the human skin and hard palate and its response to injury (Leonardo et al, 2022). Since genes that are regulated during the wound healing process in response to skin injury are likely to be functionally important for the wound repair process, gene array studies were conducted to identify the expressed genes across the genome. Gene expression analysis was performed for 30 genes that are closely related to the ECM and scar formation in wound healing. This was undertaken to compare the expression of these genes between human oral and skin wounds. These genes were grouped into families according to their structure and function, and include MMPs (MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, MMP12, MMP13, MMP19), TIMPs (TIMP1, TIMP2, TIMP3), collagens (COL1A1, COL3A1, COL4A4, COL5A1, COL6A1, COL7A1, COL11A1, COL14A1, COL16A1, COL17A1), growth factors (TGFß1, TGFß3, CTGF, FGF2), MFAPs (MFAP2, MFAP5), and other relevant genes (ACTA2 and LOX). Figures 4-9 show the comparisons of the relative expression of these genes in skin and oral wounds, which are discussed below.

MMPs

The gene expression of MMP8 and MMP13 had no significant difference between skin and oral wounds ($p > 0.05$). All other MMPs that were examined showed significant difference in their expression in skin when compared to the oral wounds at one or more time points (Figure 4). MMP1 was found to have significantly higher expression in skin over oral wounds at the 72-hour

mark (p **<0.01). MMP2 showed significantly higher expression in skin over oral wounds at baseline only (p *<0.05). MMP3 significant expression was limited to the 72-hour mark with significantly higher expression in skin over oral wounds at the 72-hour mark (p *<0.01). Interestingly, MMP9 showed significantly lower expression in skin versus oral wounds at the 24-hour mark (p *<0.05), which reversed to significantly higher expression at the two following time points 72- and 168-hours post-wounding (p**<0.01, *** <0.001). Similarly, MMP10 was found to have significantly lower expression in skin versus oral wounds at the 6- and 24-hour marks (p *<0.05 and **<0.01), which reversed to significantly higher expression at 72 hours post-wounding (p *<0.05). MMP12 showed significantly higher expression in skin versus oral wounds at two time points, the 6- and 168-hour marks (p **<0.01). Lastly, MMP19 showed significant expression differences with significantly lower expression in skin at the 6-hour mark (p *<0.05) but significantly higher expression at the 72-hour mark (P ****<0.0001).

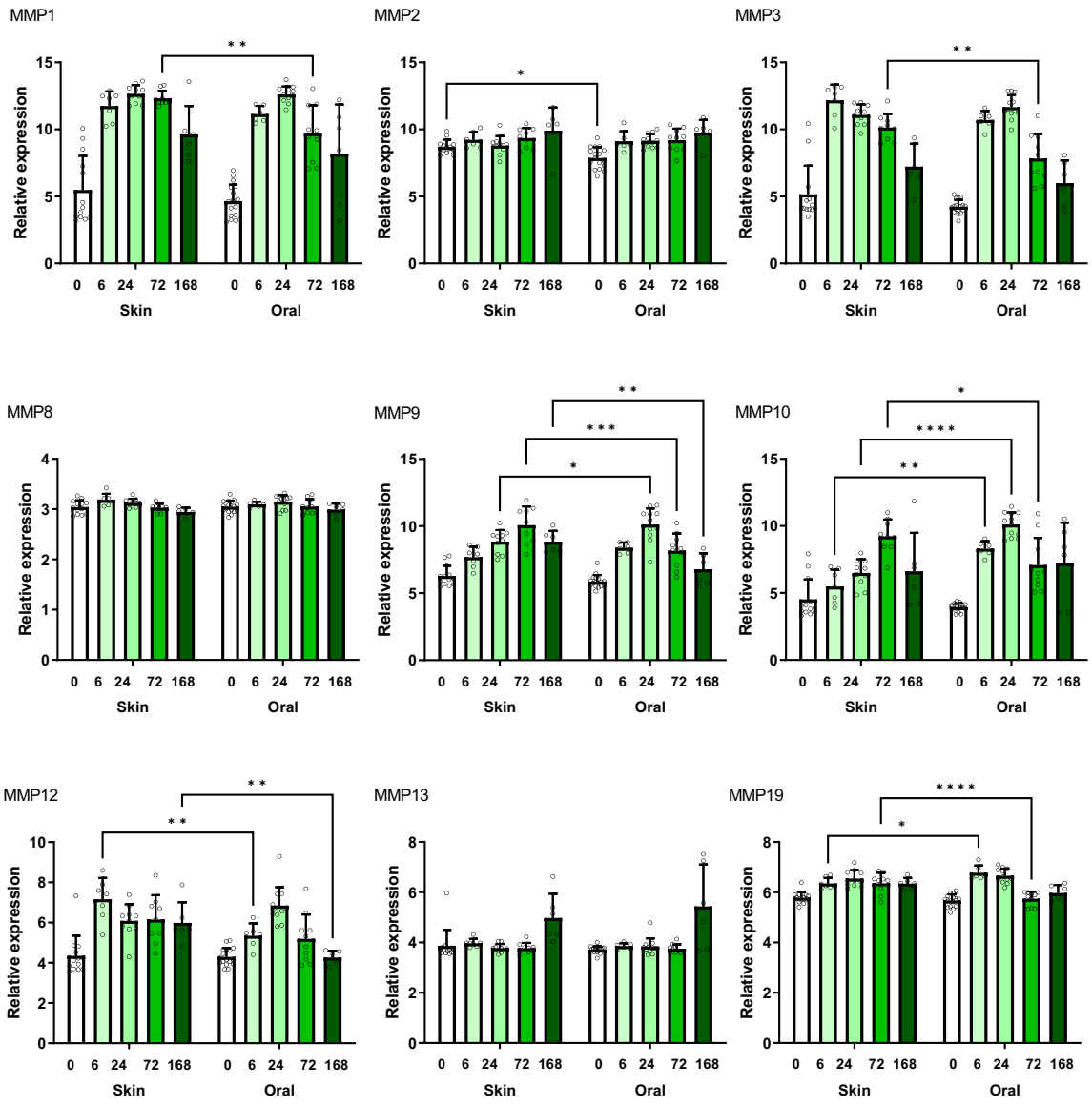

MMP1
MMP2
MMP3
MMP8
MMP9
MMP10
MMP12
MMP13
MMP19
Relative expression
0 6 24 72 168 0 6 24 72 168
Skin
Oral

Figure 4: MMPs (MMP1, MMP2, MMP3, MMP8, MMP9, MMP10, MMP12, MMP13, MMP19) genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. P *<0.05, **<0.01, *** <0.001, ****<0.0001.

TIMPs

Relatively consistent findings were seen among the three studied TIMPs. Both TIMP1 and TIMP2 were found to show significantly higher expression in skin wounds at 72-hour time point as compared to oral wounds (Figure 5) (****, p<0.0001). On the contrary, TIMP3 showed significantly lower expression in skin versus oral wounds (***, p<0.001).

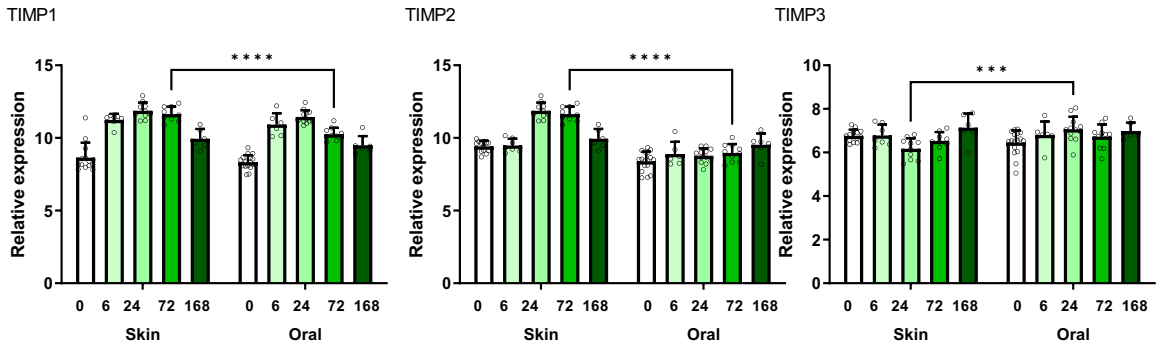


Figure 5: TIMPs (TIMP1, TIMP2, TIMP3) genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. *** $p<0.001$, **** $p<0.0001$.

Collagens

Several collagen genes were found to have no significant difference between skin and oral wounds including COL3A1, COL5A1, COL6A1, and COL16A1 (Figure 6) ($p >0.05$). COL1A1 showed significantly lower expression in skin over oral wounds at the 6-hour time point but this reversed showing higher expression of COL1A1 at 168 hours (****, $p<0.0001$). COL4A4 only showed lower expression in skin compared to oral wounds at baseline (***, $p<0.001$). COL7A1 was found to have significantly lower expression in skin over oral wounds at the 6- and 24-hour time points (* $p<0.05$ and *** $p<0.0001$). Overall, COL11A1 had the highest expression with lower expression in skin versus oral mucosa at 6-, 24-, 72-, and 168 time points (*$p<0.05$, **$p<0.01$, *** $p<0.001$, ****$p<0.0001$). COL14A1 showed significantly lower expression at two time-points the 72- and 168-hour marks (**** $p <0.0001$). Lastly, COL17A1 showed significantly lower expression in skin versus oral wounds at two time-points the 0- and 16-hour marks (** $p<0.01$).

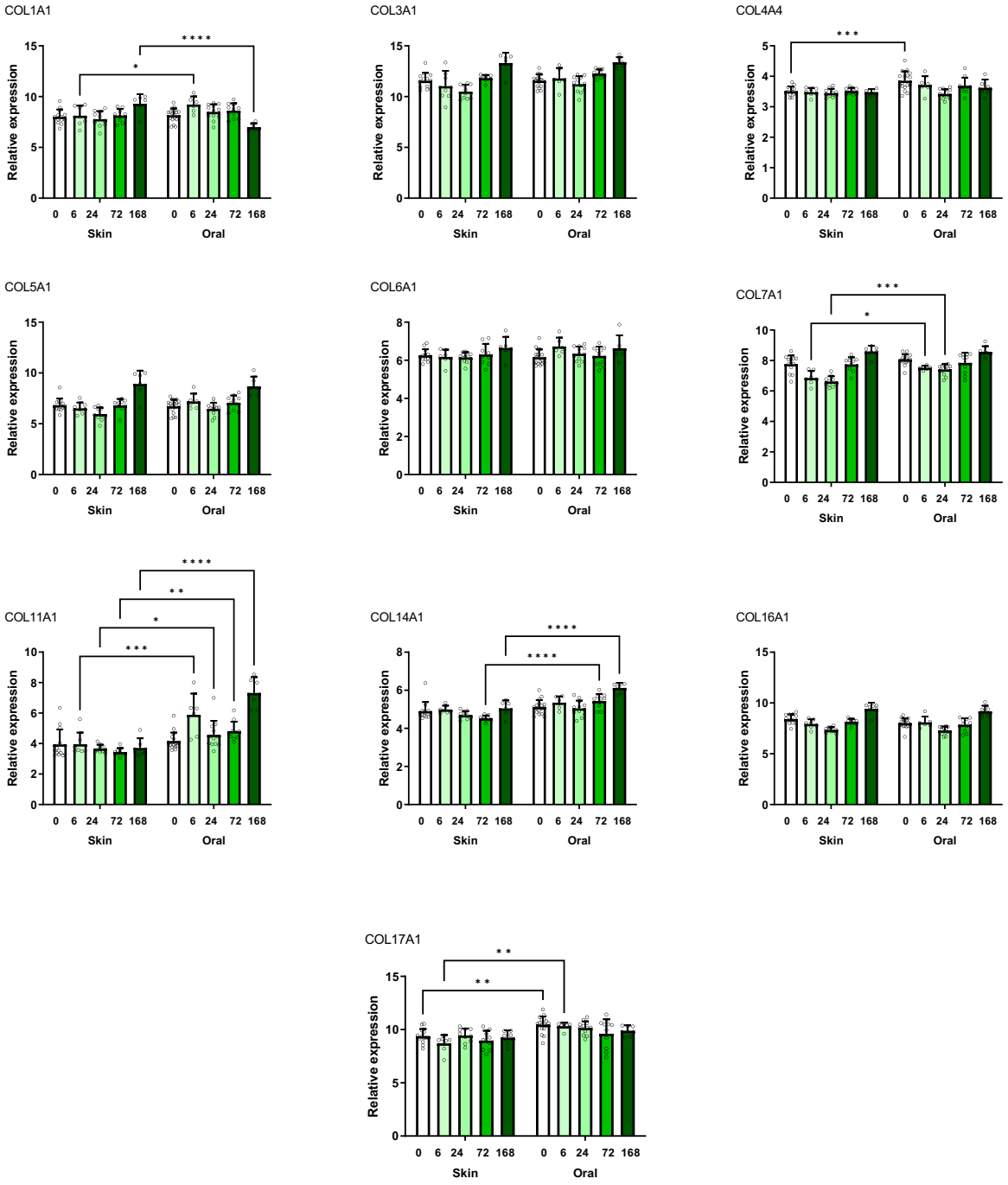

COL1A1
COL3A1
COL4A4
COL5A1
COL6A1
COL7A1
COL11A1
COL14A1
COL16A1
COL17A1
Relative expression
0 6 24 72 168 0 6 24 72 168
Skin
Oral

Figure 6: Collagens (COL1A1, COL3A1, COL4A4, COL5A1, COL6A1, COL7A1, COL11A1, COL14A1, COL16A1, COL17A1) genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. P *<0.05, **<0.01, *** <0.001, ****<0.0001.

Growth Factors

Interestingly, no significant difference was found between skin and oral wounds for TGFß1 gene at any of the time points (Figure 7) ($p > 0.05$). TGFß3 gene showed significantly lower expression in skin when compared to oral wounds only at the 24-hour (*** $p<0.001$). CTGF showed significantly higher expression in skin over oral wounds at baseline, 72- and 168-hour time points (* $p<0.05$, ** [<0.01, **** $p<0.0001$). Lastly, FGF2 showed significantly lower expression in skin versus oral wounds at two time-points 6- and 24- hour marks (**** $p<0.0001$).

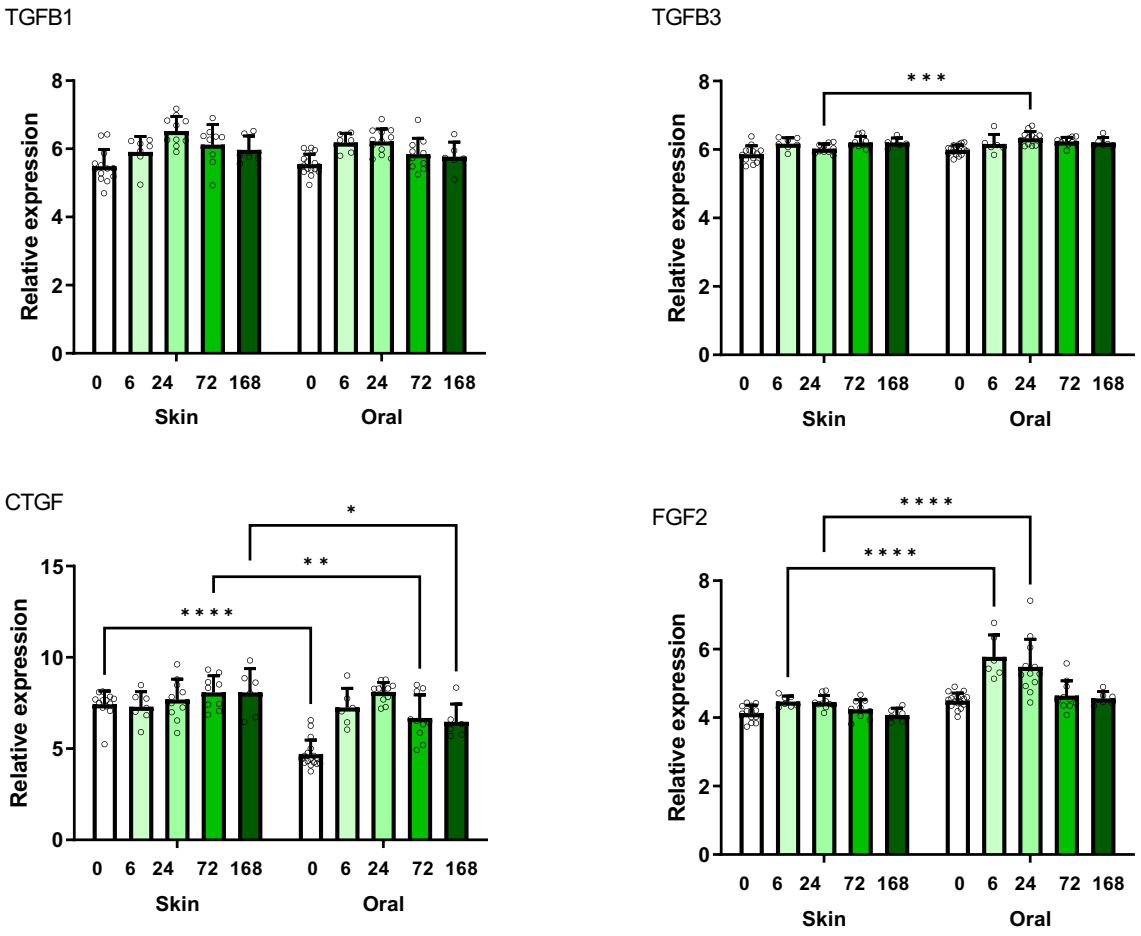


Figure 7: GFs (TGFß1, TGFß3, CTGF, FGF2) genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. P *<0.05, **<0.01, *** <0.001, ****<0.0001.

ACTA2 and LOX

The gene expression of ACTA2 had no significant difference between skin and oral wounds (Figure 8) ($p>0.05$). However, LOX expression was lower in the skin as compared to oral wounds at all time points except the (** $p<0.01$ and **** $p< 0.0001$).

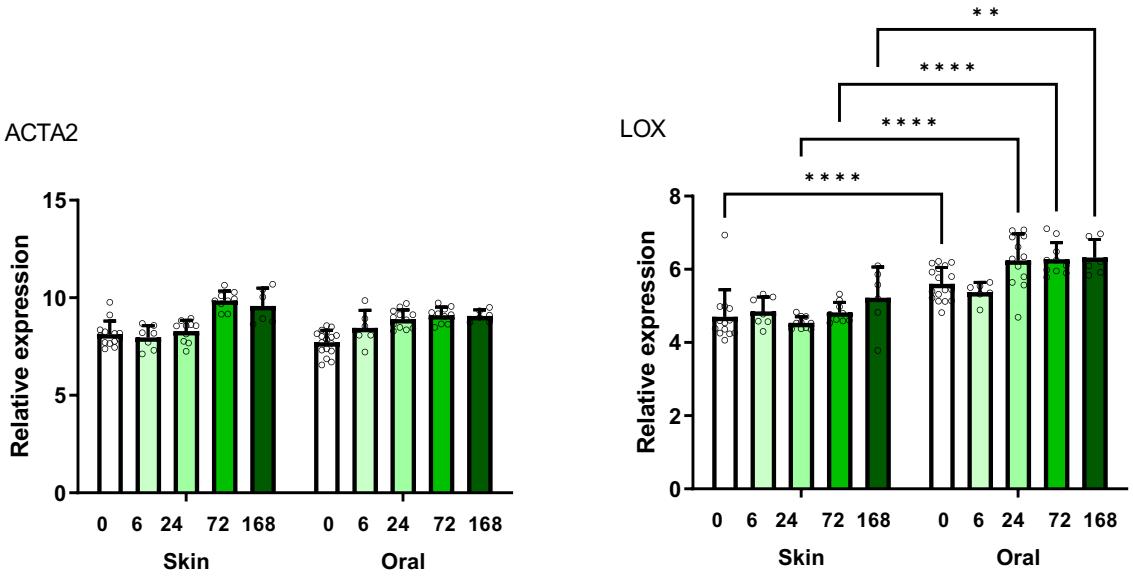


Figure 8: ACTA2 and LOX genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. P **<0.01 and ****<0.0001.

MFAPs

The gene expression of MFAP2 had no significant difference between skin and oral wounds (Figure 9) ($p>0.05$). However, MFAP5 expression was significantly higher in the skin wounds at all time points as compared to the oral wounds (Figure 9, **** $p<0.0001$). Among the genes that were analyzed, MFAP5 was identified as the most differentially expressed gene exceeding all other genes analyzed, creating high plausibility of its connection to fibrosis.

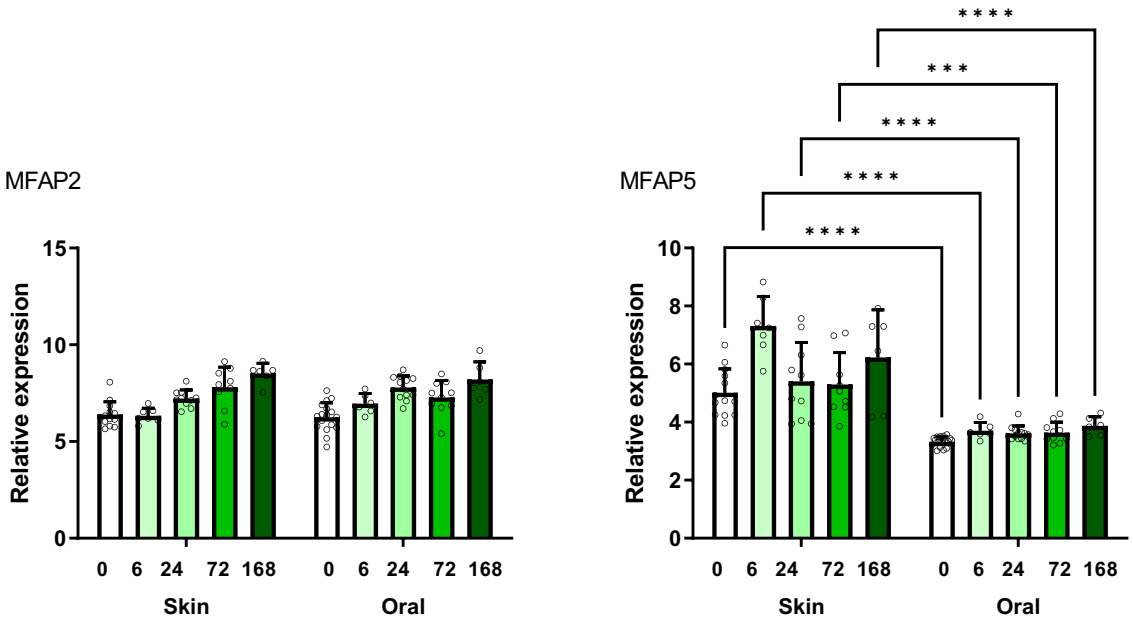


Figure 9: MFAP2 and MFAP5 genes expression in human skin and oral wounds. The X axis is representative of hours post-wounding and the Y axis is representative of the relative expression of genes at investigated time points. P ****<0.0001.

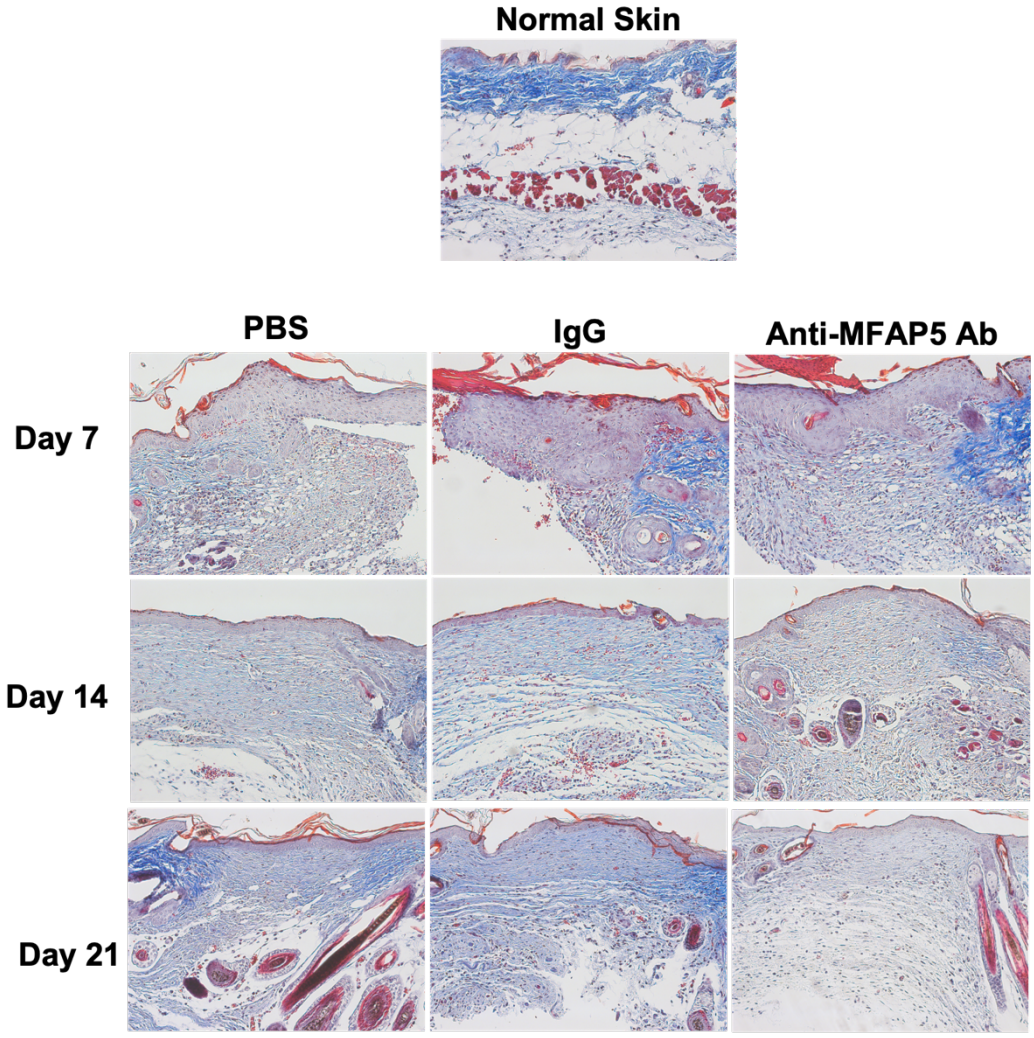
B. MFAP5 and scar formation

In order to elucidate the roles of MAFP5 in collagen deposition and composition in skin wounds, we treated skin wounds with an MFAP5 neutralizing antibody and then performed Masson's Trichrome and Picrosirius red staining to assess collagen content and structure in treated and control wounds.

Total collagen deposition

Findings from the Masson's Trichrome staining indicated higher total collagen deposition (blue stained structures) in PBS and IgG groups compared to anti-MFAP5 group in Day 7, 14, and 21 wounds, this difference reached statistical significance between IgG and anti-MFAP5 at day 21 (Figure 10 A&B and Table 2).

A.



B.

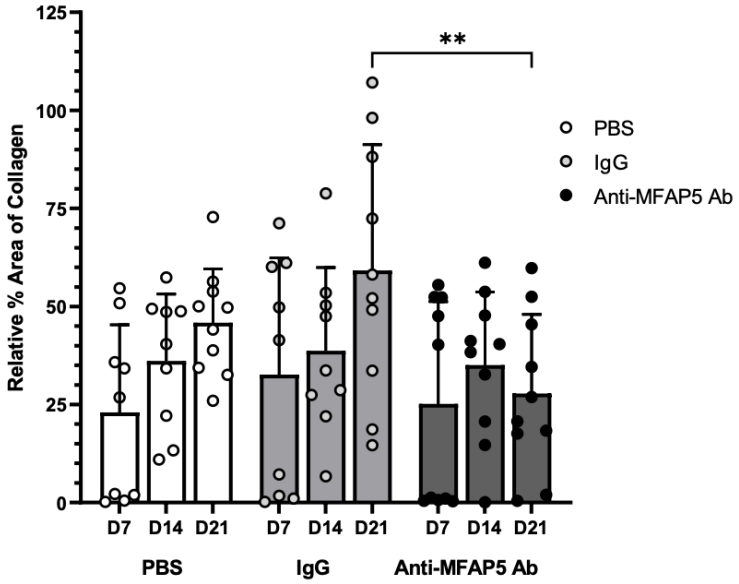
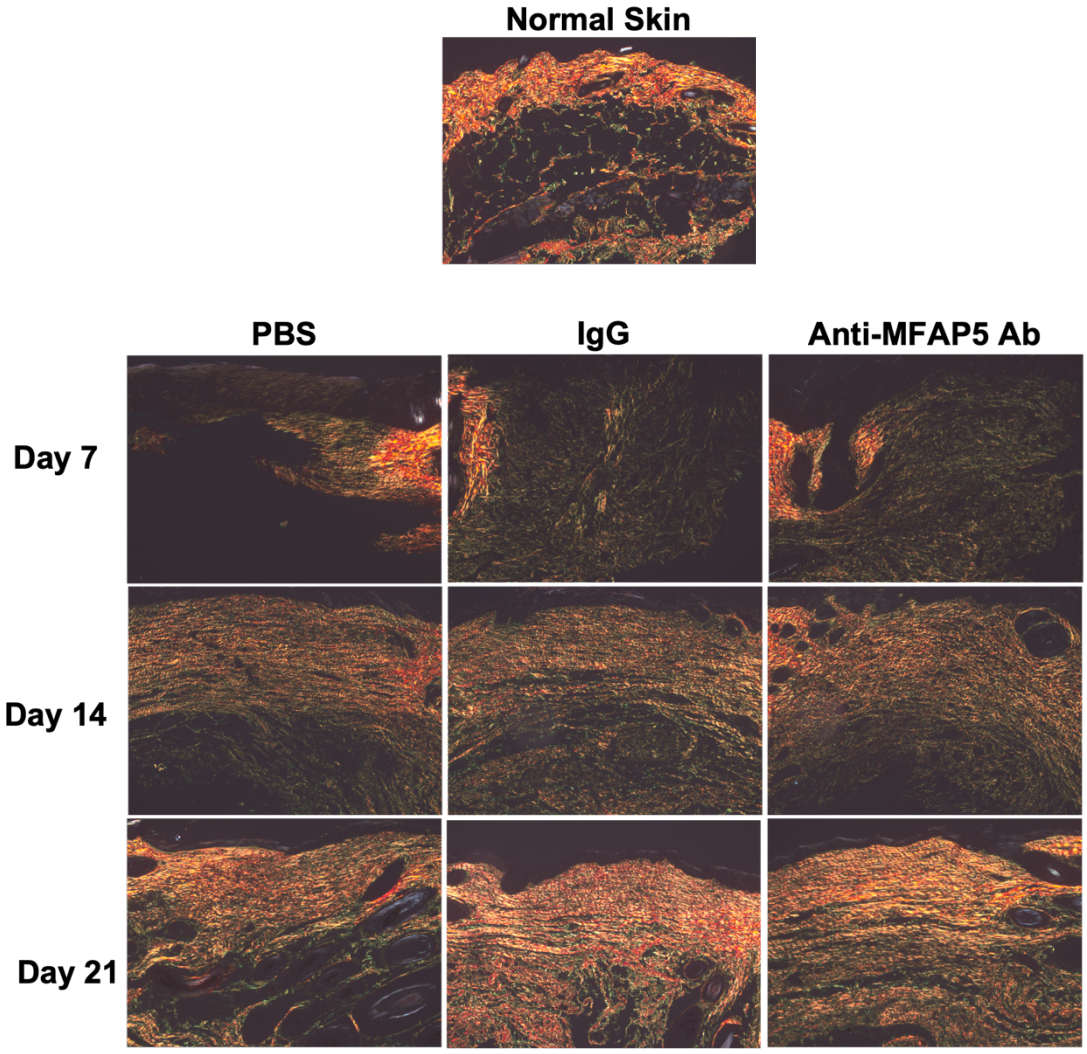


Figure 10. A) Microscopic images representing collagen histological slides stained with Masson's Trichrome staining at days 7, 14, and 21 post- wounding along with normal (unwounded) skin. Blue: stained collagen. Varying degrees of blue color density can be interpreted as a representative of collagen quantity. B) MFAP5 blockade decreases total collagen deposition. Quantification of blue-stained collagen comparing the three studied groups. P **<0.01.

Mature and Immature Collagen Composition

Picrosirius red staining was used to determine the ratio of mature (collagen I-red/orange color) and immature collagen (collagen III-green color) in wounds of anti-MFAP5 antibody treated mice as compared to PBS and IgG treated groups. Findings from the Picrosirius red staining indicate significantly higher collagen III in anti-MFAP5 compared to IgG and PBS at day 7 post-wounding but leveled down to no significant difference at both day 14 and day 21 post-wounding (Figure 11 A&B, Table 3-7). Therefore, it is apparent that the blockade of MFAP5 affects the early stage of wound healing with effects mainly seen at day 7. This blockade resulted in higher expression of collagen III and lower expression of collagen I.

A.



B.

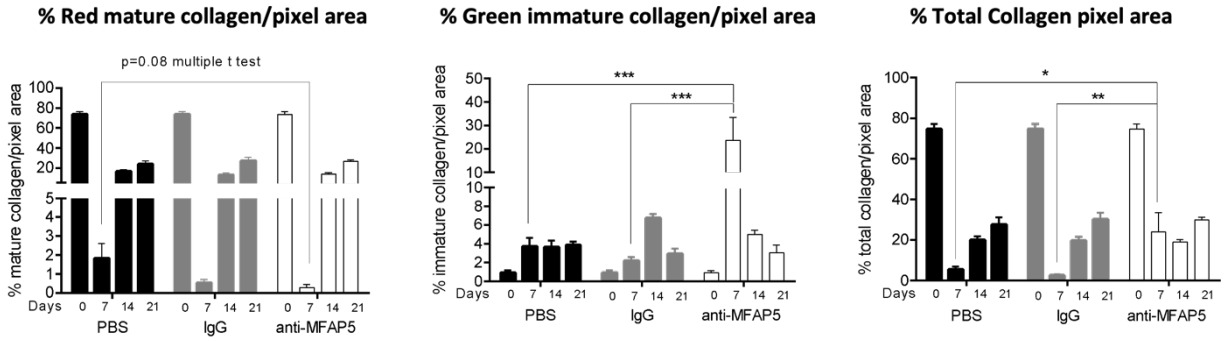


Figure 11. A) Microscopic images representing histological slides of mature (collagen I, red/orange) and immature collagen (collagen III, green) stained with Picrosirius red staining at days 7, 14, and 21 post-wounding along with normal (unwounded) skin. Red/orange: stained collagen I, green: stained collagen III. Varying degrees of red and green color density can be interpreted as a representative collagen quantity I and III, respectively. B) MFAP5 blockade increases collagen III and decreases collagen I deposition. Percent of mature (collagen I, red/orange), immature collagen (collagen III, green), and total collagen based on Picrosirius red staining comparing the three studied groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

V. DISCUSSION

A. Discussion

This study investigated the role of MFAP5 in skin wound healing, and provides new information about the function of this protein in the context of healing wounds. Prior research has revealed a functional role for MFAP5 in other systems, particular in cancer. MFAP5 is highly expressed in stromal fibroblasts in several different human cancers with higher expression linked to poorer outcomes (Chen et al., 2020; Leung et al., 2014; Leung et al., 2018; Li et al., 2018; Mok et al., 2009; Wu et al., 2019; Yeung et al., 2019; Zhou et al., 2020). In these cancers, MFAP5 has been found to influence various cell phenotypes in the tumor microenvironment and promote cancer fibrosis, angiogenesis, and chemoresistance. Despite the relatively extensive investigation of MFAP5 in diverse cancer types and human diseases, its involvement in wound healing was not previously examined (Craft et al., 2018; Vaittinen et al., 2011; Zhu et al., 2021).

The complexity and intricate nature of wound healing led us to investigate the expression of 30 genes that are closely related to collagen deposition and fibrosis during wound healing. This allowed us to identify several candidate genes that are closely related to wound healing and fibrosis. Using a microarray gene expression analysis of tissue samples from humans, we compared the expression of these genes in the skin and oral mucosa as these two tissue types have distinct regenerative and scarring phenotypes. The candidate genes included the MMP family, TIMP family, collagen family, GF family, MFAP family, and other fibrosis related genes like LOX. Through the analysis of these gene expression data, MFAP5 was identified as the

most highly differentially expressed gene among those we examined in skin wounds as compared to oral wounds.

In the mouse skin wound model, we further found that the inhibition of MFAP5 had a significant impact on collagen deposition compared to control wounds treated with PBS and mouse IgG. This was evidenced by the increased expression of collagen III (immature collagen) and decreased expression of collagen I (mature collagen) in the early stages of wound healing (day 7) in the anti-MFAP5 treated group. Also, the anti-MFAP5 antibody treated group had decreased collagen deposition during the later stage of wound healing (day 21). These findings suggest that MFAP5 plays a pro-fibrotic role in the wound healing process through increasing collagen deposition. This study, therefore, contributed to the current understanding of wound healing by exploring the potential relationship between MFAP5 and fibrosis through its effect on collagen deposition. In addition, our findings suggest that MFAP5 may be a key factor that determines the differential outcomes in skin and oral mucosal healing.

We did not yet study the mechanism by which MFAP5 regulates collagen synthesis and deposition. According to previous investigations (Mecham and Gibson, 2015), MFAP5 has the ability to bind to fibroblast αvß3 integrin via the RGD motif. This binding can activate the αvß3 integrin, which has been shown to stimulate collagen gene expression and drive fibroblast contraction and extracellular matrix (ECM) stiffening, contributing to the progression of fibrosis (Asano et al., 2005; Fiore et al., 2018; Leung et al., 2014; Mecham and Gibson, 2015). It is possible that MFAP5 binds to fibroblast αvß3 integrin during wound healing to promote ECM deposition and remodeling, which may explain why MFAP5 expression increases significantly

during the later stages of wound healing when fibroblasts produce a fibrous scar and differentiate into myofibroblasts to exert contractile force. In line with this theory, observations in our lab have demonstrated that treatment of dermal fibroblasts with rMFAP5 leads to significantly increased expression of ACTA2 and collagen genes (COL1A1, COL6A3, and COL11A1), a marker of myofibroblast differentiation, and facilitates fibroblast collagen gel contraction (Chen et al., submitted).

B. Limitations of this Study

One limitation of this study involves the analysis of collagen content using Picrosirius red staining. We used a conventional polarized microscope for this study. However, others have reported that using the four-axis Universal Stage to analyze collagen deposition and structure in picrosirius red stained tissue sections is more accurate than our use of a conventional polarized microscope (Canham et al., 1991). The Universal Stage has four axes of rotation, which allow the microscopist to align the tissue sample on the slide with the microscope's optical axis either parallel or perpendicular to it. By doing so, any birefringent element in the tissue can be centered and rotated on the stage, allowing for the recording of its three-dimensional alignment. This enables the user to view and study the tissue from different angles. Additionally, the use of thinner tissue sections has been shown to improve the resolution and accuracy of the staining results. Furthermore, it is important to note that while previous research (Lattouf et al., 2014) has taken into account the orientation of collagen fibers, fiber orientation was not considered in our study. Together, our use of conventional polarized microscopy limits the data that was derived in the current study.

A second limitation of this study is the use of antibody neutralization to block MFAP5 function. While the antibody that was employed has been shown to be an effective blocking antibody, we cannot be sure that the treatment blocked all MFAP5 molecules. Further studies that employ genetic deletion of MFAP5 might circumvent this limitation by creating an absolute absence of MFAP5 in wounds.

C. Future Directions

The results of our study provide evidence for the role of MFAP5 contributing to fibrosis. The findings of this study have the potential to serve as a starting point for further research and discovery in the role of different molecules in fibrosis of the healing wound, with the potential to significantly impact national health. Our findings suggest that MFAP5 could serve as a target for the development of novel therapeutic strategies aimed at preventing scarring, which has been linked to numerous medical complications and places a significant burden on national health systems. Furthermore, it may be of significance to explore the role of other microfibril associated proteins and ECM molecules, along with examining other factors that contribute to fibrosis, such as insufficient cell migration, phagocytosis of fibroblasts, and impaired angiogenesis.

VI. CONCLUSION

The gene expression analysis conducted in our lab indicated that the expression of MFAP5 was considerably higher in skin as compared to oral mucosal wounds at every time point. Therefore, MFAP5 was identified as the most differentially expressed gene when compared to all other genes analyzed, with a high probability of its involvement in fibrosis. Hence, MFAP5 was considered the candidate gene for further investigation.

Currently, there is limited research examining the impact of MFAP5 on fibrosis in wound healing. The research conducted in our lab is therefore novel in its effort to elucidate the function of MFAP5 in wound healing and the role it plays in scarring. Our investigations have revealed that MFAP5 expression increases in the later stages of wound healing, implying that MFAP5 likely participates in the proliferative and tissue remodeling phases characterized by greater collagen deposition and angiogenesis. The modulatory effect of MFAP5 on collagen and blood vessels formation contributes to a more fibrotic phenotype.

With the identification and acknowledgment of the role of MFAP5 and potentially other ECM proteins in fibrosis and scarring, this study offered novel insights into the involvement of microfibril-associated proteins in skin wound healing and fibrosis.

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APPENDIX

Table 2: Analysis of the percentage of total collagen deposition in wound bed. The table is illustrating the difference between the three studied groups regarding the total collagen deposition stained with Trichrome at days 7, 14, and 21 post-wounding. A significant difference seen between IgG compared to anti-MFAP5 compared to anti-MFAP5 at day 21 (ns = not significant, P *<0.05).

		Trichrome – %Collagen pixel area		
Day	**Test**	**PBS vs. IgG**	**PBS vs. anti-MFAP5**	**IgG vs. anti-MFAP5**
7	multiple t-test	ns	ns	ns
14	multiple t-test	ns	ns	ns
21	multiple t-test	ns	ns	*

Table 3: Analysis of the percentage of mature collagen stained with PS. The table is illustrating the difference between the three studied groups regarding the percentage of mature (red/orange) collagen stained with Picrosirius at days 7, 14, and 21 post-wounding. No significant difference between the three groups at all times (ns = not significant).

Day	Test	PS – %R Pixel Area		
		PBS vs. IgG	**PBS vs. anti-MFAP5**	**IgG vs. anti-MFAP5**
7	2-way ANOVA	ns	ns	ns
14	2-way ANOVA	ns	ns	ns
21	2-way ANOVA	ns	ns	ns

Table 4: Analysis of the percentage of immature collagen stained with PS. The table is illustrating the difference between the three studied groups regarding the percentage of immature (green) collagen stained with Picrosirius at days 7, 14, and 21 post-wounding. A significant difference seen between PBS compared to anti-MFAP5 and IgG compared to anti-MFAP5 at day 7 (ns = not significant, P *** <0.001).

Day	Test	PS – %G Pixel Area		
		PBS vs. IgG	**PBS vs. anti-MFAP5**	**IgG vs. anti-MFAP5**
7	2-way ANOVA	ns	***	***
14	2-way ANOVA	ns	ns	ns
21	2-way ANOVA	ns	ns	ns

Table 5: Analysis of the percentage of total collagen stained with PS. The table is illustrating the difference between the three studied groups regarding the percentage of total (red/orange and green) collagen stained with Picrosirius at days 7, 14, and 21 post-wounding. A significant difference seen between PBS compared to anti-MFAP5 and IgG compared to anti-MFAP5 at day 7 (ns = not significant, P *<0.05 and ** <0.01).

		PS – %Total Collagen Pixel Area		
Day	**Test**	**PBS vs. IgG**	**PBS vs. anti-MFAP5**	**IgG vs. anti-MFAP5**
7	2-way ANOVA	ns	*	**
14	2-way ANOVA	ns	ns	ns
21	2-way ANOVA	ns	ns	ns

Table 6: Analysis of the percentage of mature collagen over the percentage of total collagen stained with PS. The table is illustrating the difference between the three studied groups regarding the percentage of mature (red) collagen stained with Picrosirius over the percentage of total collagen (red/orange and green) at days 7, 14, and 21 post-wounding. A significant difference seen between PBS compared to anti-MFAP5 at day 7 (ns = not significant, P ** <0.01).

Day	Test	PS – %R/%Total		
		PBS vs. IgG	PBS vs. anti-MFAP5	IgG vs. anti-MFAP5
7	2-way ANOVA	ns	**	ns
14	2-way ANOVA	ns	ns	ns
21	2-way ANOVA	ns	ns	ns

Table 7: Analysis of the percentage of immature collagen over the percentage of total collagen stained with PS. The table is illustrating the difference between the three studied groups regarding the percentage of immature (green) collagen stained with Picrosirius over the percentage of total collagen (red/orange and green) at days 7, 14, and 21 post-wounding. A significant difference seen between PBS compared to anti-MFAP5 compared to anti-MFAP5 at day 7 (P ** <0.01). (ns = not significant, P ** <0.01).

Day	Test	PS – %G/%Total		
		PBS vs. IgG	PBS vs. anti-MFAP5	IgG vs. anti-MFAP5
7	2-way ANOVA	ns	**	ns
14	2-way ANOVA	ns	ns	ns
21	2-way ANOVA	ns	ns	ns

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