Ankrd1, a Modulator of Matrix Metabolism and Cell-Matrix Interactions

By

Karinna Almodóvar García

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

For the degree of

DOCTOR IN PHILOSOPHY

in

Cellular and Molecular Pathology

August, 2014

Nashville, Tennessee

Approved:

David M. Bader, Ph.D.

Jeffrey M. Davidson, Ph.D.

Linda J. Sealy, Ph.D.

Alissa M. Weaver, M.D., Ph.D

Pampee P. Young, M.D., Ph.D.

ABSTRACT

Normal tissue repair involves a series of highly coordinated events that include inflammation, granulation tissue formation, revascularization, and tissue remodeling. The transcriptional co-factor, ankyrin repeat domain protein 1 (Ankrd1), is rapidly and highly up regulated by wounding and tissue injury in mouse skin. Ankrd1 is also strongly elevated in human wounds. Overexpression of Ankrd1 in wounds by adenoviral gene transfer enhances wound healing. Ankrd1 has dual roles: a transcriptional co-regulator of several genes and a structural component of the sarcomere, where it forms a multi-component complex with the giant elastic protein, titin. Deletion of *Ankrd1* results in a wound healing phenotype characterized by impaired wound closure and reduced granulation tissue thickness. *In vitro* studies confirmed the importance of Ankrd1 for proper cell-matrix interaction. We identified two Ankrd1-target genes, Collagenase-3 (MMP-13) and Stromelysin-2 (MMP-10). Both, MMP-13 and MMP-10 are important players in matrix turnover during physiological and pathological events. In summary, Ankrd1 regulates genes involve in remodeling of the extracellular matrix and is essential for proper interaction with the extracellular matrix *in vitro.*

Dedicated to my loving and supportive family.

My parents, Elvin and Carmen, who always encouraged me to pursue my dreams.

My siblings, Roxanna, Elvin, and Carmillia, for believing in me.

My husband, Servio, who has been a constant source of support.

ACKNOWLEDGEMENTS

Completing this education journey is a true blessing. Through this long journey I have met many talented and kind individuals who did not hesitate to devote their valuable time to me when it was needed. I would like to thank sincerely, from the bottom of my heart, all those who have crossed my path during my life and believed one day that I would become a doctor.

My experience at Vanderbilt started in June 2007, when I joined the Initiative for Maximizing Student Diversity (IMSD) program. The IMSD program helped me ease into the larger university research setting. The program ensured I was prepared for graduate school and a professional career in science-related research. Special recognition to Dr. Victor Torres and Dr. Eric Skaar for outstanding mentoring during my first year in the IMSD program.

My work on this dissertation could not have been possible without the support and guidance of many individuals. I am honored and deeply appreciative to Dr. Jeff Davidson, my mentor, for his continuous support, encouragement, and guidance during the doctoral process. His assistance, knowledge, and dedication greatly contributed to my success and helped me persevere through the hard times and celebrate the good times. On a daily basis my lab has been a great place to work and learn. I thank my co-mentors, Dr. Susan Samaras and Dr. Susan Opalenik, who provided prayers, support, contribution of time, and guidance during the doctoral journey. I am grateful also to former and present members of the Davidson Lab: Minjae Kwon, Fang Yu, Steve Koch,

Angi Li, and Christina Lipscombe, for their friendship and attention. A special thanks to Maria Gabriella Giro for being more like family to me.

I would like to proudly acknowledge the support of my committee members: Dr. Young, Dr. Bader, Dr. Weaver, and Dr. Abdulkadir, who provided me thorough, meaningful, and constructive feedback throughout the Ph.D. program. I will be forever thankful to my thesis committee member and IMSD director, Dr. Linda Sealy, for giving me an opportunity and providing advice many times during my graduate school career.

I also thank Dr. Roy Zent and Dr. Ambra Pozzi, for their suggestions, feedback, expertise and advice. I am grateful to Dr. Lillian Nanney and her lab members for providing human samples and help me with histology staining of so many samples. I would also like to acknowledge Dr. Chee Lim for reading my manuscripts and provide insightful comments. Thanks to the entire faculty involved in the Cellular and Molecular Pathology Program for their invaluable support and suggestions throughout my studies.

This would not have been possible without the constant support of my family. I would like to extend my greatest appreciation and gratitude to those family and friends who have been encouraging along the way. I must acknowledge my parents and siblings: Carmen, Elvin, my oldest sister Roxanna, my brother Elvin Orlando (Bebo), and my beloved youngest sister Carmillia (Bimbi). Thanks for your continuous love and for supporting me throughout my life. They have taught me about hard work, self-respect, persistence, strength, and character. I am so thankful for my niece, Anna, and my nephew, Aaron, for bringing joy into my life. I am also grateful to my extended García family. They have always expressed how proud they are of me and how much they love me. They have been a great inspiration to achieve my goals.

A good support system is important to surviving and staying sane in grad school. My deepest appreciation goes to my closest friends: Reinaldo, Yeritza, Marian, María, and Patricia. Thanks for helping me think rationally and even for hearing my problems. Special thanks to Yanice Mendez, for being a great reliable person to whom I could always talk about my problems and excitements. Another supporter and fan is my special friend, Caridad Lopez. Her love, support, and belief in me were a treasure.

I want to give an extra special thank you to my husband, Servio. His support and understanding during this project definitely deserves my recognition, he is my soul mate, and my rock. Much love.

I recognize that this research would not have been possible without the financial assistance of the NIH/NRSA fellowship 1F31GM101947. My thesis work was also supported by my mentor's grant 5R01DK065656.

Thank you, God, for giving me the strength to continue on this journey when I felt that I could not finish.

PREFACE

The healing process is initiated at the moment of injury, and it produces a dynamic interactive response that involves the complex, overlapping interaction of various cell types, extracellular matrix molecules and soluble mediators. Normal tissue repair consists of four phases: hemostasis, inflammation, repair, and remodeling. Chronic wounds failed to proceed through the repair phases in an orderly and timely reparative way, resulting in delay or lack of repair. These chronic wounds are characterized by aberrant inflammation, and abnormal deposition of extracellular matrix molecules and cellular infiltrates, leading to impaired repair and regeneration. Correctly identifying the etiology of a chronic wound is central to successful wound treatment. Novel treatment strategies that will promote faster wound healing are necessary for chronic wounds, especially in diabetic patients, where wound repair is severely compromised.

The nuclear transcription co-factor, Ankrd1, is highly induced after wounding, and its over-expression promotes tissue repair. This thesis focuses on understanding the role of Ankrd1 during tissue repair. Global deletion of *Ankrd1* did not affect mouse viability or development. Nevertheless, *Ankrd1*-null mice showed delayed excisional wound closure characterized by decreased contraction and reduced granulation tissue thickness. Cells isolated from *Ankrd1*-deficient mice did not spread or migrate on collagen- or fibronectin-coated surfaces as efficiently as fibroblasts isolated from control mice. More importantly, *Ankrd1*-null fibroblasts failed to contract 3D floating collagen

gels. Reconstitution of Ankrd1 by viral infection stimulated collagen contraction. These data suggest that Ankrd1 is critical for proper interaction with the matrix.

In the second part of this thesis we focus on investigating the mechanisms underlying gene regulation by Ankrd1. Using analysis of cells from *Ankrd1* knockout mice, transient overexpression, and RNAi knockdown studies, we provide evidence that Ankrd1 is a negative regulator of matrix metalloproteinase (MMP) gene expression. We demonstrated that Ankrd1 associates with nucleolin, and this interaction increases the repression of MMP genes. Together, these studies highlight the importance of understanding how Ankrd1 works. Understanding the mechanisms that Ankrd1 utilizes during skin repair can lead to the development of a therapy that improves wound healing in chronic wounds.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

Ankrd1	Ankyrin Repeat Domain 1; Cardiac Ankyrin Repeat Protein
AARP/Ankrd2	Ankyrin Repeat Domain 2
ADAM	A disintegrin and metalloprotease
ANF	Atrial natriuretic factor
AP-1	Activator protein-1
BRMS1	Breast Cancer Metastasis-Suppressor 1
CASQ1	Calsequestrin 1
CDK	Cyclin dependent kinase
c-Fos	FBJ Murine Osteosarcoma Viral Oncogene Homolog
c-Jun	Jun proto-oncogene
cTNC	Cardiac troponin C
DARP/Ankrd23	Ankyrin Repeat Domain 23
DEJZ	Dermal-epidermal junction zone
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDS	Ehlers-Danlos Syndromes
FAK	Focal adhesion kinase
FLOX	*Ankrd1*$^{fl/fl}$; control
FN	Fibronectin
FPLC	Fibroblasts-populated collagen lattice
GATA4	GATA-Binding Protein 4
IL-1	Interleukin-1
KO	*Ankrd1*$^{-/-}$; knockout
Luc	Luciferase
MAP kinases	Mitogen-activated protein kinases
MARP	Muscle ankyrin repeat proteins
MCAT	Malonyl CoA:ACP Acyltransferase
MLC2v	Myosin light chain-ventricular
MMP	Matrix metalloproteinase
MMP-10	Matrix metalloproteinase-10; Stromelysin 2
MMP-13	Matrix metalloproteinase-13; Collagenase 3
Ncl	Nucleolin
Nkx2.5	NK2 Transcription Factor Related, Locus 5
PBD	Post burn day
PDGF	Platelet derived growth factor

PEST	Peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine
PMA	Phorbol-12-myristate-13-acetate
SP-1	Sp1 transcription factor
TNF-α	Tumor necrosis factor
TCA	Trichloroacetic acid
YAP/TAZ	Yes-Associated Protein/tafazzin
YB-1	Y Box Binding Protein 1

Chapter I

INTRODUCTION

Skin Anatomy

The skin is the first line of defense against external insults. It is the largest organ of the body and its function includes protection, maintenance of body temperature, and defense (Hussain, Limthongkul, & Humphreys, 2013). The thickness and structure of the skin varies over the surface of the body. In particular, the skin is both grossly and histologically different at two locations, the palms of the hands and the soles of the feet. These areas are subject to the most abrasion, are hairless, and have a much thicker epidermal layer than skin in any other location.

The skin consists of three layers: *epidermis*, *dermis*, and *subcutaneous tissue* (**Figure 1**). The *epidermis* is composed of layers of the epithelium that exhibits progressive differentiation in a basal to superficial direction. It is comprised of multiple cell types that are derived from different embryonic origins: keratinocytes, melanocytes, Langerhans cells, and Merkel cells, while the keratinocytes are the mayor epidermal cell type (Kanitakis, 2002). The dermo-epidermal junction zone (DEJZ) connects the epidermis with the dermis. DEJZ is composed of a network of structural proteins, which provides a firm connection between the basal keratinocytes and the dermis

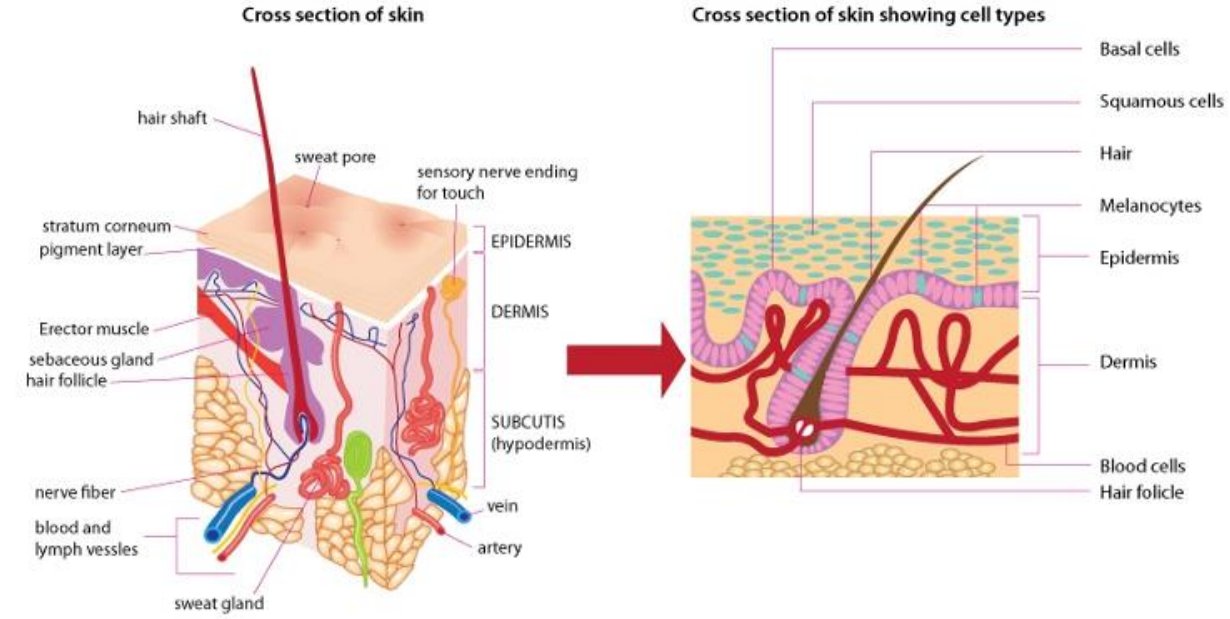


Figure 1. Cross-section of the skin.
Diagram showing a cross section of skin on the left and on the right a cross section showing the cell types. Image taken from: http://www.sciencelearn.org.nz/Cross-section-of-skin

(Wysocki, 1999). The structural network is made up: hemidesmosomes, lamina densa, lamina lucida, and anchoring fibrils. Type IV collagen, laminin, and perlecan are major components of the basement membrane in the DEJZ. The DEJZ provides enormous mechanical stability to the epidermis. Mutations or disruptions of the structures in the DEJZ present clinically with skin fragility and blistering disorders (Burgeson & Christiano, 1997). The *dermis* is the connective tissue component of the skin, and it is divided into two layers: the papillary dermis and the reticular dermis. The papillary dermis is a relatively thin layer of loose connective tissue that lies immediately beneath the epidermis; collagen fibers are thinner and loosely packed. It not only binds the epidermis to deeper tissues, but also supports the microcirculation and nerve supply of the epidermis. The deeper reticular dermis is a relatively thick layer of dense irregular connective tissue; collagen fibers are thicker and more densely and irregularly arranged. The reticular dermis also supports the larger blood vessels and nerves that supply the microcirculation and nerve supply penetrating the upper papillary layer.

The dermis protects the body from mechanical injury, control of water output, and temperature regulation. The connective tissue of the skin is composed of extracellular matrix molecules such as collagen and elastic fibers, which play an important role in the elasticity of the skin. Collagen is the main source of structural support of the skin, while elastin is responsible for the ability to recoil after stress is applied to the skin (Hussain et al., 2013). Collagen types I and III are the most prominent in human skin. Another component of the connective tissue is the proteoglycans, which are made of glycosaminoglycans, hyaluronic acid, chondroitin, keratin, dermatan, and heparins.

The dermis does not undergo differentiation, although the matrix components undergo turnover and remodeling in normal skin and during pathological process such as wound healing (Nizet & Pierard, 2009). The *subcutaneous tissue,* also called hypodermis, serves as a region for the storage of fat and it supports vessels and nerves that supply the dermis. The function of the major components of the extracellular matrix is discussed in the next topic.

Extracellular Matrix of the Skin

The extracellular matrix (ECM) consists of a complex array of distinct components including glycoproteins, collagens, glycosaminoglycans and proteoglycans that are secreted locally and assembled into a complex network. The composition and architecture of the ECM determine the biophysical properties of tissues such as stiffness, compliance and resilience (Bruckner, 2010). Deposition and assembly of extracellular matrix proteins into insoluble and complex polymers in skin are regulated processes adapted to development, tissue remodeling, repair, ageing and wound healing (Rock & Fischer, 2011). Conversely, stiffness and compliance of tissues are important factors regulating the functions of the cells embedded into the matrices. This regulation proceeds either directly because proteins within the networks interact with cell surface receptors to initiate specific signaling pathways or indirectly because activity and availability of compounds such as cytokines and growth factors are controlled through transient sequestration within the networks (Buxboim, Ivanovska, & Discher, 2010; Eckes & Krieg, 2004).

Table 1. Most frequent extracellular matrix proteins

Family name	Number of genes and name of the proteins
Collagens (COL)	44 genes, 28 proteins (COL I to XXVIII)
Elastin	1 gene
Fibronectin	1 gene, 20 splice variants
Fibulins	7 genes, 7 proteins (fibulin-1 to 7)
Fibrillins	3 genes, 3 proteins (fibrillin-1 to 3)
Latent TGF-β–binding proteins (LTBPs)	4 genes, 4 proteins (LTBP-1 to 4)
Laminins (LMs)	11 genes, 16 proteins (LM-111, LM-332, LM-511,...)
Matrilins	4 genes, 4 proteins (matrilin-1 to 4)
Nidogens	2 genes, 2 proteins (nidogen 1 and 2)
Tenascins	4 genes, 4 proteins (tenascin-C, -X, -R and-W)
Thrombospondins (TSPs)	5 genes, 5 proteins (TSP-1 to 5)
Vitronectin	1 gene, 1 protein
Proteoglycans	>40 different species

Most extracellular proteins form families. For each protein family, the family name, the number of genes and the denomination of the family members are indicated.

Table 1: Structural components of the extracellular matrix relevant to skin.
These are the major, relatively well-characterized components in the skin. Table taken from: (Krieg & Aumailley, 2011)

Some of the specific, well-characterized molecules of the ECM are: collagens, elastin, fibronectin, laminins, fibrillins, and proteoglycans (**Table 1**). Collagen is the predominant matrix component of the dermis; it is designed to provide structure and resiliency. To date, 28 distinct genetic collagen types have been identified. The characteristic molecular form of collagen is a triple helix made up of three polypeptides, called α chains, which coil into a right-handed triple helix (Ricard-Blum & Ruggiero, 2005). Elastin is the main protein component of the elastic fiber. Elastic fibers are of particular importance to the structural integrity and function of skin in which reversible extensibility or deformability is crucial (Halper & Kjaer, 2014). Fibronectin (FN) is a widely distributed multidomain glycoprotein present in most extracellular matrices. FN is expressed especially in regions of active morphogenesis, cell migration and inflammation (Singh, Carraher, & Schwarzbauer, 2010). FN levels are high in tissues undergoing repair (i.e., wound healing) and/or fibrosis (Schwarzbauer & Sechler, 1999; Singh et al., 2010). Two classes of fibronectin are formed by a single gene but from different sources: (1) the soluble form is called *plasma FN*, is produced by hepatocytes in the liver and circulates in the bloodstream, and (2) the *cellular* form is produced by a variety of cells, and gets incorporated into the matrix (Hynes, 1990). Many fibronectin variants may be formed by alternative splicing. Laminins are a family of large multidomain, heterotrimeric glycoproteins that interact with both cells and ECM. They constitute a family of basement membrane glycoproteins that affect cell proliferation, migration, and differentiation (Halper & Kjaer, 2014; Hamill, Kligys, Hopkinson, & Jones, 2009). Fibrillins are a group of large extracellular glycoproteins. They provide structural integrity of specific organ systems, and serve as a scaffold for elastogenesis in elastic

tissues such as skin, lung, and vessels (Wagenseil & Mecham, 2009). Thus, fibrillin is important for the assembly of elastin into elastic fibers. Proteoglycans are a diverse family of proteins with varying numbers, types and sizes of attached glycosaminoglycans chains linked by O-glycosidic bonds to serine or threonines. They participate in matrix organization, structural integrity and cell attachment. Other important components of the ECM of the skin are the matricellular proteins: tenascins, fibulins and thrombospondins.

A number of serious human diseases are associated with abnormal extracellular matrix in the skin (**Table 2**). Mutations in the collagen genes, or deficits in collagen-modifying enzymes or proteins involved in the architecture and biomechanical function of collagen fibrils are associated with Ehlers–Danlos syndromes (EDS) (Callewaert, Malfait, Loeys, & De Paepe, 2008). EDS are disorders of collagen fibrils, with skin hyper-extensibility, with velvety texture and easy bruising. Mutations that affect the structure or lead to a reduced synthesis of fibrillins are the cause of Marfan's syndrome (Callewaert et al., 2008). The disease is characterized by thin skin and abnormalities affecting the ocular, skeletal and cardiovascular systems. Several other acquired and inherited skin blistering disorders originate from autoantibodies targeting proteins involved in the anchorage of basal keratinocytes or from mutations in the genes encoding the components of the anchoring complexes. A whole range of severe inherited skin blistering disorders characterized by trauma-induced epidermal detachment from the dermis are caused by mutations in the genes coding for laminin 332, its α6β4 integrin receptor, collagen VII or collagen XVII (Carulli, Contin, De Rosa, Pellegrini, & De Luca, 2013; Cooper & Bauer, 1984; Coulombe, Kerns, & Fuchs, 2009).

Moreover, fibrosis and inflammatory pathologies are associated with dysfunction in extracellular matrix protein expression, function and metabolism (T. R. Cox & Erler, 2011; Korpos, Wu, & Sorokin, 2009; Krieg & LeRoy, 1998; Sorokin, 2010).

In addition to providing mechanical and structural support throughout the body; the ECM is implicated in cell migration, proliferation, embryonic development, stem cell niches, and signaling events. The expression, interaction and arrangement of the different matrix components are necessary to maintain normal physiological properties of the skin. Synthesis of ECM is a key feature of wound healing, especially when there has been a significant loss of tissue. The optimal care and treatment of wounds requires an understanding of how extracellular matrix affects the healing processes. The wound healing process is discussed in more detailed in the next subject.

Wound Healing

Wound healing consists of an orderly progression of events that attempts at repairing damaged tissue (**Figure 2**). A number of processes are involved including coagulation, inflammation, proliferation, and tissue remodeling. Immediately after injury, hemostasis is triggered, diminishing blood loss, followed by an inflammatory phase. Inflammation begins early, hours after injury. Damaged cells release chemical signals that stimulates the migration of cells into the wound bed (Gillitzer & Goebeler, 2001). Days after the initial injury, the fibroblasts and endothelial cells begin proliferating and

Table 2. Disorders with skin manifestations caused by mutations in the genes encoding extracellular matrix proteins

Collagen I	Achondroplasia EDS (EDS VIIa and VIIb)
Collagen III	Vascular EDS (EDS IV)
Collagen IV	Familiar porencephaly, hereditary angiopathy Alport syndrome, leiomyomatosis
Collagen V	Classical EDS (EDS I and II)
Collagen VI	Bethlem myopathy Ulrich muscular dystrophy
Collagen VII	Dystrophic epidermolysis bullosa
Collagen XVII	Epidermolysis bullosa junctionalis
Elastin	Cutis laxa
Fibrillin 1	Marfan's syndrome Weill-Marchesani syndrome
Fibrillin 2	Contractural arachnodactyly
Fibulin-4	Cutis laxa
Fibulin-5	Cutis laxa
Laminin 332	Epidermolysis bullosa junctionalis
Tenascin-X	Hypermobility EDS (EDS III)

For the Ehlers–Danlos syndromes (EDS), both the new and old (between parenthesis) nomenclatures are used.

Table 2: Extracellular matrix dysfunction and cutaneous diseases.
Many inherited diseases with skin involvement that are caused by mutations in the genes coding for extracellular matrix proteins, or proteins and small molecules participating in the supramolecular assembly of extracellular matrix proteins into networks. Table taken from: (Krieg & Aumailley, 2011)

forming a specialized type of tissue, called granulation tissue (Singer & Clark, 1999). As repair progresses, the number of proliferating endothelial cells and fibroblasts decreases and fibroblasts deposit increased amounts of ECM, mostly collagen. Net collagen accumulation depends on synthesis and degradation. Synthesis versus degradation of the ECM results in remodeling. Ultimately the granulation tissue scaffolding is converted into a scar. The replacement of the granulation tissue with a scar requires changes in the composition of the ECM (Gurtner, Werner, Barrandon, & Longaker, 2008). Degradation of collagen and other ECM proteins is modulated by a family of matrix metalloproteinases (MMPs) and also members of the “a disintegrin and metalloprotease” (ADAMs) family.

Complications in wound healing can arise from abnormalities in any of the repair phases. In some cases, there are wounds that heal too much and this can lead to the formation of keloids, hypertrophic scars, pyoderma gangrenosum (proud flesh), and fibroses (McNaughton & Brazil, 1995; Ogawa, Akaishi, & Izumi, 2009). On the other hand, poor healing of the wound may result in ulcer formation, tendon rupture, hyperplasia, neoplasia, and tumor development (Medina, Scott, Ghahary, & Tredget, 2005). Persistent inflammation is observed in chronic wounds and can lead to tissue destruction (K. Moore, 1999). Uncontrolled contraction of the wound can result in deformities of the wound and surrounding tissues (Bullard et al., 1999). Therefore, a controlled, dynamic, and changing process is required for proper tissue repair.

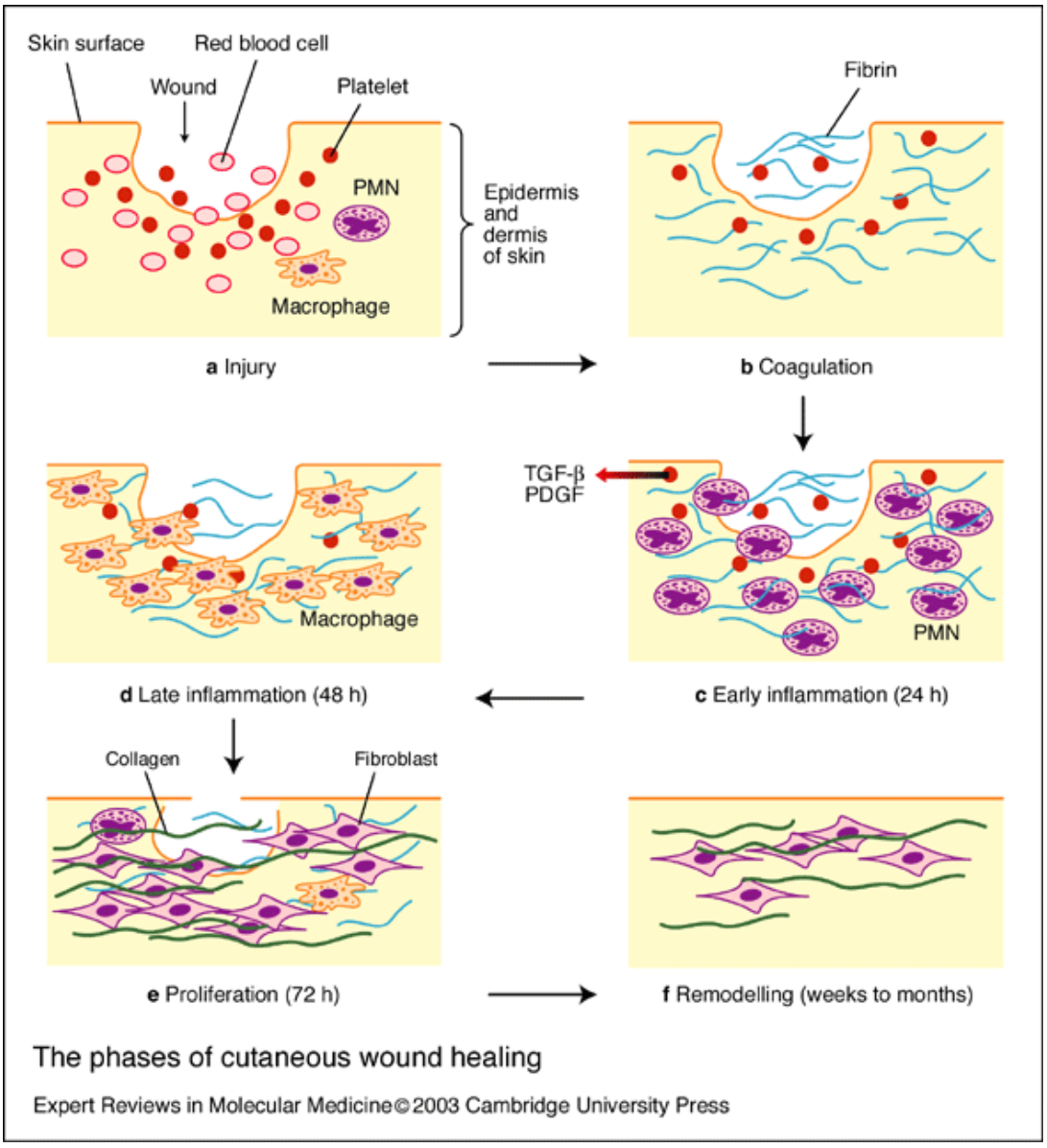


Figure 2: Classic stages of wound repair.
The normal response to injury consists of overlapping but distint stages: hemostatis, inflammation, new tissue formation, and remodeling. Image taken from: http://www.pilonidal.org/wound_healing_indepth.php

The co-transcription factor, Ankyrin Repeat Domain 1, was shown to be sharply expressed and to affect the wound healing process (Shi et al., 2005a). It is necessary for proper wound closure and cell-matrix interaction (Chapter II; Samaras, Almodovar-Garcia, et al. Submitted for publication), and it regulates matrix-related proteins (Chapter III) (Almodovar-Garcia, Kwon, Samaras, & Davidson, 2014). Understanding the mechanisms of action of this protein could lead to the development of a therapy that improves tissue repair.

Ankyrin Repeat Domain 1 (Ankrd1, *Ankrd1;* Cardiac Ankyrin Repeat Protein, CARP)

A cDNA subtraction hybridization study between intact and day 1 wounded skin identified the *Ankrd1* gene to be strongly induced after wounding (Almodovar-Garcia et al., 2014; Samaras, Shi, & Davidson, 2006b). Total RNA and protein expression showed a dramatic increase in Ankrd1 hours after initial wounding with levels remaining up for two weeks (Shi et al., 2005a). Increased *Ankrd1* expression is also observed during heart failure (Zolk et al., 2002), muscular dystrophy (Nakada et al., 2003; van Lunteren, Moyer, & Leahy, 2006), atrial fibrillation (Aihara, Kurabayashi, Saito, et al., 2000; Aihara, Kurabayashi, Tanaka, et al., 2000), re-innervation of skeletal muscle (Zhou, Cornelius, Eichner, & Bornemann, 2006), hypoxia tolerance (Avivi, Brodsky, Nevo, & Band, 2006), lupus nephritis (Matsuura, Uesugi, Hijiya, Uchida, & Moriyama, 2007), mammary and intestinal tumorigenesis (Labbe et al., 2007), and arteriogenesis (Boengler et al., 2003).

Ankrd1 was originally identified as a cytokine-inducible nuclear protein in human endothelial cells (Chu, Burns, Swerlick, & Presky, 1995a). The gene is located on chromosome 10 in humans and 19 in mouse, and is highly conserved among vertebrates. All necessary regulatory sequences appear to be within 10 K bp 5' of the *Ankrd1* transcription start site. The 5' sequence includes many canonical response elements including: GATA sites, E-box elements, CCAC box, CAGA box, MCAT, AP-1, and SP-1 binding sites (**Figure 3**) (Chu et al., 1995a; Jeyaseelan et al., 1997a). The *Ankrd1* proximal promoter contains a canonical TATA box. No alternative splicing of the RNA has been described.

The *Ankrd1* transcript encodes a 319 amino acid protein with a molecular weight of 36 kDa. Ankrd1 protein sequence and domain organization is highly conserved among mammalian species. Analysis of the peptide sequence revealed a number of putative motifs, including a bipartite nuclear localization signal that mediates active nuclear transport of proteins from cytoplasm and nucleus, a PEST-sequence that is a short sequence typical of short-lived, rapidly degraded proteins, four and a half ankyrin repeat domains that are involved in protein-protein interaction, and multiple potential phosphorylation, glycosylation, myristylation, and amidation sites (**Figure 3**). Ankyrin repeats are amongst the most frequent motifs found in proteins with different functions including: CDK inhibitors such as p16, p18, and p19, developmental regulators such as Notch, IκBα, and transcription factors Swi6, GABPα,β (Sedgwick & Smerdon, 1999). Proteins with ankyrin repeats have special importance in systems characterized by intensive communication between proteins (Mosavi, Cammett, Desrosiers, & Peng, 2004).

Ankrd1 belongs to a three-member family of muscle ankyrin repeat proteins or MARPs, which also include AARP/Ankrd2 and DARP/Ankrd23. AARP/Ankrd2 is expressed in heart, kidney and predominantly in the skeletal muscle, and DARP/Ankrd23 is present in similar amounts in the heart and skeletal muscle (Miller et al., 2003). MARP proteins have similar structures, sharing several common domains: tandem ankyrin repeats, PEST motifs and nuclear localization signals. AARP/Ankrd2, which is predominantly expressed in skeletal muscle, is induced in response to various forms of stress and is highly responsive to muscle mechanical status both *in vitro* and *in vivo* (Miller et al., 2003). DARP/Ankrd23 expression is altered by the change of energy supply induced by excess fatty acid treatment of skeletal myotubes *in vitro* (Ikeda, Emoto, Matsuo, & Yokoyama, 2003). MARPs have dual intracellular localization, found both in sarcomeric I-band and in the nuclei. In the sarcomere they bind titin and, in response to mechanical stimuli, they appear to relocalize to the nuclei where they participate in reprograming gene expression occurring during cell response to the structural or functional changes of contractile machinery (Snezana Kojic, Dragica Radojkovic, & Georgine Faulkner, 2011). Their localization in the cell is subjected to change due to physiological and pathological conditions. Recently, it was published that deletion of the MARP family (MARP triple knockout; Ankrd1, Ankrd2, and Ankrd23) results in viable mice that had normal cardiac function both at basal levels and in response to mechanical pressure overload (M.-L. Bang et al., 2014). However, Bang et al. did not provide evidence of a generation of a real triple knockout mouse. In addition, our lab recently identified *Ankrd1*-dependent abnormalities in mice after myocardial infarction and α-adrenergic stimulation (Zhong, Chiusa et al., submitted for publication).

Ankrd1 is downstream of Nkx2.5 and GATA-4 signaling pathways (Zou et al., 1997). Ankrd1 is induced during cardiomyogenesis and vascular injury (Kuo et al., 1999; Zou et al., 1997). Ankrd1 promoter-lacZ transgenic mice displayed cardiac specific expression in early embryonic development. Ankrd1 is highly enriched in adult heart and is detectable in skeletal muscle and lung after birth. Ankrd1 is a nuclear and cytoplasmic protein. Nuclear Ankrd1 is primarily recognized as a co-transcriptional factor and a negative regulator of cardiac genes, expressed during cardiogenesis and cardiomyopathy. In developing myocardium, Ankrd1 is a negative regulator of myosin light chain-ventricular (MLC2v) acting through the HF-1 site in collaboration with transcription factor YB-1 (Zou et al., 1997) . Several cardiac-specific genes including atrial natriuretic factor (ANF) and cardiac troponin C (cTNC) are also repressed following Ankrd1 overexpression (Jeyaseelan et al., 1997a). In cardiomyocytes, cytoplasmic Ankrd1 associates with sarcomeric proteins titin, desmin, myopalladin, CASQ, and p94/calpain to form a complex important for muscular stress (Bang et al., 2001b; Miller et al., 2003). It has been hypothesized that Ankrd1 may be a mediator of mechanical stress, where it could link myofibrillar stretch signals to the transcriptional regulation of muscle gene expression (B. Chen et al., 2012; Samaras et al., 2006b), possibly by shuttling between cytoplasm and nucleus (Snezana Kojic et al., 2011).

Ankrd1 is up-regulated in animal models of cardiac hypertrophy induced by pressure overload and adrenergic stimulation, and in patients with dilated cardiomyopathy (Aihara, Kurabayashi, Saito, et al., 2000; Nagueh et al., 2004; Zolk, Marx, Jackel, El-Armouche, & Eschenhagen, 2003). In cardiomyocytes, Ankrd1 and GATA4 signaling pathways regulate sarcomere gene expression and maintain

sarcomere organization (B. Chen et al., 2012). Recently, Ankrd1 has been shown to mediate agonist-induced myocardial hypertrophy. Under conditions of α-adrenergic stimulation, Ankrd1 serves as a sarcomere scaffolding protein to induce ERK-GATA4 phosphorylation. Ankrd1 recruits and localizes ERK1/2 and GATA4 in a sarcomeric complex to enhance GATA4 phosphorylation, followed by translocation of the Ankrd1/GATA4 complex to the nucleus to induce gene activation and cardiac hypertrophy (Zhong, Chiusa et al., submitted for publication). The exact functional role of Ankrd1 in the heart remains to be elucidated.

Very little is known about the expression, localization, and importance of Ankrd1 in skin and tissue repair. In situ hybridization studies during wound healing showed *Ankrd1* expression in skeletal muscle, epidermis, vasculature, keratinocytes, vessel wall, and inflammatory cells. Overexpression of Ankrd1 improved angiogenesis and blood perfusion in several animal models (Shi et al., 2005a). Additionally, overexpression of Ankrd1 results in increased vascularization in wounds and experimental granulation tissue, but it does not affect proliferation (Shi et al., 2005a). Global deletion of *Ankrd1* did not affect mouse viability or development, although *Ankrd1*$^{-/-}$ mice had at least two significant wound healing phenotypes: extensive necrosis of ischemic skin flaps that was reversed by adenoviral expression of *Ankrd1* (data not shown); and delayed excisional wound closure characterized by decreased contraction and reduced granulation tissue thickness (Chapter II). A serious gap in our knowledge of Ankrd1 is its downstream effects during tissue repair. This work shows that Ankrd1 negatively regulates matrix metalloproteinases-13 and -10 during wound

healing (Chapter III) (Almodovar-Garcia et al., 2014). Further studies presented in this thesis characterize the mechanism of action of Ankrd1.

Matrix Metalloproteinases

Precise removal of connective tissue is critical to several physiological (development) and pathological (wound healing) processes that require cell movement and tissue remodeling (Vu & Werb, 2000). In order for these processes to occur, the ECM must be degraded to allow free movement of cells, and processing and deposition of new matrix. A family of proteases involved in the degradation of components of the ECM is the matrix metalloproteinases (MMPs). These extracellular matrix-degrading enzymes that share common functional domains and activation mechanisms (Takino, Sato, & Seiki, 1995). They are synthesized as secreted or transmembrane pro-enzymes and processed to an active form by the removal of an amino-terminal pro-peptide (Mott & Werb, 2004). There are several distinct subgroups based on preferential substrates: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, and -10), stromelysin-like MMPs (MMP-11 and -12), matrilysins (MMP-7, and -26), membrane-type MMPs (MMP-14, -15, -16, and -24), and other less characterized (MMP-17, -18, -19, -20, -23, 25, and -28). Collagenases are the principal enzymes capable of degrading native fibrillar collagens such as type I, type II and type III. In skin, the interstitial collagens (type I, II, and III) are the principal targets of destruction and the secreted collagenases, MMP-1, MMP-8 and MMP13, have a major role in this process (Armstrong & Jude, 2002b).

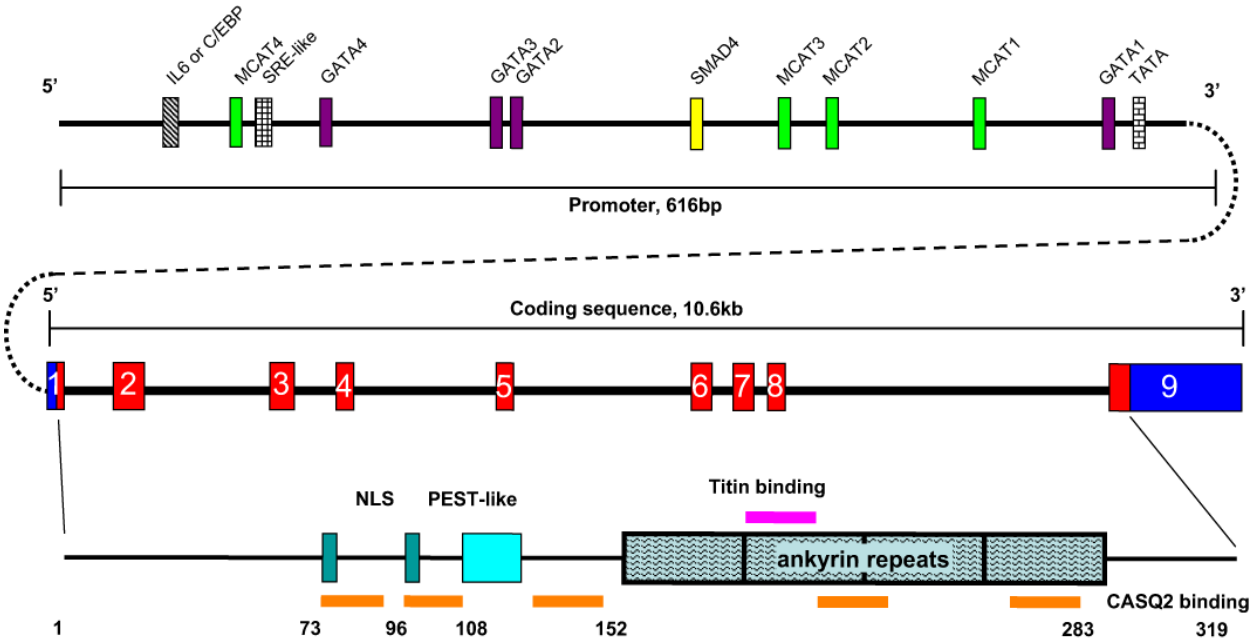


Figure 3: ***Ankrd1* gene and protein structure**.
Graphic representation of *Ankrd1* gene structure shows the 600-bp proximal promoter with known transcription factor binding sites and the structural gene. All 9 exons of the structural gene encode for some portion of the protein. Protein encoding regions in all 9 exons are shown in red, with the domain organization shown below. Adapted from: (Samaras et al., 2006b).

MMP expression and activity is highly regulated, since inappropriate degradation of the ECM could damage the integrity of the skin and other tissues. Excessive collagen degradation during tissue repair might result in formation of non-healing chronic wounds (Martins, Caley, & O'Toole, 2013). MMP levels are controlled at multiple levels: the expression/synthesis, activation, and inhibition of the active enzyme. In general, most MMPs are secreted as inactive zymogens and are subsequently activated by proteolytic cleavage at the N-terminus in the extracellular space. The activation of proMMP requires disruption of the cysteine switch. Several proteinases, *e.g.* plasmin, trypsin, kallikrein, mast cell tryptase and other MMPs (MMP-14 and MMP-2) can cleave the propeptide and result in disruption of the cysteine switch. Once activated, the activity of MMPs is regulated by general protease inhibitors: α2 macroglobulin, α1-antiprotease, and by specific inhibitors, *i.e.* tissue inhibitors of metalloproteinases (TIMPs). The TIMP family consist of four members (TIM-1,-2,-2, and-4), which competitively and reversibly inhibit the activity of all MMPs (Baker, Edwards, & Murphy, 2002).

While potent inhibitors of MMP enzymatic activity have been developed, their use has been limited due to safety issues and lack of selectivity (Brown, 2000). Inhibition of MMP expression at the transcriptional level may be a viable alternative option. The promoter of most of the MMPs contains a TATA box, the core transcriptional unit, at approximately -30 bp, an AP-1 site at approximately -70 bp, multiple PEA3 sites (Benbow & Brinckerhoff, 1997) (**Figure 4**). The proximal AP-1 site (5'-TGAG/CTCA-3'), which binds heterodimers of the Fos and Jun families, is considered a major player in transcriptional activation of MMPs (Auble & Brinckerhoff, 1991; Auble, Sirum-Connolly, & Brinckerhoff, 1992). Transcriptional activation by the potent protein kinase C agonist,

phorbol myristate acetate (PMA), strongly activates the AP-1 site. Numerous growth factors and cytokines, including EGF, PDGF, TNF-α, and lL-1, induce expression of most MMPs and are also dependent on the AP-1 site (Benbow & Brinckerhoff, 1997; Birkedal-Hansen et al., 1993).

Proteolysis is an important feature of wound repair. The cells participating in tissue repair specifically produce several MMPs and regulate their activity. MMP-1 and MMP-9 play a role in re-epithelization in tissue repair in skin and lung (Olsen et al., 2008; Pilcher et al., 1997). MMP-7 also plays an important role in tissue repair in airway epithelium (Dunsmore et al., 1998). MMP-3 and -13 are also involved in cutaneous wound repair. MMP-13 in wound healing coordinates cellular activities important in the growth and maturation of granulation tissue, including myofibroblast function, inflammation, angiogenesis, and proteolysis (M. Toriseva et al., 2012). MMP-13 is also expressed by fibroblasts in chronic dermal ulcers characterized by persistent inflammation (Vaalamo et al., 1997a). MMP-13 plays a predominant role in the pathogenesis of joint inflammation (Konttinen et al., 1999; B. A. Moore, Aznavoorian, Engler, & Windsor, 2000). Proper regulation of MMPs can be employed in development of novel therapeutic modalities for promoting wound repair and inhibiting excessive scar formation.

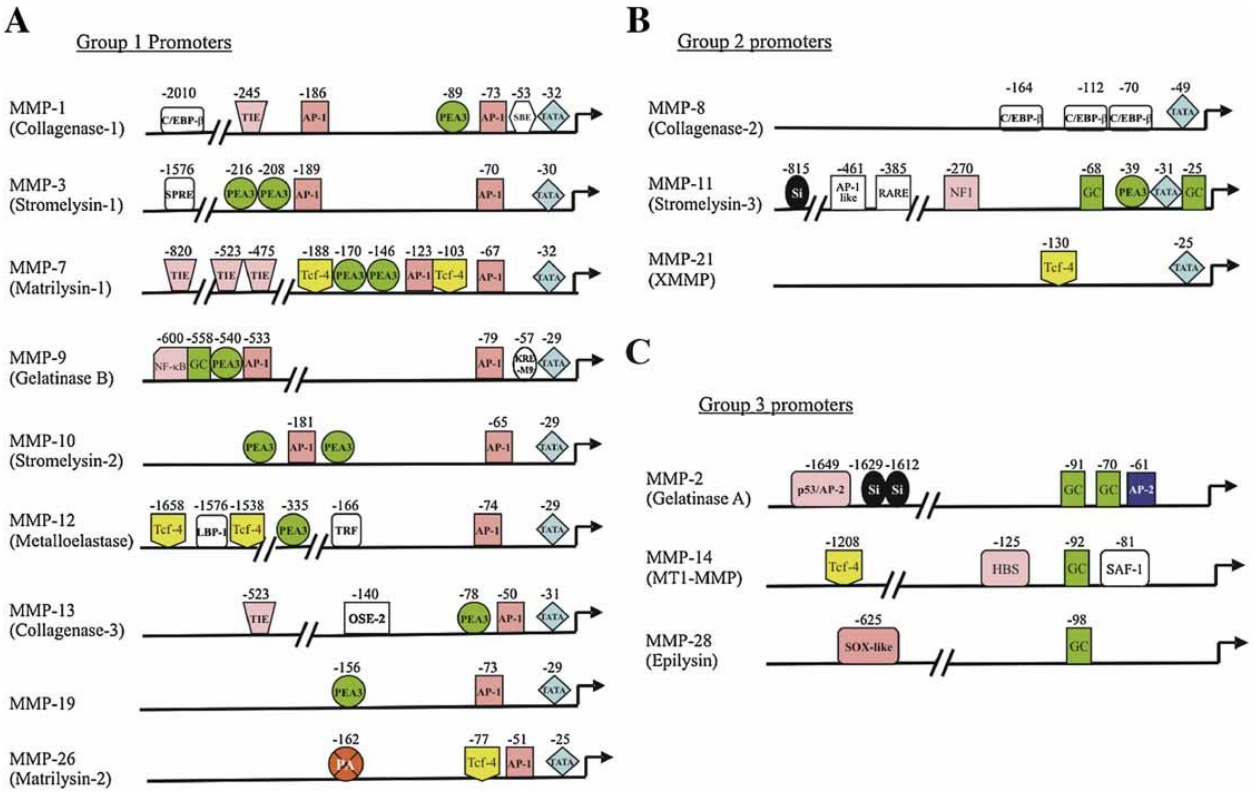


Figure 4: *Cis*-elements in human MMP promoters.
The MMP promoters harbor several *cis*-elements allowing for the regulation of MMP gene expression by a diverse set of *trans*-activators including AP-1, PEA3, Sp-1, β-catenin/Tcf-4, and NF-κB. Several of the MMP promoters are strikingly similar and, in fact, share several *cis*-elements. Adapted from: (Yan & Boyd, 2007).

Cell-Matrix Interaction

The extracellular matrix helps to hold cells and tissues together. It provides an organized lattice where cells can migrate and interact with one another. Cells can also interact with the surrounding extracellular matrix by connecting their actin filaments to the matrix. The principal receptor on cells responsible for binding to the extracellular matrix proteins are the integrins. Integrins are heterodimers comprised of an α and a β subunit that mediate cellular attachment to the extracellular matrix. They also function as signal transducers, activating various intracellular signaling pathways through their cytoplasmic tails following activation by matrix binding; this is known as “outside-in” signaling. Signals arising from the cytoskeleton modulate integrin conformation (“inside-out” signaling) (Pozzi & Zent, 2003).

Cellular activities controlled by cell-ECM matrix interaction are required for normal development, but they are also crucially involved in several physiological and pathological processes, in particular wound healing, scarring and fibrosis. Integrins mediate cell adhesion, migration, survival, and also specific differentiation programs relevant to development, tissue maintenance, and repair (Paller, 1997). Usually, integrins are expressed at the cell surface in an inactive form. Activation results in increased affinity for ECM molecules and clustering into focal adhesions (Eble, 2001).

An important role for integrins is to provide a link between the ECM and the actin cytoskeleton, and the best described actin-binding proteins that associate with integrin cytoplasmic tails in focal adhesions include talin, filamin, vinculin, tensin and α-actinin (MacPherson & Fagerholm, 2010). Talin binds directly to integrins and is a major component of the formation of focal adhesions (Bouaouina, Harburger, & Calderwood,

2012). These assemblies of structural proteins are believed to play important roles in stabilizing cell adhesion and regulating cell shape, morphology, and mobility (**Figure 5**). They may also serve as a framework for the association of signaling proteins that regulate signal transduction pathways leading to integrin-induced changes in cell behavior.

Many signaling pathways downstream of integrins have been identified. Integrins regulate many protein tyrosine kinases and phosphatases, such as FAK and Src, to coordinate many of the cell processes, including spreading, migration, proliferation and apoptosis. The regulation of MAP kinases by integrins is important for cell growth or other functions. Phosphatidylinositol lipids and their modifying enzymes, particularly PI3-kinase, are strongly implicated as mediators of integrin-regulated cytoskeletal changes and cell migration. Similarly, actin cytoskeleton regulation by the Rho family of GTPases is coordinated with integrin signaling to regulate cell spreading and migration. Rho can alter myosin light chain phosphorylation, inhibit actin depolymerization and activate phosphoinositide-dependent F actin formation all of which alter the ability of the cell body to contract and migrate (E. A. Cox, Sastry, & Huttenlocher, 2001).

Interaction of cells with the surrounding ECM is an important modulator of all cellular activities. Cells respond to external stimuli by converting mechanical force into biochemical signals. Stress applied to the ECM would be transmitted via integrin receptors to the cytoskeleton throughout the cells and even into the nucleus, where changes in gene expression occur (Chiquet, Gelman, Lutz, & Maier, 2009). This complex interplay controls specific gene expression program at any given time. Events that disrupt cellular mechanosensing, intracellular mechanotransduction signaling or

intracellular or extracellular force distribution, can result in clinical phenotypes (Jaalouk & Lammerding, 2009). The role of the different ECM proteins, the function of the integrins, their modulation forces, and their interplay remains to be fully described.

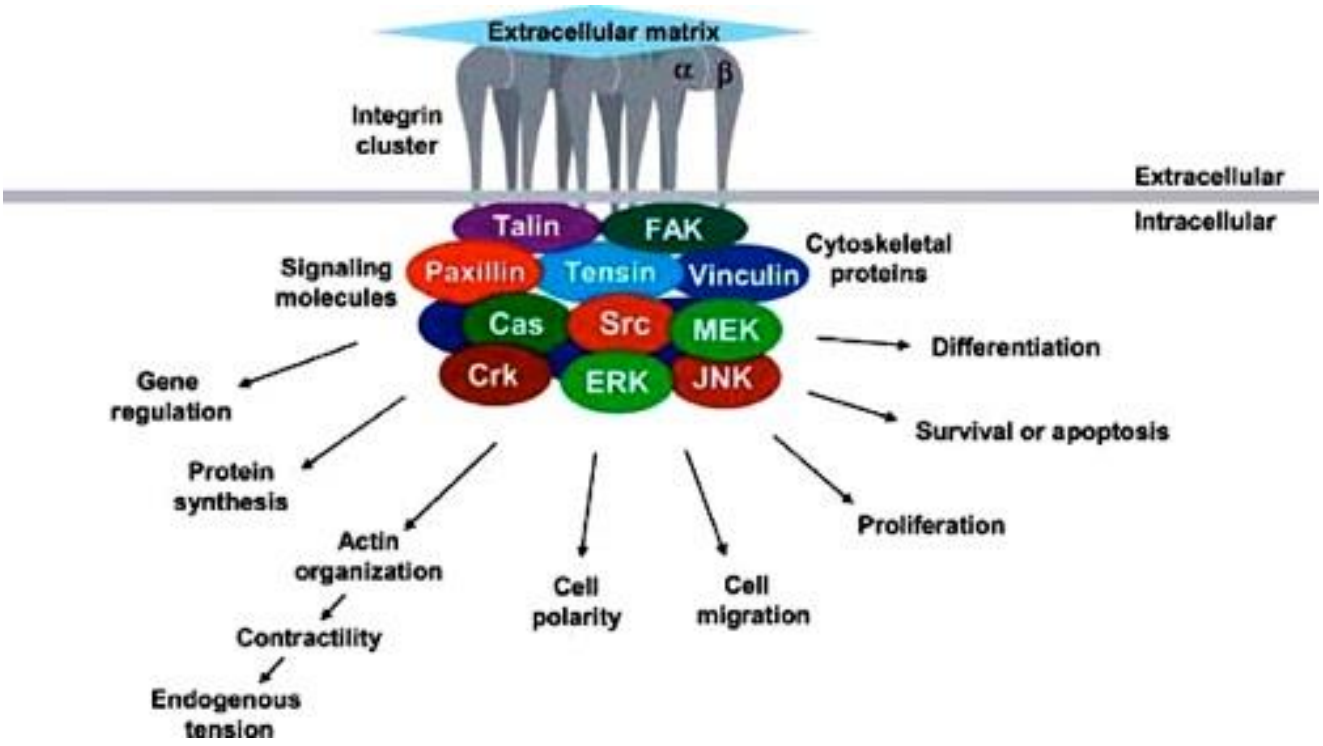


Figure 5: General model of cell matrix interaction and their downstream regulation.
Cell-extracellular matrix adhesions containing clusters of integrins recruit cytoplasmic proteins, which in cooperation with other cell surface receptors control diverse cellular processes and functions. Adapted from: (Berrier & Yamada, 2007).

Summary and Dissertation Goals

A collaborative study early in 2005 identified *Ankrd1* to be highly regulated by wound healing. Eight years later, our understanding of *Ankrd1* in tissue repair is in its early stages. The goal of this thesis was to enhance our knowledge of the role of *Ankrd1*. These studies focused on identifying *Ankrd1* binding partners and characterize target genes that are responsive to *Ankrd1*-associated transcription complexes during skin repair. The following chapters discuss: (a) a phenotype resulting from *Ankrd1* global deletion that causes dysfunctional cell-matrix interaction in fibroblasts; (b) the mechanisms underlying gene regulation by Ankrd1; and (c) expression of Ankd1 in human burn tissue.

Deletion of the *Ankrd1* gene results in dermal fibroblast dysfunction

Targeted deletion of *Ankrd1* resulted in an excisional wound healing phenotype marked by slow wound closure and reduced granulation tissue. These studies were based on our previously developed knockout *Ankrd1* mouse model. We demonstrated that Ankrd1 is necessary for proper interaction of fibroblasts with a collagenous matrix *in vivo* and *in vitro*. *Ankrd1*-null fibroblasts showed reduced cell migration, adhesion, and impaired cell contraction and spreading in the absence of *Ankrd1*. This study serves as a foundation for future studies on the role of *Ankrd1* in wound healing.

Ankrd1 acts as a transcriptional repressor of MMP-13 via the AP-1 site

This study aimed to determine Ankrd1 binding partners and its target genes. We found that Ankrd1 repressed transactivation of *MMP13* through interaction with the negative regulator, nucleolin, and that genetic deletion or suppression of *Ankrd1* resulted in

overexpression of *MMP13* while *Ankrd1* reconstitution decreased *MMP13* levels in null fibroblasts. *Ankrd1* deletion additionally relieved *MMP10* transcriptional repression.

Increased Ankrd1 expression after burn injury in humans

A total of 29 burn patients and 5 normal skin samples were analyzed by immunohistochemistry to determine Ankrd1 expression. Results from this study emphasize the prevalence of Ankrd1 during human skin wound healing.

Chapter II

DELETION OF THE *ANKRD1* GENE RESULTS IN IMPAIRED CELL-MATRIX INTERACTION

Introduction

As part of a gene expression profiling program to identify genes altered following dermal wounding in mice, we reported that ankyrin repeat domain protein 1 (Ankrd1; also called cardiac ankyrin repeat protein, CARP) mRNA and protein levels were highly induced and remained elevated during the healing process (Shi et al., 2005a). Cells of the epidermis and dermis that showed induced expression included vascular endothelial cells, cells of the hair follicle bulb and the panniculus carnosus, keratinocytes, monocytes, and fibroblasts, indicating increased Ankrd1 may be a generalized stress response in many cells types of the skin. Overexpression of Ankrd1, a member of the muscle ankyrin repeat protein (MARP) family, which includes Ankrd2 and Ankrd23 (Miller et al., 2003) also improved many aspects of wound healing in several animal models (Shi et al., 2005a).

The dynamics of MARP gene expression during normal muscle development and function as well as those seen under pathological stress conditions in many tissues, including muscle, (S. Kojic, D. Radojkovic, & G. Faulkner, 2011; Mikhailov & Torrado, 2008; Samaras et al., 2006b) suggested that disruptions in normal MARP function could

have dire consequences to the organism. In a unique report, Barash et al. observed that deletion of the MARP family, both individually and in combination, produced a much milder phenotype (Barash et al., 2007). Based on skeletal muscle response to eccentric contraction, the authors suggested that the MARPs are not essential for development or basal function of skeletal muscle, but that they play a role in mechanical behavior and stability of muscle following exercise. It was also concluded that the three MARP proteins are structurally and functionally redundant because it required deletion of all three genes to see a significant effect. A recent study with the same strains further concluded that the MARPs were not involved in the response to pressure overload (M. L. Bang et al., 2014).

Our lab developed a conditional *Ankrd1*$^{fl/fl}$ from which we created a global deletion for the purpose of studying Ankrd1 function in wound healing. We now report that deletion of *Ankrd1* results in delayed excisional wound closure characterized by decreased contraction and reduced granulation tissue thickness. Results of cell culture studies suggest that part of this phenotype results from dysfunctional cell-matrix interaction in fibroblasts.

Materials and Methods

Surgical wounding. Studies were carried out in the AAALAC approved facilities of Vanderbilt University, Nashville, Tennessee, under approval of the Institutional Animal Care and Use Committee at Vanderbilt University School of Medicine. 15-20 week old *Ankrd1*$^{fl/fl}$ (**FLOX**; control) *and Ankrd1*$^{-/-}$ (**KO**) male mice (n=8) in the mixed background, received two 6mm, full-thickness excisional wounds on the dorsum. Wounds were photographed (Canon PC1234) at the times indicated in the figures and legends. The wound areas were determined using ImageJ (National Institutes of Health, Bethesda, MD) and expressed as percentage of initial wound size. At completion of the experiment, mice were euthanized, and the complete wounds, including 2 mm of the wound margin, were harvested and prepared for histology.

Histology. Mice were euthanized by CO_2 asphyxiation at the times indicated in the figure legends. The wounds plus surrounding skin were excised and fixed in neutral buffered formalin overnight at 4°C, embedded in paraffin, sectioned, stained with hematoxylin and eosin or Masson's trichrome green stain, and photographed (Olympus BX50 microscope with Olympus DP71 camera, Software CellSens Standard 1.6, Olympus Corporation). Digital images of each excisional wound were used to determine the distance between the edges of the panniculus carnosus as a measure of original wound gap using ImageJ (National Institutes of Health, Bethesda, MD). Digital images of the wounds were also used to measure the granulation tissue thickness and cross-sectional area under 4x magnification.

Cells. Mouse dermal fibroblasts were isolated from *Ankrd1*$^{fl/fl}$ (FLOX) and *Ankrd1*$^{-/-}$ (KO) neonatal skin as previously described(Normand & Karasek, 1995). Early-passage populations of the isolated skin fibroblasts were immortalized with a SV40 large T-antigen plasmid (Chang, Pan, Pater, & Di Mayorca, 1985). Briefly, 100 mm dishes of primary cells at 80-90% confluence were transfected with 8 µg of plasmid using Lipofectamine (Life Technologies, Grand Island, NY) overnight at 37^{o}C in 5% CO_2, after which the transfection reagent was removed and growth medium added(Chang et al., 1985). Both primary and immortalized cells were grown in DMEM containing 10% FBS, 100 Units/ml penicillin–streptomycin (P/S), 1% Antibiotic-Antimycotic (AA) and 2 mM L-Glutamine. Mouse vascular smooth muscle cells were isolated from FLOX and KO neonates as described (Ray, Leach, Herbert, & Benson, 2001), and half of the cells were stably transformed with SV40 large T antigen plasmid (Chang et al., 1985). Vascular aortic smooth muscle cells were grown in Medium 231 (Invitrogen, Grand Island, NY) supplemented with smooth muscle growth supplement (Invitrogen, Carlsbad, CA) and 2 mM L-Glutamine.

Migration assay. Four wells of a 12-well tissue culture plate were coated with extracellular matrix (ECM) molecules by incubation in a tissue culture hood for 2h at RT. In each plate, 2 wells were coated with collagen (rat tail type I, 100µg/ml BD Biosciences, Bedford, MA) and 2 wells with fibronectin (10µg/ml, Santa Cruz, Santa Cruz, CA), both in PBS. ECM solutions were removed, the wells washed with sterile PBS and incubated with serum-free DMEM at 37^{o}C in 5% CO_2. FLOX and KO fibroblasts at 80% confluence were trypsinized from flasks, counted and diluted to a concentration of 6x10^{5} cells/ml, a concentration that permitted cells to reach confluence

24h after plating. ECM-coated cell culture plates were secured onto a magnetic array that aligned magnets in the center of each of four coated wells. The media were removed from the four coated wells, 500 µl of fresh growth medium added, and sterile, magnetically-adherent stencils (Ashby, Wikswo, & Zijlstra, 2012) (MAtS) were placed in the center of each well. 500 µl of each cell suspension was equally distributed around the MAtS, one cell type per each ECM, and the plates were incubated for 24h to allow cells to attach and reach confluence. Plates were removed from the magnetic arrangement and the medium was carefully aspirated to remove floating cells and replaced with fresh growth medium. The MAtS were carefully removed to leave a cell free, undisturbed, 4-arm ECM-coated area in the center of each well. The cell-free wells were filled with warm PBS to help prevent drying of open plates, and the plate was placed in a humidified stage incubator (Bioscience Tools, San Diego, CA) infused with 5% CO_2, attached to a Zeiss Axiovert 200M microscope with an automated stage driven by the Ludl Mac 2000 driver module. Images were acquired every 10 min for 5 h with a Tucsen 3.3MP cooled CCD digital microscope camera (OnFocus Laboratories, Lilburn, GA) using a 10X objective. ImagePro Plus 3D with StagePro software (Media Cybernetics Inc., Bethesda, MD) for time lapse image acquisition was used to acquire and process images. Data analysis to determine percent of total pixels that were not covered (percent open area) was done using TScratch software (Geback, Schulz, Koumoutsakos, & Detmar, 2009).

Collagen gel contraction. Three-dimensional collagen lattices were prepared as previously described (Ngo, Ramalingam, Phillips, & Furuta, 2006). Briefly, type I rat tail collagen (BD Biosciences, San Jose, CA) was diluted with 20mM acetic acid to 3mg/ml.

Primary or immortalized skin fibroblasts isolated from FLOX and KO neonatal mice were cultured in DMEM medium (10% FBS, 1% AA, 1% P/S), trypsinized and counted. 200µL of collagen neutralized to pH 7.0 with 1*N* sodium hydroxide was loaded into each well of a 24-well plate. Cells (1.5×10^5) were suspended in 400µl of DMEM (10% FBS, 1% AA, 1% P/S) and added to the wells containing 200µl of collagen solution to yield a final concentration of 1mg/ml. The gels were then incubated at 5% CO_2 and 37°C for 30min to allow the collagen to polymerize. After collagen polymerization, 500µl of DMEM (10% FBS, 1% AA, 1% P/S) was added to each well. Using a sterile spatula, each gel was mechanically released from the wall and bottom of the wells. Collagen lattices were imaged (GelLogic200 Imaging System, Molecular Imaging System, Carestream Health; Woodbridge, CT) every 24h and lattice area was analyzed using the Molecular Imaging software (Carestream Health, Inc., Woodbridge, CT). Reduction in lattice area due to contraction was determined at daily intervals up to 7 days.

Adenovirus infection/reconstitution. FLOX and KO immortalized skin fibroblasts were infected with an adenovirus expressing *Ankrd1* (adAnkrd1-GFP) or luciferase (adLuc-GFP) as a control with a multiplicity of infection of 100. Cells were harvested 48h after infection, and used for collagen gel contraction assays or protein isolation.

Protein preparation. Whole cell protein extracts were made by lysing the cells in RIPA buffer (Sigma, St Louis, MO) containing a protease inhibitor cocktail (Complete Mini Protease Inhibitor Tablets; Roche, Mannheim, Germany) followed by sonication using a Bronson 250 Sonifier with a water bath cup horn attachment. Protein concentration in cleared lysates was determined by BCA protein assay kit (ThermoScientific, Rockford, IL) and lysates were stored at -80^{o}C.

Western Blots. Thirty µg of cell extracts were separated by 10% acrylamide SDS-PAGE and transferred to PVDF membrane (Immobilon, Millipore, Billerica, MA) using the NuPage (Life Technologies, Carlsbad, CA) blotting apparatus, following the manufacturer's protocol. After blocking with a solution of 10mM Tris-HCl pH 8, 150mM NaCl, 0.05% Tween-20, and 5% milk powder, the membrane was incubated with anti-Ankrd1 antibody (1:2000) and anti-Cyclophilin (1:20,000; BML-SA296, Enzo, Farmingdale, NY) at 4°C overnight, followed by incubation with anti-rabbit IgG (C2609; Santa Cruz) at room temperature (RT) for 30min. The membrane was washed, incubated with Western Lightning Plus Enhanced Chemiluminescent Reagent (Perkin Elmer, Waltham, MA), and protein bands were visualized and quantified using a Kodak Image Station 4000MM Pro with Kodak MI software (Carestream Health, Inc., Woodbridge, CT). For the virus reconstitution studies, protein was isolated from cells 48 hours after infection.

Phalloidin staining. Collagen lattices were fixed in 4% paraformaldehyde in phosphate buffer after 3 days of culture. Gels were stained following manufacturer's instructions. Briefly, fixed collagen gels were treated with 0.1% TritonX100 in PBS for 5min and washed twice with PBS. To reduce non-specific staining, 1% BSA in PBS was added to the gels for 30min prior to staining with rhodamine phalloidin (Invitrogen) and sytox green nucleic acid staining (Invitrogen). 1µM sytox green nucleic acid staining and 12.5µl of rhodamine phalloidin were diluted in 500 µl PBS per well and incubated with the gels for 30min at room temperature. Gels were washed twice with PBS followed by confocal analysis under 20X magnification using the Perkin Elmer Opera QEHS Automated Confocal Microscopy System (PerkinElmer, Waltham, Massachusetts) at the

Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE) Core Facility. Confocal images were analyzed using Columbus Software (PerkinElmer, Waltham, Massachusetts).

Polyacrylamide (PA) gels. Preparation of PA hydrogels was adapted from a previously described protocol (Tse & Engler, 2010). Briefly, 18-mm cover slips were animo-silanated by treating them first with 0.1 M NaOH and then with 3-aminopropyl-triethoxysilane (APES) (Sigma, St Louis, MO). Coverslips were washed with dH_2O and treated with 0.5 % glutaraldehyde (Sigma, St Louis, MO) in PBS. Dry coverslips were then used for gel preparation. Gels with gradients in stiffness (2, 4, 8, 10, and 20 kPa) were prepared using the exact following protocol: (Tse & Engler, 2010). Mixtures of acrylamide and bisacrylamide were polymerized by adding 1.0µl TEMED and 10µl of 10% ammonium persulfate. 25µl of the gel solution was pipetted into the amino-silanated coverslip. Another coverslip is also placed on top of the polyacrylamide solution. The polymerization was completed in about 30 min and the top coverslip was peeled off. The bottom cover slip with the attached polyacrylamide gel is immersed in a multi-well plate (6-well MATtek dish).

For cell seeding, collagen I protein or fibronectin was conjugated to the surface of the hydrogel using the heterobifunctional linker Sulfo-SANPAH (Pierce, Rockford, IL). 1 mg/ml solution of Sulfo-SANPAH is dissolved in dH2O, and 200ul of this solution is pipetted onto the gel surface. The polyacrylamide gel is then placed under an ultraviolet lamp and irradiated for 10 min. The gels were then washed twice with 50mM HEPES in PBS. The gels were then coated with 0.10mg/ml of rat type I collagen (BD Biosciences, Bedford, MA) or 10 ug/ml fibronectin (Santa Cruz, Santa Cruz, CA) overnight at 4^0C.

Coated gels were washed three times with PBS followed by addition of FLOX or KO fibroblasts.

Results

In vivo analysis of *Ankrd1* deletion

Loss of Ankrd1 produces a wound contraction phenotype. The biological role of Ankrd1 was only partially implicated by the effects of its over-expression (Shi et al., 2005a). Based on preliminary observations in 18h wounds, we analyzed wound closure in FLOX and KO mice to the point of near-closure. Bilateral, full-thickness, excisional wounds (6mm) were made on the dorsum and photographed daily for 8d (**Figure 6A**). The wounds of the KO mice closed more slowly than those of the FLOX mice. Measurements of the wounds showed a particularly significant delay in closure between 6d and 8d (**Figure 6B**). Histomorphometric analysis of wound sections revealed both a significant reduction in granulation tissue thickness **(Figure 6D)** and an increased wound gap between the edges of the intact panniculus carnosus of KO wounds **(Figure 6E)**. The change in the shape, rather than the implied volume of granulation tissue, suggested that the delay in closure of KO wounds resulted from decreased contraction.

In vitro analysis of *Ankrd1* deletion

Dermal fibroblasts from KO *mice have an altered phenotype.* Since granulation tissue morphology was differentially affected by *Ankrd1* deletion, dermal fibroblasts were isolated from FLOX and KO mice and immortalized with the SV40 large T antigen.

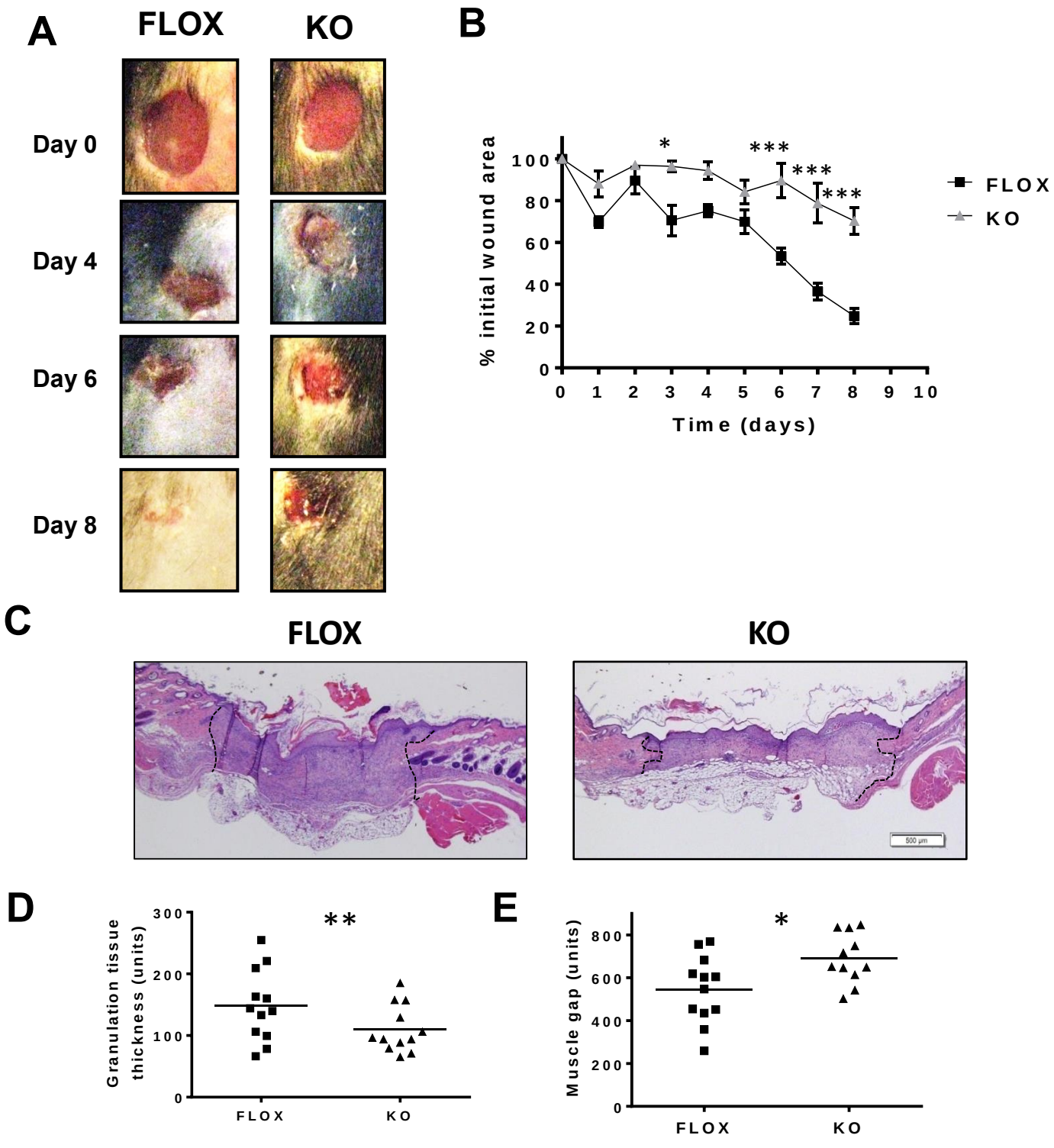

A
FLOX
KO
Day 0
Day 4
Day 6
Day 8
B
% initial wound area
Time (days)
FLOX
KO
*
***
***
***
C
FLOX
KO
500 µm
D
Granulation tissue thickness (units)
**
FLOX
KO
E
Muscle gap (units)
*
FLOX
KO

Figure 6: Delayed excisional wound closure in KO mice.
A. *Left panel*: Gross images at days 0, 4, 6 and 8 after biopsy showed larger wound areas for KO mice compared to FLOX mice. *Right panel*: The relative open wound area decreased more rapidly in FLOX mice than KO mice. **C**. H&E staining in 2 representative wounds showed thinner granulation tissue in KO wounds (area marked by dotted lines). **D**. Granulation tissue thickness was measured in histological sections and it was significantly reduced in KO wounds compared to wounds in FLOX mice (n= 12) **E**. A larger gap between the cut edges of the panniculus carnosus (muscle gap, $p < 0.05$, n=12) was measured in KO compared to FLOX wounds. Student t-test was used to determine statistical significance (*<p0.05, **p<0.01, ***p< 0.001).

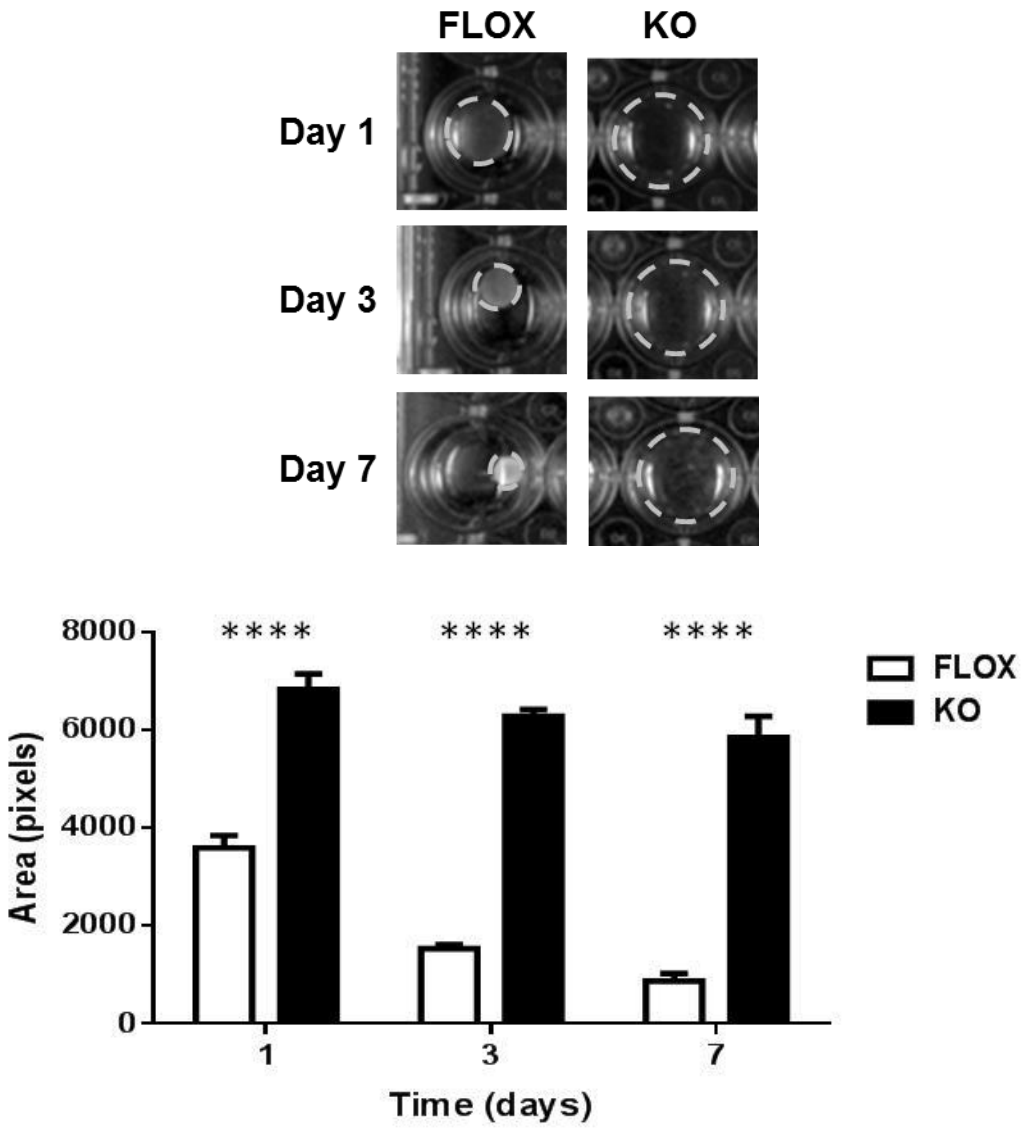


Figure 7: *Ankrd1*-null fibroblasts show impaired collagen contraction over time. Collagen gels (1mg/ml) containing $1.5x10^5$ cells/well were formed in 24-well culture plates and detached at 24h. *Upper panel*: Collagen lattices, containing FLOX fibroblasts contracted over time, while KO fibroblast lattices had impaired ability to contract up to d7. *Lower panel*: Quantification of collagen gel area was measured over a 7-day interval. FLOX fibroblasts produced rapid, time-dependent contraction, while little contraction was observed in KO cells up to d7. Student t-test; (****p< 0.0001).

Experiments were carried out with cells between passage 2 and 10 as the cells exhibited morphological changes at higher passage (data not shown).

KO fibroblasts fail to contract collagen lattices. Wound contraction plays a significant role in closure of dermal wounds in loose-skinned rodents. We used both primary and immortalized FLOX and KO mouse fibroblasts to compare their ability to contract fibroblast-populated collagen lattices (FPCL). One day following FPCL release from the wall of the tissue culture plate well, there was significant ($p < 0.0001$) contraction of FLOX fibroblasts (**Figure 7**). KO populated FPCL failed to contract FPCL even when incubated for up to a week (**Figure 7**) or with increasing serum concentration in the medium (**Figure 8B**). FLOX fibroblasts showed significant collagen contraction even with the lowest number of cells, but little or no FPCL contraction by KO cells, irrespective of the cell number (**Figure 8A**). Similar results were found when using non-immortalized cells. Inhibition of contraction was also observed with vascular smooth muscle cells (**Figure 9**).

Reconstitution of *Ankrd1* by viral transduction was used to show that the absence of Ankrd1 was central to the contraction failure phenotype of the KO cells. Western blot analysis showed that transduction with adLuc-GFP had no effect on cellular Ankrd1 in FLOX and KO cells (**Figure 10A**, lanes 1 and 3 respectively). Transduction with adAnkrd1-GFP increased Ankrd1 in FLOX cells (**Figure 10A**, lane 2) and reconstituted expression in KO cells (**Figure 10A**, lane 4). Restoration of Ankrd1 in KO fibroblasts improved their ability to contract the FPCL and increased the rate of contraction by FLOX cells (**Figure 10B**). Phalloidin staining of F-actin in FPCL after infection with

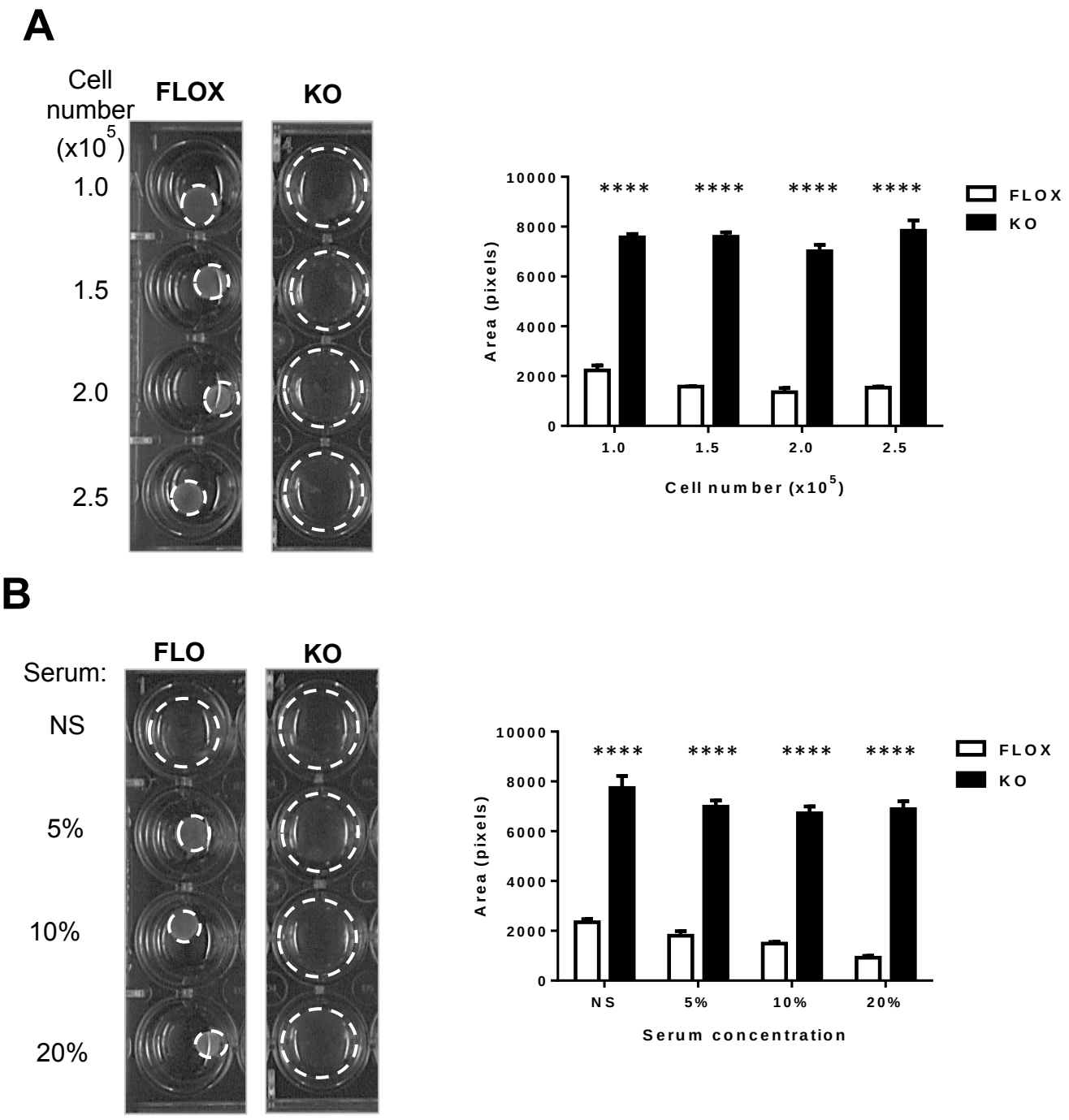


Figure 8: KO fibroblasts fail to contract collagen gels.
A. Cells (1 x10^5, 1.5x10^5, 2 x10^5, or 2.5x10^5 per well) were plated in 1mg/ml collagen gels in a 24 well tissue culture plate. After 24h detached gels containing FLOX fibroblasts demonstrate increased collagen contraction in a cell-number dependent fashion whereas no contraction occurred in collagen gels containing KO fibroblasts regardless of cell number. Cell density-dependent collagen gel contraction at day 1 was significantly different between FLOX and KO fibroblasts **B**. Increasing serum concentrations do not improve the ability of KO fibroblasts to contract collagen gels. At day 1 of culture with no serum (NS), 5%, 10% or 20% serum, there was no significant contraction of collagen by KO fibroblasts (open bars). FLOX fibroblasts (solid bars) contracted collagen at all serum concentrations. Student t-test (****, p<0.0001).

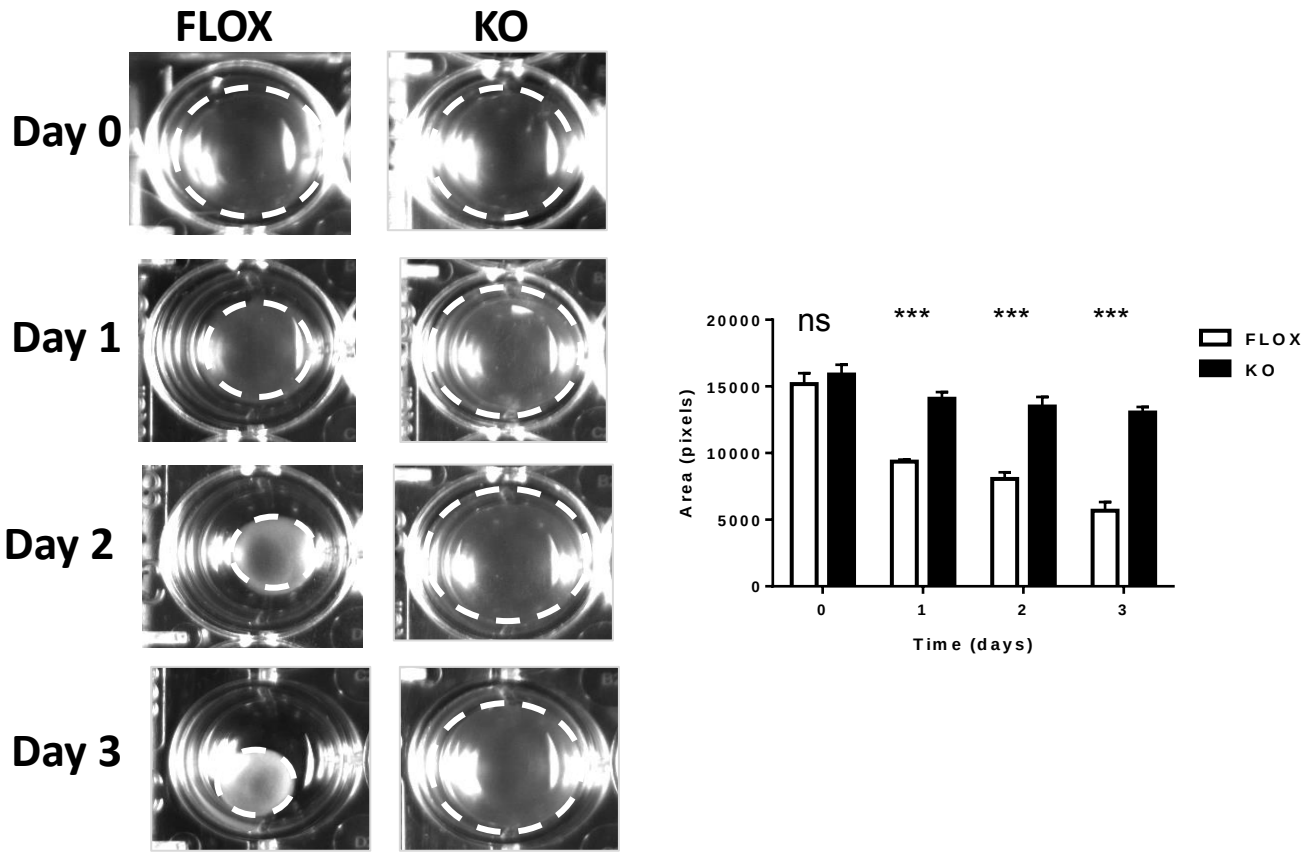


Figure 9: *Ankrd1* is required for contraction of a smooth muscle cell-populated collagen lattice.

Aortic smooth muscle FLOX cells (1.5 x 10^5 per gel) produced time-dependent contraction of collagen gels while no contraction was observed, even at day 3, with KO fibroblasts. Student t-test (***p<0.001).

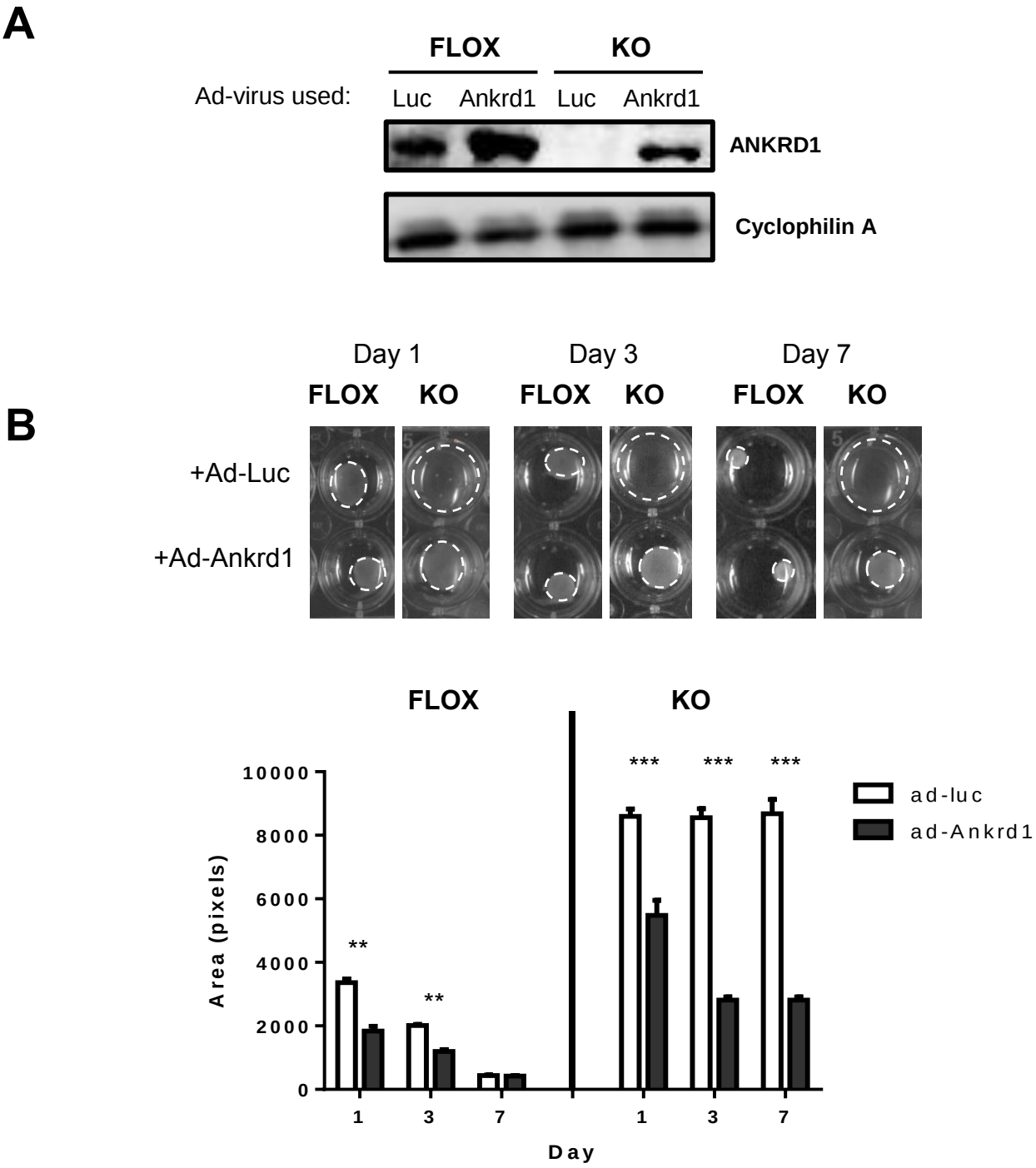


Figure 10: Reconstitution of Ankrd1 in KO fibroblasts restores their ability to contract collagen gels.

A. Western blot analysis of FLOX fibroblasts infected with adLuc-GFP (lane 1) and adAnkrd1-GFP (lane 2) showed overexpression of Ankrd1 after infection with adAnkrd1-GFP. Ankrd1 protein was absent in extracts from KO fibroblasts infected with adLuc-GFP (lane 3) but was easily detectable after infection with adAnkrd1-GFP (lane 4). **B.** Overexpression of Ankrd1 in FLOX cells increased contraction on d1 and d3 ($p<0.01$) and reconstitution of Ankrd1 in KO cells restored their ability to contract ($p<0.001$) at all-time points.

adLuc-GFP FLOX revealed abundant filamentous actin network, while there was very poor actin assembly in control-transduced KO fibroblasts (**Figure 11**). Reconstitution of Ankrd1 in KO fibroblasts increased the staining of an F-actin network. These data were consistent with our *in vivo* wound closure studies, and they suggest that Ankrd1 is necessary for proper interaction of fibroblasts with a collagenous matrix *in vivo* and *in vitro*.

Adhesion to, and migration on, extracellular matrix were analyzed using a magnetically attached stencil (MAtS) assay with both collagen I and fibronectin substrates (see Methods). We observed that KO cells took longer than FLOX cells to attach to the collagen matrix, but both fibroblast genotypes were stably attached before initiating stencil removal that initiates cell migration. Both FLOX and KO cells closed the open area more completely on fibronectin (open area: FLOX = 54 ± 1.75%, KO = 72 ± .24%) than on collagen coated substrate (open area: FLOX = 62 ± 3.36%, KO = 80 ± 2.71%) (**Figure 12A and 12B**). Both cell types migrated to a significantly lesser extent on collagen than fibronectin ($p < 0.001$ at 5h; **Figure 12 C and 12D**), KO fibroblast migration was significantly slower than FLOX migration on either substrate. In summary, KO fibroblasts had an impaired ability to interact with and a migrate upon two common matrix substrates.

KO fibroblasts fail to spread on polyacrylamide gels coated with collagen regardless of stiffness. To investigate whether cell spread was affected by the substrate or the stiffness of the substrate, we studied cell behavior on polyacrylamide hydrogels spanning a range of stifnesses. The PA gels we prepared represent a range of elastic moduli, from 2 kPa to 20 kPa. The PA surface was covalently functionalized with

collagen I or fibronectin using Sulfo-SANPAH. We studied how FLOX and KO cells responded to these substrates. FLOX fibroblasts adopted spindle-shape morphology on PA hydrogels, from 4 kPa to 20 kPa, coated with collagen, while KO fibroblasts were rounded up on all PA hydrogels that were coated with collagen I, regardless of stiffness (**Figure 13A**). Interestingly, both FLOX and KO fibroblasts were able to spread starting at an elastic modulus of about 8 kPa (**Figure 13B**) on hydrogels that were coated with fibronectin. Thus, FLOX and KO fibroblasts did not respond similar when cultured on collagen-coated substrates as on fibronectin-coated PA hydrogels. Together these data suggest that Ankrd1 is critical for the proper interaction of cells with a compliant collagenous matrix *in vitro*.

Discussion

Elevated *Ankrd1* expression marks numerous developmental and pathological events, including cardiomyogenesis (Kuo et al., 1999; Zou et al., 1997) and neovascularization during tissue repair, and overexpression of *Ankrd1* can enhance healing in several animal wound models (Shi et al., 2005a). Despite the resultant implication that Ankrd1 could be critical to development, we found that global deletion of murine *Ankrd1* yielded no abnormalities in a mixed genetic background. In contrast to developmental effects, the physiological stress of a standardized model of wounding clearly discriminated one of the *Ankrd1* phenotypes. *Ankrd1* deletion retarded excisional wound closure and the granulation tissue response; furthermore, the matrix-dependent migration, contractility, and spreading of KO skin fibroblasts were markedly impaired

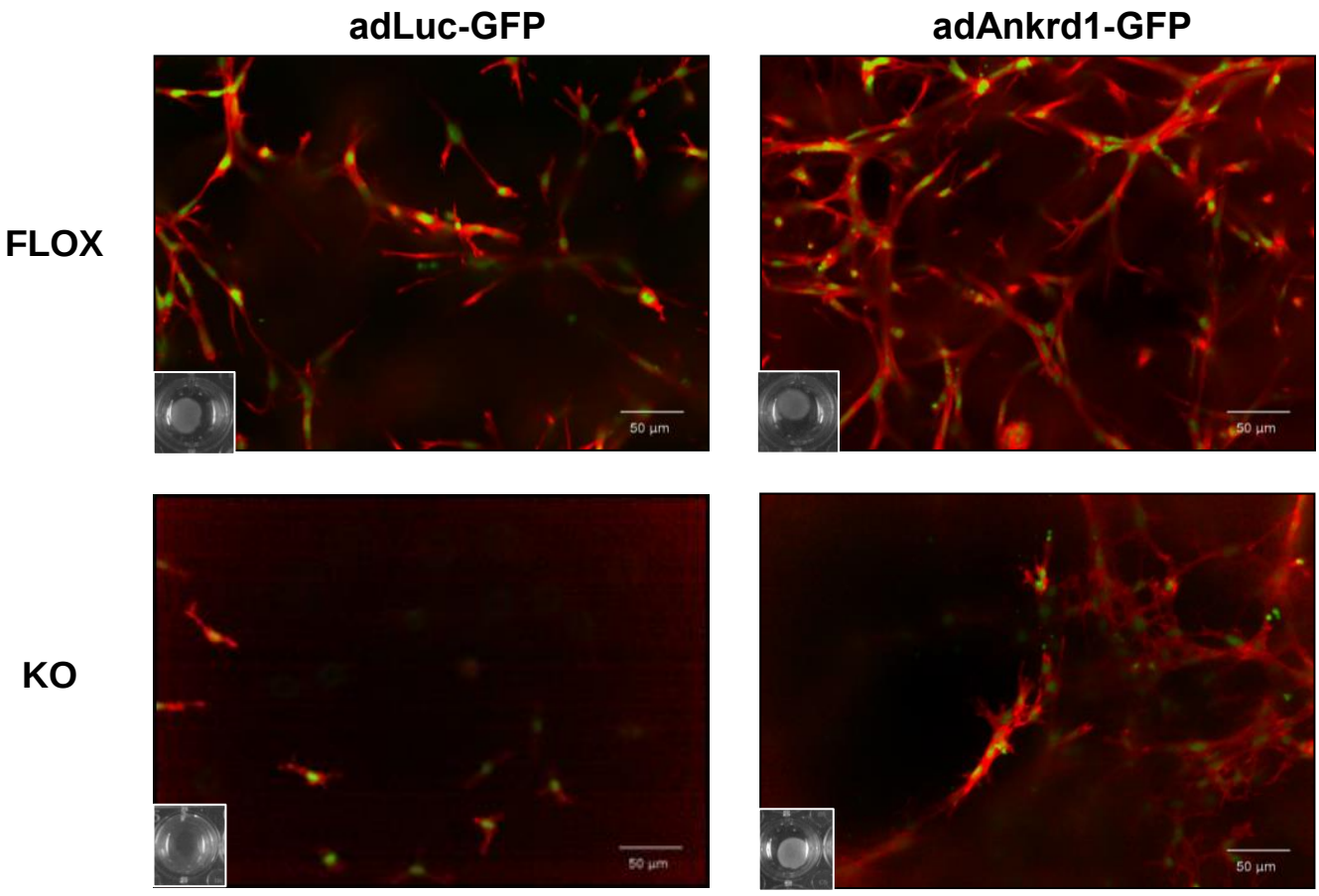


Figure 11: Actin networks formed after reconstitution of Ankrd1 in *Ankrd1*-deficient fibroblasts.
Collagen gels were loaded with equal numbers of the indicated cell types and infected with either adenoviral luciferase or Ankrd1 expression vectors. Actin fibers were present in collagen gels populated with FLOX fibroblasts infected with adLuc-GFP and were more abundant after Ankrd1 overexpression. Actin fibers were nearly absent in similarly treated KO cells and markedly increased with Ankrd1 overexpression.

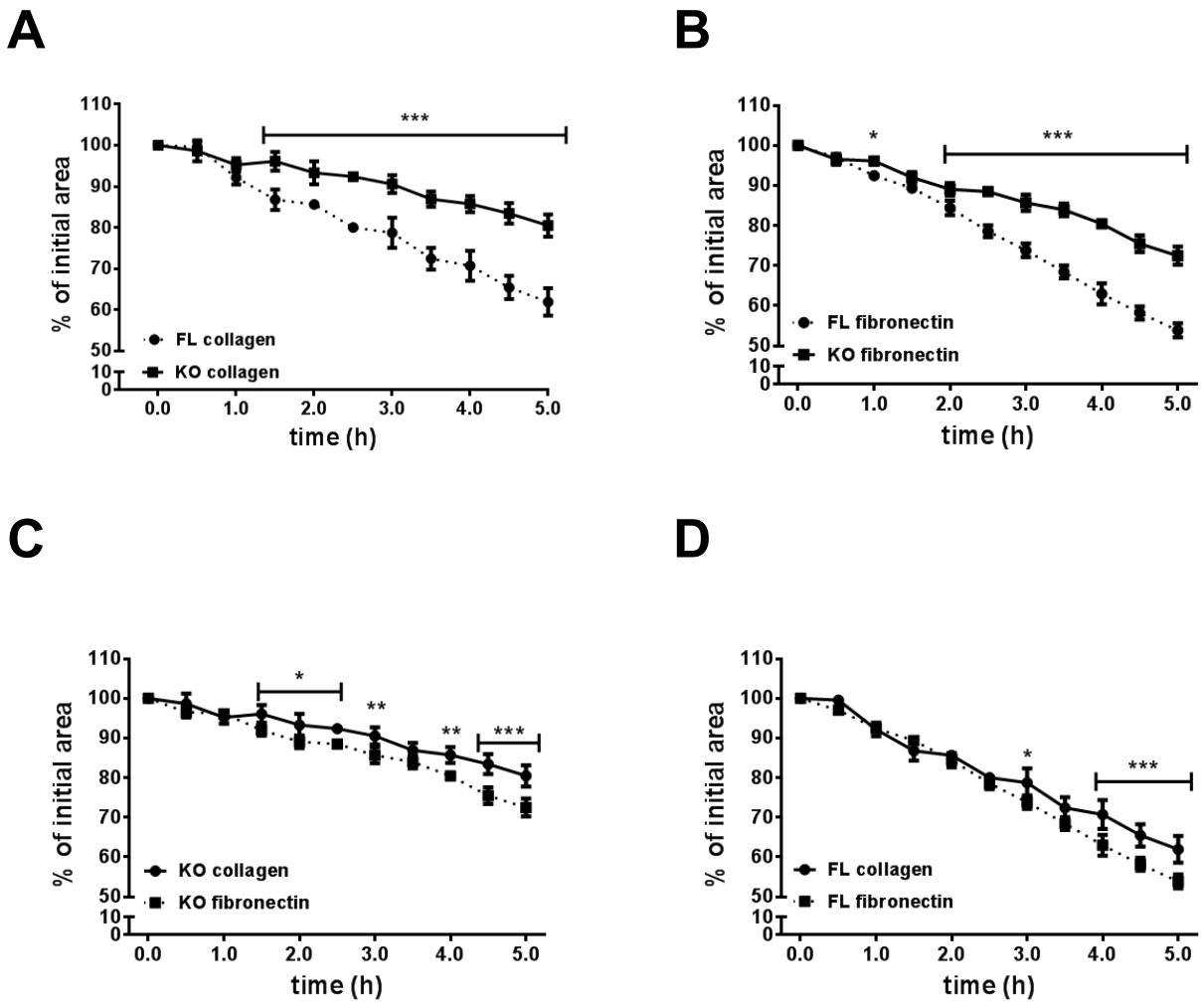


Figure 12: Deletion of *Ankrd1* slows fibroblast migration on collagen and fibronectin substrates.

Migration of FLOX and KO fibroblasts was studied on fibronectin or collagen using the MAtS approach. **A**. KO fibroblasts migrated significantly slower on collagen than FLOX cells, with differences seen as early as 1.5 h ($p<0.001$) **B**. KO fibroblast migration on fibronectin was significantly slower than FLOX f broblasts at 1.0h ($p< 0.05$) and from 2h to 5h ($p<0.001$) **C.** KO fibroblasts migrated more rapidly on fibronectin than on collagen (1.5-2.5h, $p<0.05$; 3.0h, $p< 0.01$; 4.0h, $p< 0.01$; 4.5-5.0h, $p<0.001$). **D**. FLOX fibroblasts migrated at similar rates on fibronectin and collagen, although significant differences were seen at later time points (3.0h, $p<0.05$; 4.0-5.0h, $p<0.001$).

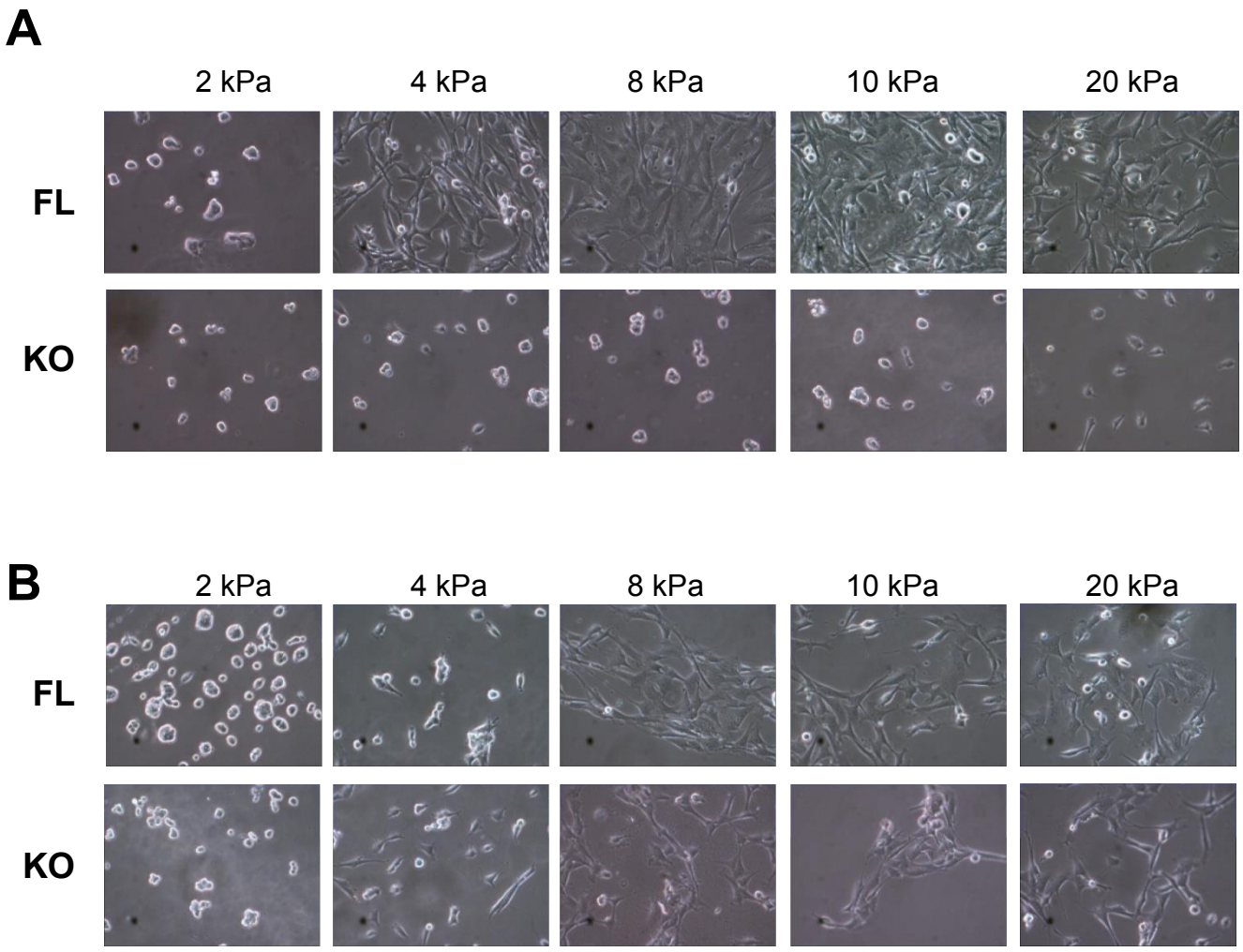


Figure 13: *Ankrd1*-null cells fail to spread on collagen-coated low rigidities polyacrylamide gels

FLOX and KO fibroblasts were plated on polyacrylamide (PA) gels ranging from 2 to 20 kPa coated with **A**. collagen or **B**. fibronectin. Cell spreading was dependent on rigidity and the substrate. **A**. FLOX cells spread more on collagen-coated soft gels while KO failed to spread at all rigidities when gels were coated with collagen. **B**. FLOX and KO fibroblasts failed to spread on soft fibronectin-coated PA gels. However, both FLOX and KO cells spread in more rigid PA gels, ranging from 8 – 20 kPa, coated with fibronectin.

compared to normal cells. These findings are highly consistent with the concept that Ankrd1 abundance becomes a rate-limiting factor when cells and tissues are challenged by injury and stress.

Cellular actions of Ankrd1. The interpretation of the *Ankrd1*-null phenotype is complicated by the multiple roles of Ankrd1. In the sarcomere, Ankrd1 is a titin-associated structural component that together with myopalladin, calpain protease p94, and calsequestrin forms a complex that appears to be critical for sarcomere stability of differentiating cardiomyocytes *in vitro* (B. Chen et al., 2012; Kojic et al., 2010; S. Kojic et al., 2011; Mikhailov & Torrado, 2008). Reduction of *Ankrd1* mRNA and protein in rat cardiomyocytes by siRNA or doxyrubicin leads to sarcomere disintegration and myofibrillar disarray(B. Chen et al., 2012), presumably due to (a combination of) loss of Ankrd1 from the I-band complex and reduced expression of a range of Ankrd1 transcriptional targets. It has also been suggested that members of the MARP family serve a role in the muscle cell as part of a mechanosensory apparatus, in which MARPs acts as a shuttle to transport factors such as p53 to the nucleus (Kojic et al., 2010; S. Kojic et al., 2011; Moulik et al., 2009). The MARP proteins may also shuttle between cytoplasm and nucleus as part of a stress-related regulatory pathway. We have recently determined that Ankrd1 is involved in the transport of GATA-4 from the sarcomere to the nucleus of the cardiomyocyte (Zhong, Chiusa, et al, submitted for publication).

Based on a large number of foregoing studies, depletion of Ankrd1 could affect both cytoplasmic and nuclear processes. Several studies have suggested that a key role of sarcomeric MARPs is mechanotransduction that is initiated by transportation of the protein from the cytoplasm to the myocyte nucleus (S. Kojic et al., 2011). Desmin is

another cytoplasmic partner whose depletion leads to up regulation of *Ankrd1* (Mohamed & Boriek, 2012). The MARPs are associated with several aspects of transcriptional control in striated muscle, including associations with YB-1,(Zou et al., 1997) p53,(Belgrano et al., 2011; Kojic et al., 2010) and nucleolin (Almodovar-Garcia et al., 2014). Early developmental studies showed that the high-level expression of *Ankrd1* was associated with down-regulation of (myocyte proteins) by association with YB-1. In Chapter III, we suggest on the basis of knockout, knockdown, and overexpression studies in skin fibroblasts, that the association of Ankrd1 with nucleolin leads to the down-regulation of AP-1 stimulated *Mmp13* and *Mmp10* expression (Almodovar-Garcia et al., 2014).

Impaired wound and collagen contraction in the absence of Ankrd1. Using a standard excisional wound model with no splinting to prevent contraction, an initial observation of a slight reduction in the rate of closure was accentuated by 3 days and striking through 8 days, after which the closure defect was gradually overridden by undetermined compensatory mechanisms. Granulation tissue thickness was also significantly reduced, a finding that was in harmony with the positive effects of *Ankrd1* overexpression on this aspect of tissue repair (Shi et al., 2005a). The defect in contraction of the wound margins was certainly consistent with the inefficiency of KO skin fibroblast migration on matrix-coated substrates and the inability of these null cells to contract a fibroblast-populated collagen lattice (FPCL), a model that is often considered an *in vitro* analog of (compliant) granulation tissue.

In a functional cell spreading assay using polyacrylamide gel substrates, we observed that both FLOX and KO cells indeed sense different levels of substrate

rigidity. None of the cells could spread in 2 kPa soft gels. FLOX fibroblasts could spread on both collagen and fibronectin-coated hydrogels, ranging from 4 to 20 kPa. KO fibroblasts failed to spread on all stiffnesses when hydrogels were coated with collagen, while they successfully spread when PA gels were coated with fibronectin, ranging from 8 to 20 kPa. To spread, fibroblasts require cell–ECM binding, integrin activation, Rho GTPase signaling, and subsequent actin polymerization (Price, Leng, Schwartz, & Bokoch, 1998). Spreading cells exert contractile force on the ECM substrate to allow for focal adhesion formation (Ezzell, Goldmann, Wang, Parashurama, & Ingber, 1997) and locomotion, and sufficient ECM resiliency is required to balance cellular tension force and to support the spreading process (Chicurel, Chen, & Ingber, 1998; Ezzell et al., 1997). Thus, we speculate that Ankrd1 is required for proper binding to collagen matrices. Additional experiments are required to determine whether Ankrd1 plays an important role in integrin activation, Rho GTPase signaling, actin polymerization, and formation of focal adhesion.

The significant effect of *Ankrd1* deletion on wound contraction, together with the dramatic inability of *Ankrd1*-null fibroblasts to contract an ECM on collagen equivalent suggests that Ankrd1 may function in a pathway that normally transduces cell-matrix interaction into contractile activity, at least in the low-tension environment of the FPCL. In addition to Ankrd1 association with other components in the classic, sarcomere contractile apparatus, it also binds to desmin, (S. H. Witt, Labeit, Granzier, Labeit, & Witt, 2005) and desmin knockdown has suggested that this filamentous protein may regulate *Ankrd1* expression through an Akt/NF-kappaB pathway (Mohamed & Boriek, 2012). Equally intriguing are the reports that both *Ankrd1* and *CCN2,* which are strongly

induced during injury and repair, are downstream targets of a mechanical strain response that is mediated by the YAP/TAZ pathway (Aragona et al., 2013; Dupont et al., 2011; Halder, Dupont, & Piccolo, 2012; Piccolo, Cordenonsi, & Dupont, 2013). If cell contraction in the FPCL depends on *Ankrd1* expression, then it may be a critical mediator of mechanotransduction during wound repair, in which there is disruption of the normal mechanical environment.

Chapter III

ANKRD1 ACTS AS A TRANSCRIPTIONAL REPRESSOR OF MMP-13 VIA THE AP-1 SITE

Introduction

Dynamics of matrix metabolism, composition and organization play important roles during tissue repair, and defects in these categories have a strong relationship to the impaired healing seen in patients that suffer from arterial and venous insufficiency, lymphedema or diabetes (Midwood, Williams, & Schwarzbauer, 2004; Schultz & Wysocki, 2009). Chronic inflammation in non-healing wounds can lead to excessive fibrosis and scar formation (Saaristo et al., 2006). However, our understanding of the transcriptional regulation of extracellular matrix is incomplete. Our lab identified the transcriptional co-factor, Ankyrin repeat domain 1 (Ankrd1; cardiac ankyrin repeat protein, CARP), to be significantly elevated by wounding (Shi et al., 2005b). Ankrd1 is also induced in other forms of tissue injury, particularly those of skeletal and vascular smooth muscle. In murine wounds, *Ankrd1* mRNA and protein expression dramatically increased within hours, reaching peak levels by 15 hours and remaining elevated for 2 weeks (Samaras, Shi, & Davidson, 2006a). Immunohistochemistry and *in situ* hybridization for *Ankrd1* mRNA and protein in day 1 wounds revealed increased

expression in monocytes, cells of the epidermis and the vasculature, and striated panniculus carnosus muscle (Samaras et al., 2006a; Shi et al., 2005b).

Ankrd1 was initially discovered and characterized as a novel, cytokine-inducible nuclear protein in endothelial cells (Chu, Burns, Swerlick, & Presky, 1995b; Jeyaseelan et al., 1997b). Its protein sequence contains a nuclear localization signal, four repeats of an ankyrin motif, which appears to be involved in protein-protein interactions, a PEST-like sequence that targets ubiquitinated proteins for degradation, and multiple phosphorylation consensus sites (Chu et al., 1995b). Ankrd1 is present in both the cytoplasm and the nucleus, suggesting shuttling between cellular compartments, and Ankrd1 is a significant constituent of the cardiac sarcomere, where it is part of a multicomponent, titin-binding complex (Bang et al., 2001a).

Ankrd1 belongs to a conserved family of muscle ankyrin repeat proteins (MARPs) along with Ankrd2 and Ankrd23 (Mikhailov & Torrado, 2006). Ankrd1 is downstream of the Nkx2.5 pathway during cardiomyogenesis (Zou et al., 1997), and Ankrd1 expression is regulated by cardiac overload, hypertension, and heart failure in the adult heart (Jeyaseelan et al., 1997b; Zou et al., 1997). We previously reported that Ankrd1 overexpression induced a remarkable angiogenic response in an experimental granulation tissue model reminiscent of the action of a number of angiogenic agents (Shi et al., 2005b). In the *Ankrd1*$^{-/-}$ mouse, global deletion of *Ankrd1* resulted in increased necrosis after an ischemic insult as well as significantly reduced contraction of excisional wounds (Chapter II) (S. Samaras, K. Almodóvar, N. Wu and J. M. Davidson, submitted for publication). The latter observation in particular suggested an alteration of the wound remodeling process.

The matrix metalloproteinase (MMP) family regulates extracellular matrix remodeling in many normal processes including wound healing, and different MMPs have been shown to play major roles throughout the wound repair process (Gill & Parks, 2008; Martin, 1997; Ravanti & Kahari, 2000). Abnormal expression of MMPs may be involved in the pathogenesis of chronic ulcers (Armstrong & Jude, 2002a; Pilcher et al., 1999). Members of the MMP family can be classified into different subfamilies of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Hartenstein et al., 2006). Collagenases degrade native fibrillar collagens in the extracellular space (Hartenstein et al., 2006). MMP-13 is one of three mammalian collagenases capable of initiating the degradation of interstitial collagens during wound healing (Mariani, Sandefur, Roby, & Pierce, 1998; Wu et al., 2002). It has been reported that angiogenesis and granulation tissue development was delayed in MMP-13 knockout mice (Hattori et al., 2009b; M. Toriseva et al., 2012). MMP-13 plays a key role in keratinocyte migration, angiogenesis, and contraction in wound healing (17). Furthermore, MMP-13 has been shown to regulate multiple cellular functions including myofibroblast activity, cell motility, angiogenesis, inflammation, and proteolysis during growth and maturation of granulation tissue (M. Toriseva et al., 2012).

We hypothesized that Ankrd1 regulates the transcription of genes associated with the wound repair process. This study aimed to determine how Ankrd1 affected *MMP13* expression in the context of tissue repair. We found that Ankrd1 repressed transactivation of *MMP13* through interaction with the negative regulator, nucleolin, and that genetic deletion or suppression of *Ankrd1* resulted in overexpression of *MMP13* as well as *MMP10*. The findings reveal a novel function for Ankrd1 and an additional link

between transcriptional regulation of MMPs and wound healing. Understanding the molecular mechanisms that regulate wound matrix turnover may provide new approaches for treating chronic wounds.

Materials and Methods

Reagents. Phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) was diluted in dimethyl sulfoxide (DMSO, Fisher Scientific, Waltham, WA). Treatments with PMA (100 ng/ml) were done in serum-free Dulbecco's modified Eagle medium (DMEM) overnight. The concentration used for lipopolysacharide (LPS, Sigma, St. Louis, MO) was 100 ng/ml. Normal rabbit IgG (sc-2027), anti-c-Jun (sc-1694X), and donkey anti-rabbit IgG-HRP (sc-2313) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclophilin antibody (BML-SA296) was from Enzo Life Sciences (Farmingdale, NY), anti-nucleolin antibody (ab70493) was from Abcam (Cambridge, MA), and anti-MMP-13 antibody (AB8120) was from Millipore (Billerica, MA). Affinity-purified rabbit anti-Ankrd1 antibody was prepared by Caprologics (Hardwick, MA) against the N-terminal 104 amino acids of murine Ankrd1 as described (Shi et al., 2005b). *Ankrd1* SMARTpool™ siRNA and scrambled control siRNA were purchased from Thermo Scientific/Dharmacon (L-059054-01, Lafayette, CO). Lipofectamine 2000 (Invitrogen, Grand Island, NY) was used for transfection of plasmids or siRNA into cells. pcDNA 3.1 was obtained from Invitrogen (Grand Island, NY). Mouse monoclonal Anti-Flag M2 antibody (F3165) was purchased from Sigma (St. Louis, MO).

Cells and constructs. As reported elsewhere (S. Samaras, K. Almodóvar-García, N. Wu and J. Davidson, in revision) the *Ankrd1*$^{-/-}$ mouse was generated by mating a Sox2-Cre mouse strain with an *Ankrd1*$^{fl/fl}$ strain in which loxP sites had been inserted 600bp upstream of the transcriptional start site and in intron 2 of *Ankrd1,* contained in a 129B6 mouse-derived bacterial artificial chromosome (BAC) clone. Mouse dermal fibroblasts were isolated from *Ankrd1*$^{fl/fl}$ (FLOX) and *Ankrd1*$^{-/-}$ (KO) neonatal skin as previously described (Normand & Karasek, 1995). A portion of the isolated skin fibroblasts were immortalized with a SV40 large T-antigen plasmid (Chang et al., 1985). Both primary and immortalized cells were grown in DMEM containing 10% FBS, 100 Units/ml penicillin–streptomycin, and 2 mM L-Glutamine. Mouse vascular smooth muscle cells were isolated from FLOX and KO neonates as described (Ray et al., 2001), and half of the cells were stably transformed with SV40 large T antigen plasmid (Chang et al., 1985). Vascular aortic smooth muscle cells were grown in Medium 231 (Invitrogen, Grand Island, NY) supplemented with smooth muscle growth supplement (Invitrogen, Carlsbad, CA) and 2 mM L-Glutamine. NIH3T3 fibroblasts, and HeLa cells were grown in DMEM containing 10% FBS. Cells were maintained at 37°C in 5% $CO_{2.}$ Human microvascular endothelial cells (HMVECs) (Xu et al., 1994) were cultured in Medium 131 supplemented with microvascular cell growth supplement (Invitrogen, Carlsbad, CA) and 10 mM L-Glutamine. SV40 transformed HMVECs were maintained at 33°C in 5% $CO_{2.}$ For experiments, transformed HMVECs were grown at 37°C in 5% $CO_{2.}$, 100 Units/ml penicillin–streptomycin, and 2 mM L-Glutamine.

The construction of pGL3 p660 MMP-13 reporter construct was described previously (20). Mutant plasmids in the AP-1 site of MMP-13 promoter (-bp -46 through -41) were

prepared by using a Quick Change Site Mutagenesis Kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions (for the primer sequences, see **Table 3**). The AP-1 binding site sequence TGACTCA was changed to **G**GACTCA (5' mutant), TGACTC**T** (3' mutant), **GTC**CTCA (Mutant 1), and GA**TGAC** (Mutant 2 creating a new AP-1 site). A Flag-Ankrd1 construct was made by cloning full length mouse *Ankrd1* cDNA (NM_013468) into pCMVTag2B (Agilent Technologies, La Jolla, CA) using *BamHI* and *SalI* restriction sites. Viral infections with adenovirus constructs expressing *Ankrd1* (Ad-Ankrd1) or luciferase (Ad-Luc) used as a control were previously described (Shi et al., 2005b).

Yeast two hybrid. Yeast two-hybrid analyses were performed according to the manufacturer's instructions (Clontech, Mountain View, CA). Briefly, full-length mouse *Ankrd1* cDNA was amplified (See **Table 3** for primer sequences) and the product was cloned into *NdeI/BamHI* sites of pGBKT7 vector (Clontech). The resulting construct, pGBKT7-Ankrd1-GAL4 DNA binding vector was used as the bait plasmid in yeast two-hybrid experiments. A cDNA library was made by synthesizing first-strand cDNA from 780 ng of neonatal mouse aorta total RNA using the cDNA Library Construction kit (Clontech). The first-strand cDNA mixture was used as template to synthesize double-stranded DNA by long distance PCR, and the resulting double-stranded DNA was size fractionated using a Chroma Spin Column (Clontech, Mountain View, CA). The yeast strain AH109 was co-transformed with a mouse aorta cDNA library (Clontech, Mountain View, CA), the pGBKT7-*Ankrd1* clone, and the pGADT7-Rec vector. Cultures were grown on selective, quadruple dropout media plates: SD/-Ade/-His/-Leu/-Trp. Colonies that grew in these plates after 6 days were re-plated on

Primer sequence	Primer
Primers used to make AP-1 mutant plasmids	
5’ CCCAAAGTGGGGACTCATCACTATCAT 3’	5' mut Forward
5’ ATGATAGTGATGAGTCCCCACTTTGGG 3’	5' mut Reverse
5’ CCCAAAGTGGTGACTCTTCACTATCAT 3’	3' mut Forward
5’ ATGATAGTGAAGAGTCACCACTTTGGG 3’	3' mut Reverse
5’ CACACCCCAAAGTGGGTCCTCATCACTATCATGCTATA 3’	Mut 1 Forward
5’ TATAGCATGATAGTGATGAGGACCCACTTTGGGGTGTG 3’	Mut 1 Reverse
5’ CACACCCCAAAGTGGTGATGACTCACTATCATGCTATA 3’	Mut 2 Forward
5’ TATAGCATGATAGTGAGTCATCACCACTTTGGGGTGTG 3’	Mut 2 Reverse
Primers for qRT-PCR	
5’ GTGACTTCTACCCATTTG 3’	MMP-13 Forward
5’ GCAGCAACAATAAACAAG 3’	MMP-13 Rev
5’ CTTGTGGTCTTCTGGCACACG 3’	MMP-13 Probe
5’ AGACTCCTTCAGCCAACATGATG 3’	Ankrd1 Forward
5’ CTCTCCATCTCTGAAATCCTCAGG 3’	Ankrd1 Reverse
5’ CCCCTGCCTCCCCATTGCCATTCT 3’	Ankrd1 Probe
5’ CGTAGACAAAATGGTGAAG 3’	GAPDH Forward
5’ CCATGTAGTTGAGGTCAA 3’	GAPDH Reverse
5’ TTGATGGCAACAATCTCCACTT 3’	GAPDH Probe
5’ CACTCTTCCTTCAGACTTA 3’	MMP-10 Forward
5’ GCTGCATCAATCTTCTTC 3’	MMP-10 Rev

Table 3: Primer sequences (see methods)

fresh SD/-Ade/-His/-Leu/-Trp/X-α-Gal plates. The more intense blue colonies (10^4), indicating lacZ expression due to the activation of a third reporter gene, MEL1 were isolated and analyzed for cDNA content by PCR. Those colonies exhibiting >1 PCR product were re-plated 2-3 times to yield colonies representing individual cDNA sequences. PCR amplification and transformation of E. coli rescued the cDNAs in order to identify those sequences responsible for a positive, two-hybrid interaction. Plasmid DNA was isolated, sequenced, identified by searches with BLAST and compared to the GenBank database.

Co-immunoprecipitation. For co-immunoprecipitation, skin fibroblasts were grown in 10 cm dishes and transfected for 24 h with 20 µg of Flag-Ankrd1 plasmid DNA or pcDNA 3.1 as a control using Lipofectamine2000 (Invitrogen, Grand Island, NY). After transfection, cells were washed with PBS and solubilized in RIPA buffer. 5% of the extract was used as input. After centrifugation for 5 min at 13,000 × *g*, the supernatant was incubated with protein A/G-Sepharose beads (sc-2003, Santa Cruz, CA) for 1h followed by another centrifugation for 30sec at 13,000 × *g* to remove nonspecifically bound protein. The supernatant was then incubated overnight at 4°C with antibodies directed against nucleolin or Ankrd1 or with nonimmune IgG. Protein A/G-Sepharose beads were added to the cell lysates, followed by incubation for 1 h at 4°C, and the beads were washed five times with ice-cold RIPA buffer. The beads were centrifuged for 1 min at 11,000 _ *g*, and the supernatant was collected and used for Western blot analysis with the indicated antibodies.

Immunofluorescence. Cells were grown on glass coverslips, rinsed with PBS, and fixed for 15min with 4% paraformaldehyde. Cells were then washed three times with

PBS and permeabilized in 0.2% Triton X-100 for 5 min and blocked for 1h in blocking buffer (10% normal goat serum in PBS). Primary antibodies were diluted in blocking buffer, and incubation was performed overnight at 4°C. The following primary antibodies were used: (1) anti- Ankrd1 (1:1000), and (2) anti-nucleolin (1:100). Secondary antibodies were diluted in blocking buffer, and incubation was performed at RT for 2 hours. The following secondary antibodies were used: Alexa 488-labeled goat anti-rabbit IgG (1:400) and Alexa-546 goat anti-mouse IgG (1:200). Images were acquired at the Vanderbilt Cell Imaging Shared Resource using a confocal microscope (LSM 510; Carl Zeiss).

Luciferase reporter assay. HeLa cells were co-transfected for 24 hours with the combination of three plasmids: (1) 0.5 µg of pGL3 p660 MMP-13-luciferase reporter construct or a 5' mut p660 MMP-13-luciferase plasmid, (2) 0.4 µg of Flag-Ankrd1 plasmid or pcDNA 3.1 (Invitrogen, Grand Island, NY) as a control, and (3) 0.05 µg of pTK-RL, a *Renilla* luciferase vector, purchased from Promega (Madison, WI). 24h after the triple transfection, cells were treated overnight with PMA (100 ng/ml in DMSO) or DMSO (vehicle), followed by measurement of luciferase activity using the Dual-Luciferase Reporter Assay system kit (Promega, Madison, WI), according to manufacturer's instructions. Renilla luciferase expression was used as an internal control to normalize DNA transfection efficiency.

Mouse excisional wounds. Studies were carried out in the AAALAC approved facilities of Vanderbilt University, Nashville, Tennessee, under approval of the Institutional Animal Care and Use Committee at Vanderbilt University School of Medicine. 15-20 week old FLOX and KO male mice (n=8) in a ~80% C57bl/6J background, received two

6mm, full thickness excisional wounds on the back of each animal. To reduce wound contraction and maximize granulation tissue formation, wounds were stented (Davidson JM, 2013; Yu F, 2010). At 2 and 4 days, mice were euthanized, and the complete wounds, including 2 mm of the wound margin, were harvested. Tissue was processed for RNA and protein isolation.

RNA Isolation and Real Time PCR. For preparation of cellular RNA, cells were scraped in PBS and collected by centrifugation. RNA was isolated using the Illustra RNAspin Mini Isolation Kit (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions. For isolation of RNA from mouse skin, the freshly isolated skin was incubated with RNAlater solution (Invitrogen, Grand Island, NY) according to manufacturer's instructions and then frozen at -80°C until ready to continue with the RNA isolation. Mouse skin was homogenized in RA1 (Illustra kit, GE Healthcare, Piscataway, NJ) with β-mercaptoethanol using a Tissuelyser II (Qiagen, Newton, PA) and total RNA was isolated following manufacturer's directions (Illustra kit, GE Healthcare, Piscataway, NJ). RNA concentration was determined spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE), and samples were stored at −80°C. Taqman quantitative real time PCR (qRT-PCR) for *Ankrd1*, *MMP13*, *MMP10*, *Gapdh*, and cyclophilin B (Applied Biosystems, Foster City, CA) was performed on 80 ng of reverse transcribed RNA. Sequences for primer and probes are provided in **Table 3**.

Western blot analysis. Whole cells or skin tissue biopsies were lysed with RIPA buffer (Sigma, St Louis, MO). Forty µg of cell extract was separated by 10% acrylamide SDS-PAGE and transferred to PVDF membrane (Immobilon, Millipore, Billerica, MA) using

the NuPage (Life Technologies, Carlsbad, CA) blotting apparatus, following the manufacturer's protocol. After blocking with a solution of 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Tween-20 containing 5% milk powder, the membrane was incubated with anti-MMP-13 antibody (1:2000) at 4°C overnight, followed by incubation with anti-goat IgG (sc2056, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature (RT) for 30 min. The membranes were rinsed, incubated with Western Lightning Plus Enhanced Chemiluminescent Reagent (Perkin Elmer, Waltham, MA), and protein bands were visualized and quantified using a Kodak Image Station 4000MM Pro with Kodak MI software (Carestream Healthcare, Woodbridge, CT). After measurement of the chemiluminescent reaction, the PVDF membrane was stripped with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL). Subsequently, the membrane was incubated with anti-cyclophilin antibody (1:80,000) for 1h at RT followed by anti-rabbit IgG (1:10,000) for 1h and imaged as described above.

Adenovirus infection. FLOX and KO immortalized skin fibroblasts were infected with an adenovirus expressing *Ankrd1* (Ad-Ankrd1) or luciferase (Ad-Luc) as a control with a multiplicity of infection of 250. Cells were harvested 24h after infection, and total RNA was isolated as described above. qRT-PCR was performed using *MMP13*, *Ankrd1*, and *GAPDH* probe/primers.

siRNA Knockdown of Ankrd1. Immortalized FLOX skin fibroblasts were transfected using Lipofectamine 2000 with 25 nM, 50 nM, or 100 nM of smartPOOL siRNA against *Ankrd1* (Dharmacon, Lafayette, CO). Scrambled siRNA (100 nM) was transfected into cells as a control. Cells were harvested 48h after siRNA treatment, followed by isolation

of total RNA as described above. qRT-PCR was performed using probe/primers for *Ankrd1* and *MMP13*, as well as *GAPDH* for normalization.

Gel shift assay. A 60bp biotinylated probe containing a WT or mutated AP-1 binding site (5'mut, 3' mut, Mut 1) was made by PCR with the following biotinylated or non-biotinylated primers: forward: 5'-ACTAGGAAGTTAACACACACCCCAA and reverse : 5'-AGCATCTTCTATTTTATAGCATGAT. The annealing temperature used was 52°C. The PCR templates were the pGL3 P660 MMP-13 WT, 5' mut, 3' mut, and Mut 1 plasmids. PCR amplified biotinylated and non-biotinylated probes were run in a 12% polyacrylamide gel. The 60 bp DNA band was extracted, and DNA purified using the Qiaquick gel extraction kit (Qiagen, Germantown, MD) following manufacturer's instructions.

Nuclear extracts were prepared from FLOX and KO immortalized skin fibroblasts treated with PMA in DMSO (100ng/ml), DMSO, or LPS (100 ng/ml) in serum free medium for 24h using a NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL). Protein concentration was calculated by BCA protein assay (Pierce, Thermo Scientific Rockford, IL). Gel shift analyses were performed using the LightShift Chemiluminiscent EMSA Kit (Pierce) following manufacturer's instructions. Briefly, a 10% polyacrylamide gel was run in 0.5X TBE for 60min (100V). Binding reactions were prepared while the gel was running. Binding reactions contained the following reagents: 1X Binding Buffer, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/ml poly(dI-dC), 0.05 % NP-40, 5 µg of protein extract, and 20 fmol of biotinylated probe. The reaction was made up to 20 µl with water. For competitive EMSA reactions, 4 pmol of non-biotinylated probe was added to the binding reaction before adding the protein extract and biotinylated probes.

The nuclear protein extracts were added to the binding reaction prior to addition of biotinylated probes. Binding reactions were incubated for 20 minutes at RT. After incubation, 5 µl of 5X loading buffer was added to each sample and samples were loaded onto the pre-run polyacrylamide gel, and run for 3h at 100 V. Electrophoretic transfer of binding reactions to nylon membrane (Biodyne B Precut Nylon membrane, Thermo Scientific, Rockford, IL) was performed for 1h at 380 mA using Powerpac Basic Power Supply (Biorad, Hercules, CA). The cross-linking of transferred DNA to membrane was accomplished by incubating the membrane face down on a UV transilluminator (312nm) for 15 min. Detection of biotin-labeled DNA by chemiluminescence was performed exactly as per manufacturer's specifications. For supershift analysis, binding reactions were incubated with 5 µg of antibody without addition of biotinylated probes for 25 min at RT, followed by addition of biotinylated probe and further incubation for 25 min at RT.

Chromatin Immunoprecipitation. FLOX and KO immortalized skin fibroblasts were treated with PMA (100 ng/ml in DMSO) or DMSO vehicle in serum-free DMEM for 24 h. Cells were then washed with PBS, and fixed with 1% formaldehyde for 5 min. The crosslinking reaction was stopped by adding 2.5M glycine. Cells were washed with cold PBS and transferred to 1.5ml Eppendorf tubes on ice and centrifuged for 5 min at 5000 *g*. The supernatant was aspirated, and the cell pellet was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.0) and incubated on ice for 10 min. Resuspended cells were transferred to Eppendorf tubes containing 250 mg of glass beads (<106µM G-4649; Sigma, St Louis, MO). The samples were carefully sonicated 10 times for 30 sec at 4°C using a Branson Sonifier S-250A analog ultrasonic

Name	
Xin actin-binding repeat containing 2	Xirp2
Desmin	Des
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh
Keratin 4	Krt4
Eukaryotic translation initiation factor 3	Eif3d
Ribosomal protein S20	Rps20
Tenascin XB	Tnxb
Ferritin heavy chain 1	Fth1
SLAIN motif family, member 2	Slain2
Troponin T3	Tnnt3
Nucleolin	Ncl
Mannose phosphate isomerase	Mpi
Titin, transcript variant N2A	Ttn
Breast cancer metastasis suppressor	Brms1
Thrombomodulin	Thbd
Actinin alpha 1	Actn1
SET domain containing 6	Setd6
Late cornified envelope 3F	Lce3f
Angiotensin II type I receptor-associated protein	Agtr1
Ewing sarcoma breakpoint region 1	Ewsr1
Ribosomal protein L13a	Rp13a
Caveolin 3	Cav3
Eukaryotic translation initiation factor 5	Eif5
Myoglobin	Mb
Carboxypeptidase D	Cpd
Myogenic factor 6	Mrf4

Table 4: **Summary of yeast two-hybrid results**.
Potential Ankrd1 interacting factors are shown in table 4. Underlined factors have been previously characterized as Ankrd1 binding partners.

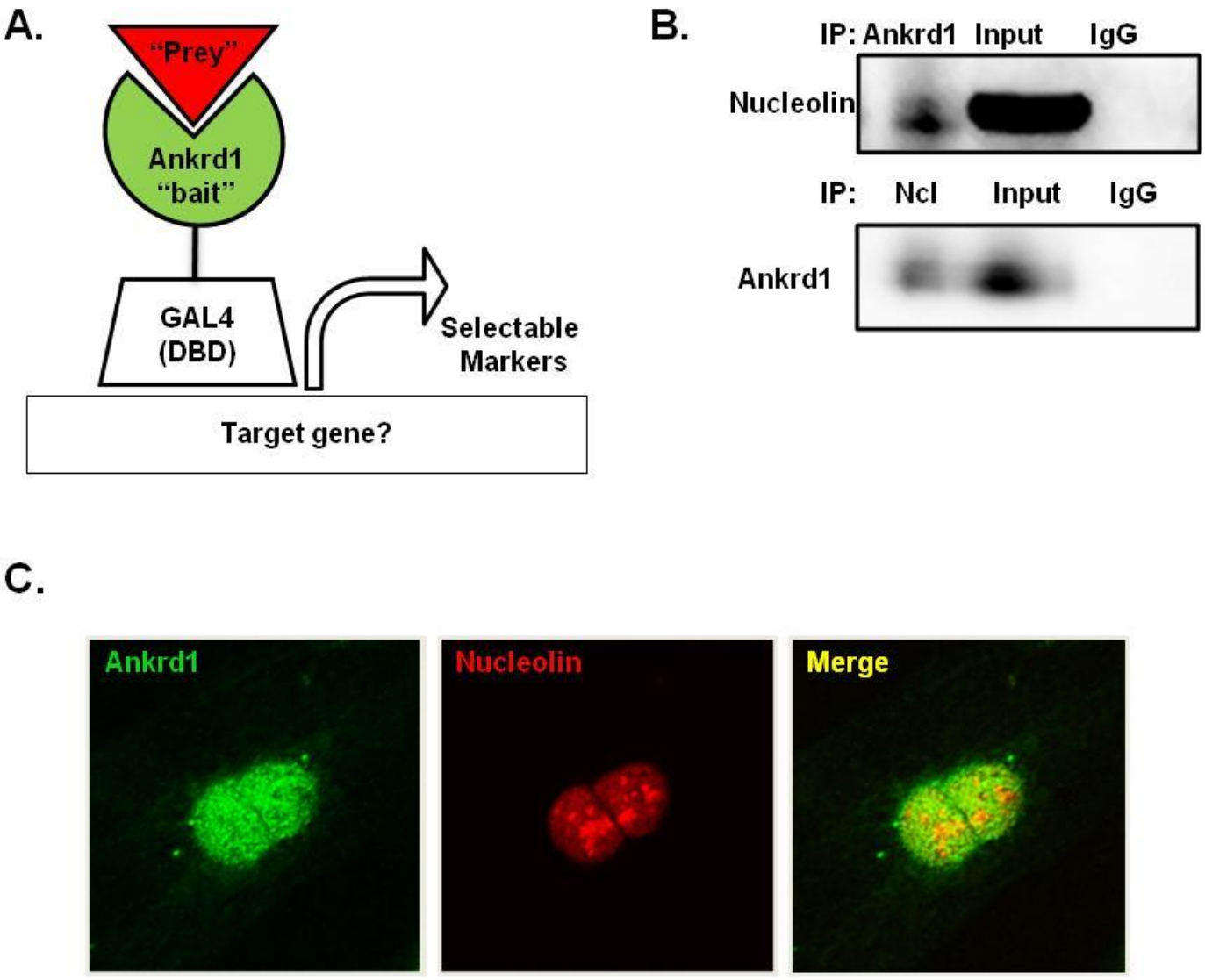


Figure 14: Ankrd1 interacts with the transcription factor, nucleolin.
A. Representation of yeast two hybrid analysis. Ankrd1 served as "bait" that only activates the transcription of the target gene when engaged by an unknown "prey" protein of sufficiently high affinity. **B**. Coimmunoprecipitation of Ankrd1-nucleolin complexes from primary FLOX (normal) mouse skin fibroblasts. Aliquots of the immunoprecipitation input were analyzed by Western blotting using the indicated antibodies. For the top panel, immunoprecipitation (IP) used antibody against Ankrd1, followed by Western analysis with antinucleolin. For the bottom panel, immunoprecipitation with antinucleolin (Ncl), followed by Western analysis with anti-Ankrd1. IgG, isotype-matched nonimmune rabbit IgG. **C**. Nuclear colocalization of Ankrd1 and nucleolin. HMVECs were immunostained for endogenous Ankrd1 (green) and nucleolin (red) and localized by confocal microscopy. The merged panel shows partial colocalization (yellow) of Ankrd1 and nucleolin, predominantly in the two adjacent nuclei.

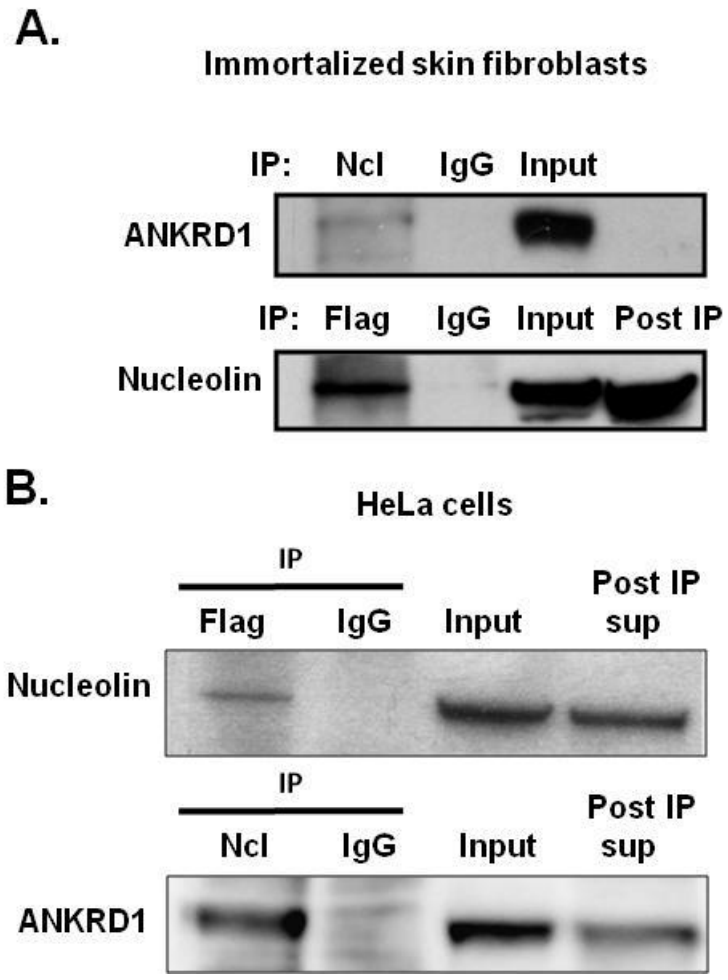


Figure 15: Co-immunoprecipitation of Ankrd1-nucleolin complexes.
A. Co-immunoprecipitation of Ankrd1-nucleolin complexes from immortalized FLOX (normal) skin fibroblasts expressing FLAG-tagged *Ankrd1*. Aliquots of the immunoprecipitation input and eluate (Post IP) were analyzed by Western blot using the indicated antibodies. *Upper panel*: immunoprecipitation (IP) reacted antibody against nucleolin (Ncl) or isotype-matched IgG to input material, followed by Western blot analysis with anti-Ankrd1. *Lower panel*: immunoprecipitation with anti-FLAG or IgG was similarly followed by Western blot analysis with anti-nucleolin. Post IP represents FLAG-Ankrd1 that was not removed by immunoprecipitation. **B**. Confirmation of protein interaction using ectopically expressed Ankrd1 in Hela cells. *Top panel*: Immunoprecipitation (IP) with anti-FLAG followed by Western blot analysis with anti-nucleolin. Nucleolin co-precipitated with transfected FLAG-Ankrd1. Nucleolin was abundant in the total cell extract (Input) as well as the supernatant after immunoprecipitation (Post-IP sup). Normal rabbit IgG immunoprecipitate (IgG) was used as a negative control. *Bottom panel*: Immunoprecipitation with anti-nucleolin was followed by Western blot analysis with anti-Ankrd1. Ankrd1 co-precipitated with nucleolin (Ncl).

cell disruptor (Fischer Scientific, Waltham, MA). Between each sonication, the samples were frozen on dry ice for 30 sec, and then returned to wet ice. After sonication, the samples were centrifuged for 10 min at 4°C at 5,000 x *g*. Cold dilution buffer (0.01 % SDS, 1.1 % Triton X100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.0, and 167 mM NaCl) containing 0.01% complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany), and protein A/G agarose (Santa Cruz Biotech, Santa Cruz, CA), was added to sonicated samples. Samples were incubated at 4°C for one hour, centrifuged at 10,000 *x g* for 30 seconds at 4°C, and the supernatant was transferred to new tubes. The following antibodies were added to the samples: normal rabbit IgG (control; Santa Cruz Biotech, Santa Cruz, CA), rabbit polyclonal c-Jun (Santa Cruz Biotech, CA), and rabbit anti-Ankrd1. Samples were incubated with or without antibody overnight at 4°C, protein A/G agarose (Santa Cruz Biotech, Santa Cruz, CA) was added, and the samples were incubated for 3h. After incubation, the samples were centrifuged for 1 min and the supernatant was carefully removed. The beads were washed for 5 min at 4°C: twice with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), twice with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), then twice with TE buffer. Elution buffer (0.2 % SDS, and 0.1M $NaHCO_3$) and 20ul NaCl (5M) were added to the washed beads. NaCl was added also to the no antibody supernatants, and all samples were then incubated overnight at 65°C. DNA identification was confirmed with qRT-PCR using primers specific for the AP-1 site. An 80bp fragment containing the AP-1 site within the MMP-13 region was amplified using the following primers: Forward – 5’ GCCTCACTAGGAAGTTAA and Reverse: 5’GCAAGCATCTTCTATTTTATAG. PCR

reactions contained: SYBR green Supermix (Biorad, Hercules, CA), 50 nM of each primer, 2 µl template DNA, in a total volume of 20 µl. The iCycler real time- thermocycler (Biorad, Hercules, CA) was used.

Mouse stress response array. A mouse stress response qRT-PCR array (96 StellArray, Lonza, Walkersville, MD) was utilized according to manufacturer's procedures. Total RNA (1.0 µg) from FLOX and KO immortalized skin fibroblasts was reverse transcribed using iScript cDNA synthesis kit (Biorad, Hercules, CA) following the manufacturer's protocol. qRT-PCR was performed using iQ SYBR Green Supermix purchased from Biorad (Hercules, CA). Potential Ankrd1 target genes were validated by qRT-PCR in separate RNA extracts using probe-primers specific for the identified gene of interest.

Results

Ankrd1 interacts with transcription factor, nucleolin

Identification of Ankrd1 interacting factors and their targets may help to understand how Ankrd1 affects tissue repair. Yeast two-hybrid analysis (**Fig. 14A**) with mouse aorta cDNA identified Ankrd1association with titin and desmin, which are both previously characterized Ankrd1 binding partners (**Table 4**) (Bang et al., 2001b; Miller et al., 2003), among a collection of candidate cytoplasmic proteins. Our analysis also provided evidence of Ankrd1 association with the transcription factors, nucleolin and breast cancer metastasis-suppressor 1 (*Brms1*). The association of Ankrd1 and nucleolin was confirmed by co-immunoprecipitating endogenous proteins from primary

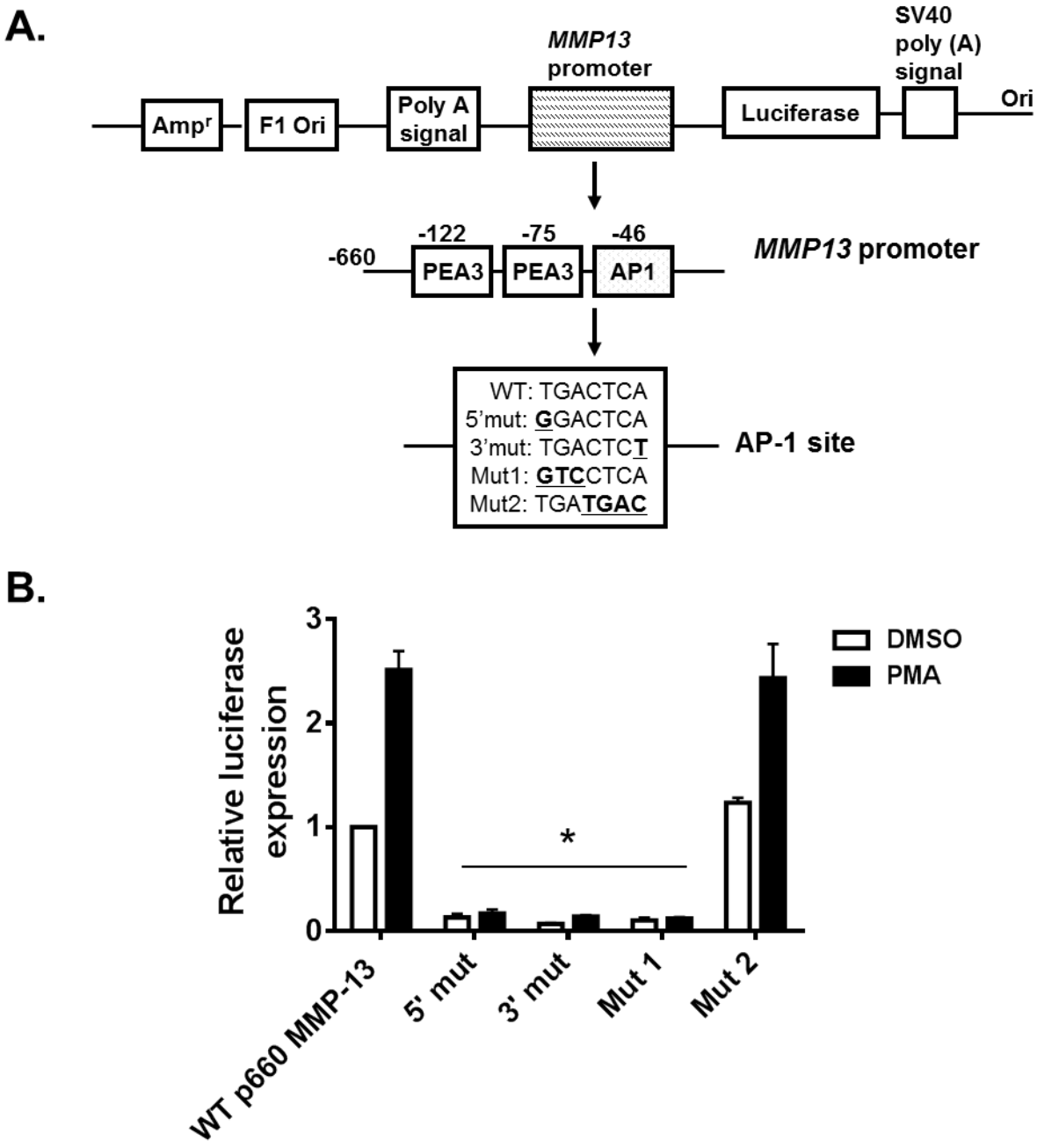
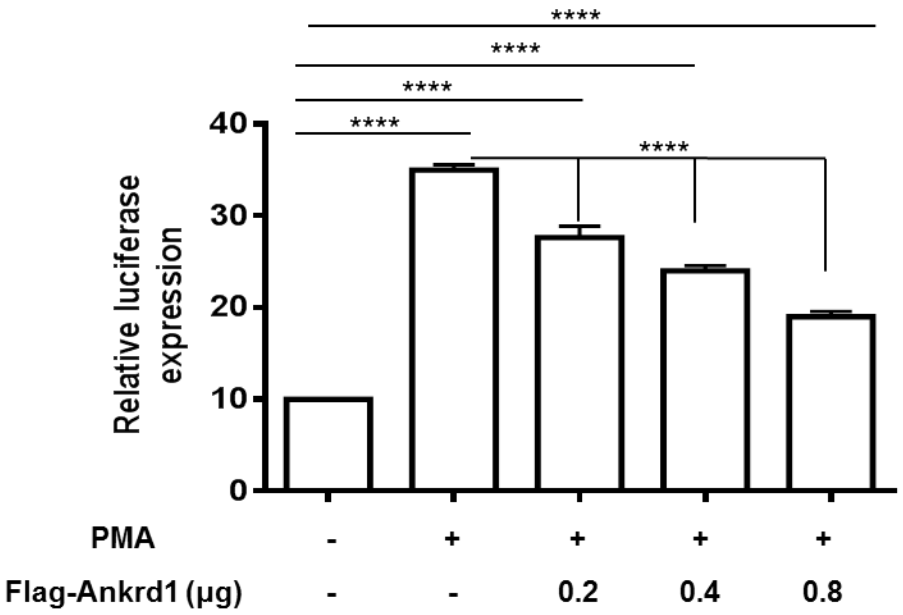

A.
MMP13 promoter
SV40 poly (A) signal
Ampr
F1 Ori
Poly A signal
Luciferase
Ori
-660
-122
-75
-46
PEA3
PEA3
AP1
MMP13 promoter
WT: TGACTCA
5'mut: GGACTCA
3'mut: TGACTCT
Mut1: GTCCTCA
Mut2: TGATGAC
AP-1 site
B.
Relative luciferase expression
DMSO
PMA
*
WT p660 MMP-13
5' mut
3' mut
Mut 1
Mut 2

Figure 16: Mutations in the AP-1 site abrogate MMP13 promoter activity.
A. Schematic of the proximal *MMP13* promoter/reporter constructs (660 bp) containing either a native (WT) or a mutated (bold, underlined) AP-1 binding site (23). **B**. Luciferase plasmids with WT *MMP13* promoter sequence (660 bp) or four different mutations within the *MMP13* AP-1 site were transfected into NIH 3T3 cells. The luciferase activity was measured in cells grown in DMSO alone or containing the AP-1 activator, PMA. Three mutants (5′, 3′, and Mut1) did not show luciferase activity. The Mut2 sequence created an additional AP-1 site that fully restored promoter activity. The data represent results of four transfection experiments. Renilla luciferase-expressing vector pTK-RL (Promega) was used as an internal control to normalize DNA transfection efficiency. Error bars indicate the standard errors of the mean (SEM; $n = 4$). 5′, 3′, and Mut1 mutant plasmids were statistically significant (*, $P < 0.001$), as determined by two-way analysis of variance (ANOVA) with Tukey's multiple-comparison test) compared to WT *MMP13* plasmid with the respective controls.

A.


****
****
****
****
****
40
30
20
10
0
Relative luciferase expression
PMA - + + + +
Flag-Ankrd1 (μg) - - 0.2 0.4 0.8

B.

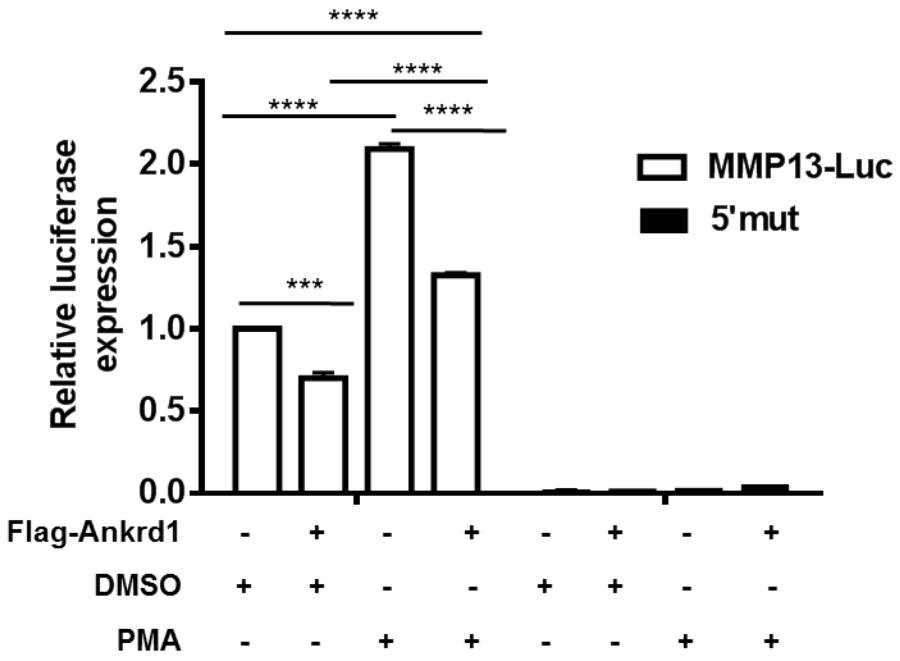

****
****
****
****
***
2.5
2.0
1.5
1.0
0.5
0.0
Relative luciferase expression
MMP13-Luc
5'mut
Flag-Ankrd1 - + - + - + - +
DMSO + + - - + + - -
PMA - - + + - - + +

Figure 17: Ankrd1 overexpression decreases basal and PMA-induced *MMP13* promoter activity.

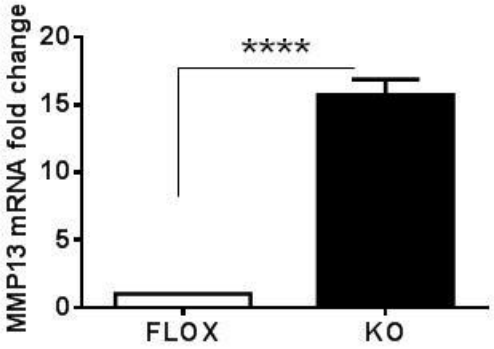
A. Ankrd1 decreases PMA-induced *MMP13* promoter activity in a dose-dependent manner. NIH 3T3 cells were cotransfected with the WT-p660 *MMP13* promoter/reporter construct and Flag-Ankrd1 plasmid or pcDNA3.1 (control) and treated with PMA or DMSO vehicle for 24 h, followed by measurement of the luciferase activity. Significance was tested by one-way ANOVA with Dunnett's multiple-comparison test (****, $P < 0.0001$). **B**. *MMP13* promoter (luciferase) activity in HeLa cells. Ankrd1 (Flag-Ankrd1) overexpression decreased *MMP13* promoter activity even in the presence of PMA. A T→G mutation at the 5′ end of the *MMP13* AP-1 site abolished luciferase expression under all experimental conditions. The data represent results of three transfection experiments performed in triplicate. Error bars indicate the SEM ($n = 9$), as determined by one-way ANOVA (***, $P < 0.001$; ****, $P < 0.0001$). Renilla luciferase-expressing plasmid pTK-RL was used as an internal control to normalize DNA transfection efficiency in both panels.

skin fibroblasts with both anti- Ankrd1 and anti-nucleolin antibodies and detecting the binding by Western blot (**Figure 14B**). Immunoblotting analyses were also performed in HeLa cells and immortalized skin fibroblasts overexpressing Flag-*Ankrd1* with anti-Flag and anti-nucleolin antibodies (**Figure 15**). In addition, double immunofluorescence showed a predominantly nuclear co-localization of Ankrd1 and nucleolin in human microvascular endothelial cells (**Figure 14C**). Nucleolin binds to an AP-1 DNA sequence in the MMP-13 promoter and represses AP-1-dependent transactivation (Samuel, Twizere, Beifuss, & Bernstein, 2008). Our findings suggested that Ankrd1 could play a role in the ability of nucleolin to affect transcription of MMP-13 from an AP-1 site.

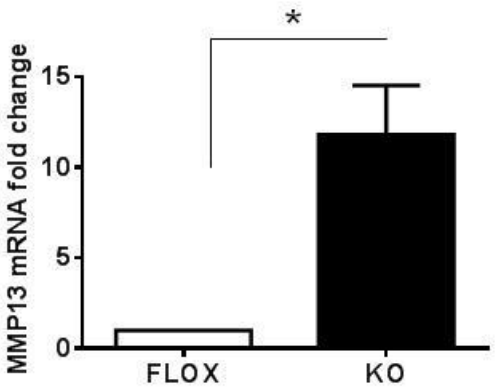
Ankrd1 decreases PMA-induced MMP-13 promoter activity via AP-1

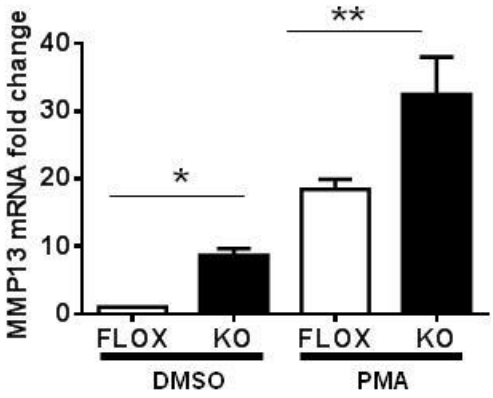
Based on our identification of nucleolin as an Ankrd1 interacting factor and evidence that it acts as a transcriptional regulator of *MMP13* (Samuel et al., 2008), we examined the effect of Ankrd1 on regulation of *MMP13* promoter activity. We previously reported that murine *MMP13* core promoter activity resides within the proximal 660 bp, a region that includes two PEA3 (−75, −122) and one AP1 (−46) consensus sequences (Wu et al., 2002) (**Figure 16A**). Therefore, we used a reporter construct containing the *MMP13* core promoter cloned upstream of the luciferase gene (p660-MMP13 Luc) as previously described (Wu et al., 2002) to study the role of Ankrd1 in *MMP13* regulation. To confirm the importance of an intact AP-1 site in the *MMP13* promoter, we developed four different mutant constructs that had mutations of the AP-1 site within the MMP-13 proximal promoter region of the p660-MMP13 Luc construct (**Figure 16A**). NIH3T3

A.


MMP13 mRNA fold change
20
15
10
5
0
****
FLOX
KO

B.


MMP13 mRNA fold change
15
10
5
0
*
FLOX
KO


MMP13 mRNA fold change
40
30
20
10
0
*
**
FLOX
KO
FLOX
KO
DMSO
PMA

C.

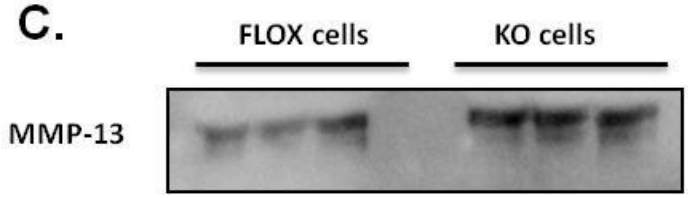

FLOX cells
KO cells
MMP-13

Figure 18: Deletion of *Ankrd1* relieves *MMP13* transcriptional repression *in vitro*. **A**. *MMP13* transcript levels were increased in primary KO skin fibroblasts. qRT-PCR analysis of *MMP13* mRNA in FLOX and KO primary skin fibroblasts was performed as described in Materials and Methods. Error bars indicate the SEM (n = 6; ****, P < 0.0001 [Student t test]). The fold change is the level of expression of *MMP13* in KO cells relative to FLOX cells. **B**. Basal and PMA-stimulated *MMP13* transcript levels are increased in immortalized KO cells. qRT-PCR analysis of *MMP13* mRNA in immortalized FLOX and KO skin fibroblasts without stimulation (left panel) and after exposure to DMSO or PMA in DMSO (right panel) for 24 h. The fold change represents the level of cellular *MMP13* expression in KO relative to FLOX (left) or FLOX-DMSO (right, control treatment). Error bars indicate the SEM (n = 12; *, P < 0.05; **, P < 0.01 [Student t test], left panel), and two-way ANOVA with the Bonferroni multiple-comparison test (right panel) were used to determine statistical significance. **C**. Evidence for elevated MMP-13 secretion in KO cells. MMP-13 protein in conditioned medium in FLOX and KO cells was detected with anti-MMP-13 antibody by Western blotting, following concentration of media by trichloroacetic acid precipitation. In the absence of a well-accepted protein loading control for secreted proteins, we used the cell number and the total protein for normalizing the total amount of conditioned medium for each sample.

fibroblasts were transiently transfected with the native and mutant constructs. All mutations within AP-1 binding site abolished *MMP13* promoter activity except for Mut2, which showed luciferase activity similar to the native sequence (**Figure 16B**); however, the three-base pair mutation in the Mut2 variant created a new AP-1 binding site. PMA was used to stimulate *MMP13* promoter activity, since it is a known effector of MMP expression acting through AP-1 (Jormsjo et al., 2000; Mittelstadt & Patel, 2012; Wu et al., 2002). NIH3T3 cells were transiently co-transfected with the p660-MMP13 Luc reporter construct and a construct expressing Flag-Ankrd1 and grown in the absence or presence of PMA. Overexpression of Ankrd1 decreased PMA-induced *MMP13* promoter activity in NIH3T3 cells in a dose-dependent manner (**Figure 17A**). Similarly, HeLa cells that were transiently co-transfected with the p660-MMP13 Luc reporter construct and Flag-Ankrd1 showed reduced *MMP13* luciferase activity (**Figure 17B)** Together, these results indicate that overexpression of Ankrd1 inhibits AP-1 dependent transactivation of the *MMP13* promoter.

Deletion of Ankrd1 relieves MMP13 transcriptional repression

To evaluate Ankrd1 involvement in the regulation of *MMP13* transcription, we analyzed *MMP13* mRNA levels in mouse dermal fibroblasts derived from FLOX and KO mice. Under basal conditions, both primary and immortalized KO skin fibroblasts showed significantly increased *MMP13* transcript levels compared to FLOX cells (**Figure 18A and 18B**). In the presence of PMA (100 ng/ml), *MMP13* mRNA expression was further enhanced ~3-fold by deletion of the *Ankrd1* gene (**Figure 18B**). Analysis of conditioned media derived from FLOX and KO skin fibroblast cultures showed

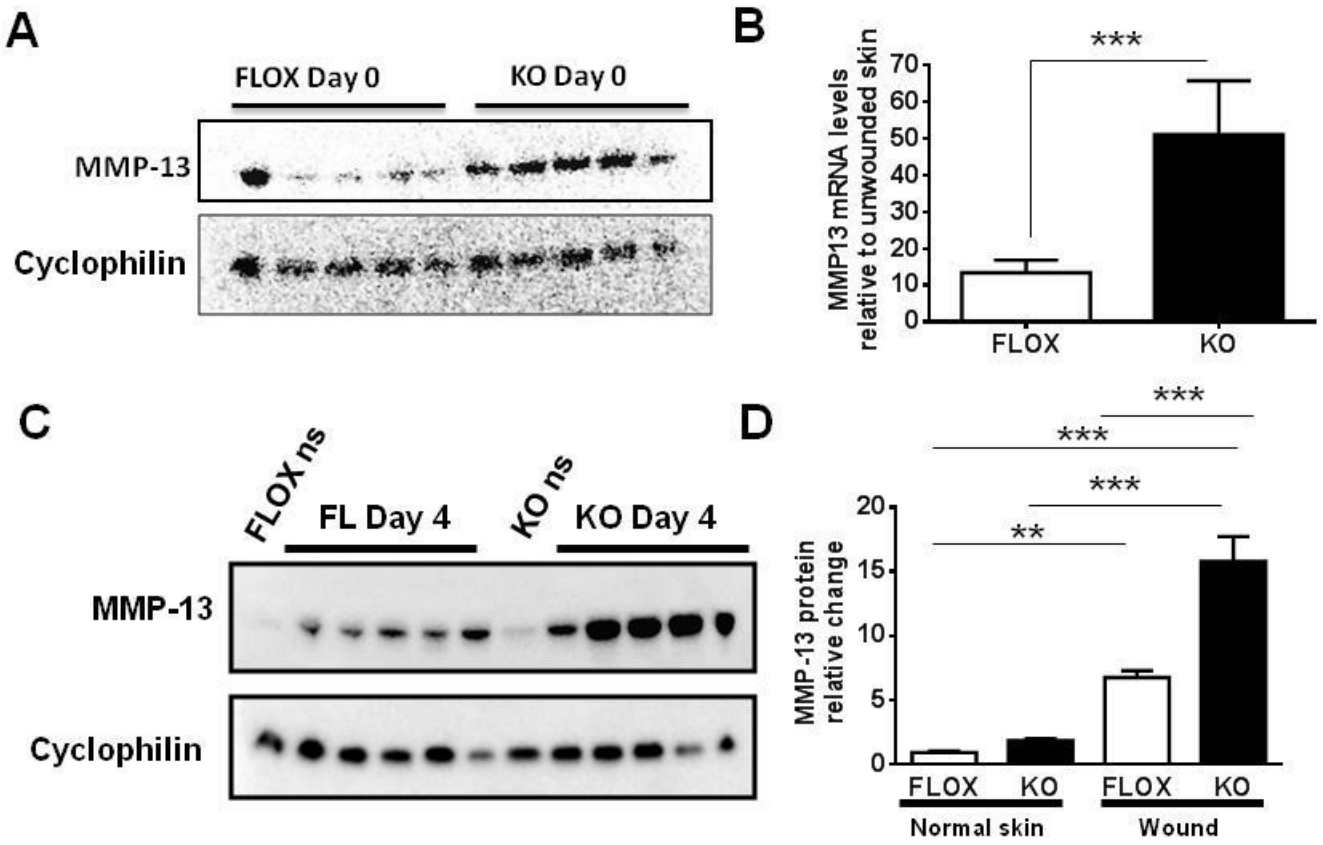


Figure 19: Deletion of *Ankrd1* elevates MMP-13 transcripts and protein in intact and wounded mouse skin.

A. MMP-13 protein levels in unwounded skin are elevated in KO mice. A total of 30 µg of skin protein extract from FLOX and KO mice was analyzed by Western blotting with anti MMP-13 and anti-cyclophilin A. **B**. Day 4 wounds in KO mice express elevated *MMP13* mRNA levels. mRNA levels in 4 day excisional wounds of FLOX and KO mice were compared to those in intact skin by qRT-PCR as described in Materials and Methods. Error bars indicate the SEM (n = 6). **C**. MMP-13 protein is elevated in *Ankrd1*-null mouse wounds. Immunoblot analysis of MMP-13 abundance in normal skin (ns) and day 4 excisional wounds from FLOX and KO mice showed increased MMP-13 protein. Cyclophilin A levels are shown in the lower panel. **D**. Digital quantification of chemiluminescence. Cyclophilin band intensities were used to normalize data. Error bars indicate the SEM (n = 5). One-way ANOVA with the Bonferroni correction and a Student t test were used to determine the statistical significance (**, $P < 0.01$; ***, $P < 0.001$).

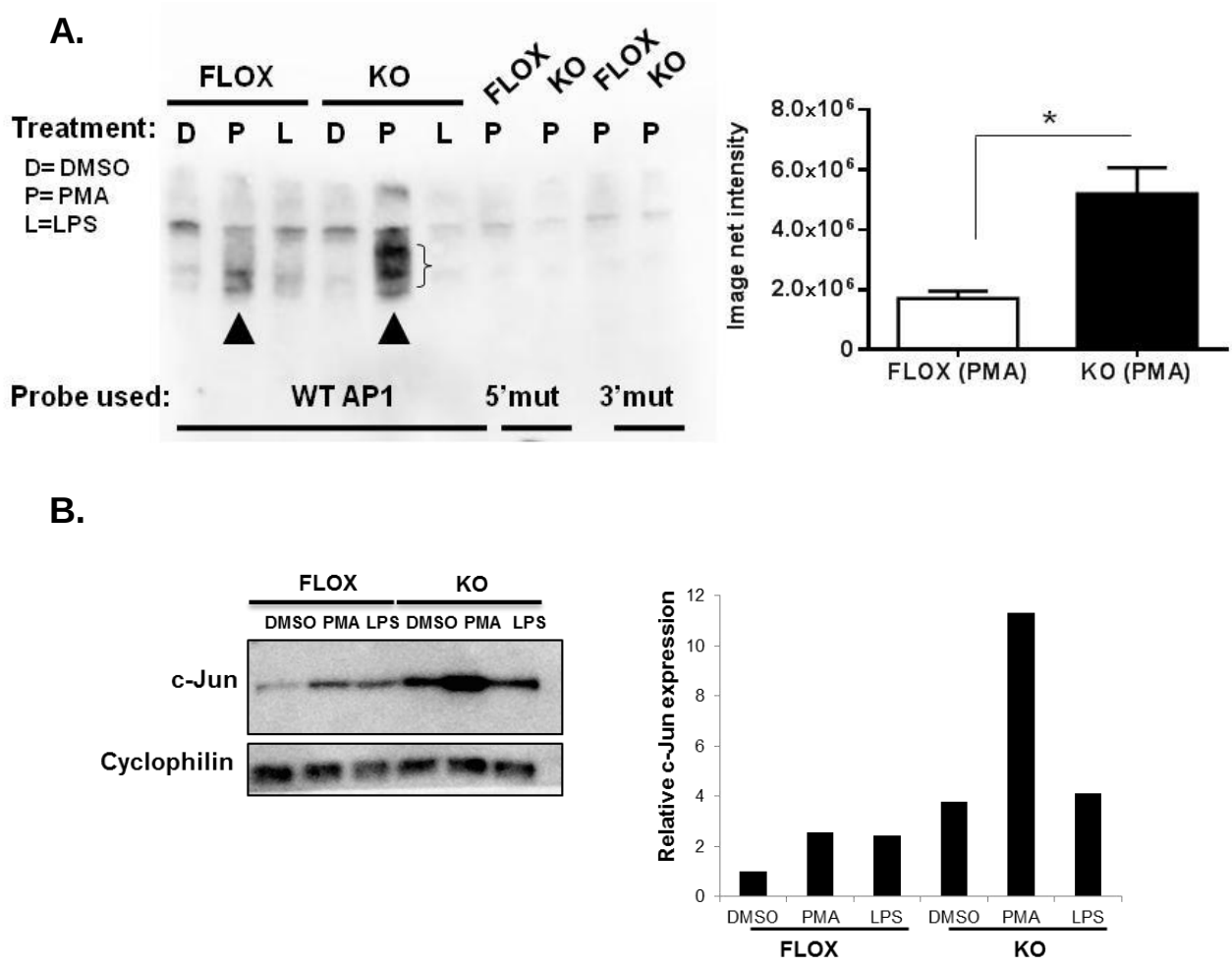


Figure 20: Mutations in the *MMP13* AP-1 site abolish the enhanced binding of transcription factors to the *MMP13* promoter caused by *Ankrd1* deletion.
A. Left panel: EMSA was performed with a biotinylated oligonucleotide containing either a WT or a mutated (5′mut or 3′mut) AP-1 site, using nuclear extracts from FLOX and KO skin fibroblasts that had been treated with either DMSO (lanes D), PMA in DMSO (lanes P), or LPS (lanes L). EMSA of nuclear extracts from FLOX and KO skin fibroblasts showed increased AP-1-binding activity after PMA treatment, and binding was elevated by *Ankrd1* deletion (compare the lanes marked by triangles). PMA stimulated the binding of additional transcription complexes to the WT MMP-13 AP-1 probe (WT AP1) in KO cells. The *MMP13* AP-1 probes that contained 5′ or 3′ point mutations showed no binding in PMA-stimulated cells. The results of a representative experiment of three are shown. Right panel: shows the digital quantification of the regions marked by the triangles and bracket in the left panel. Error bars indicate the SEM (n = 3). A Student t test was used to determine statistical significance (*, $P < 0.05$). B. Left panel: Levels of c-Jun protein were studied in nuclear extracts from FLOX and KO skin fibroblasts that were treated with DMSO, PMA, or LPS by Western Blot. Right panel: Quantification of the chemiluminescent signals.

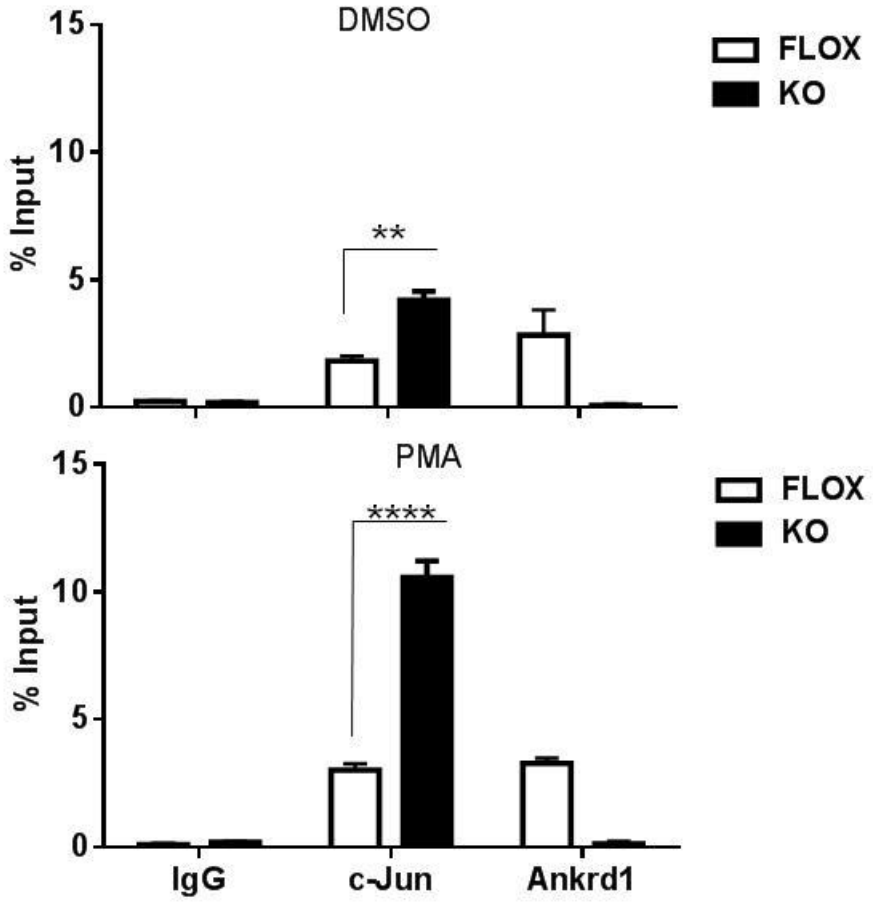


Figure 21: Deletion of *Ankrd1* increases binding of c-Jun to the *MMP13* AP-1 site.
The effect of *Ankrd1* deletion and PMA stimulation on the binding of c-Jun and Ankrd1 to the AP-1 site of the *MMP13* promoter was assessed by ChIP analysis. Using the antisera indicated n on the *x* axis, immunoprecipitated chromatin from FLOX and KO cells was analyzed by qRT-PCR. Primers were designed to amplify the AP-1 site within the *MMP13* promoter region. Values from immunoprecipitated chromatin were divided by signals obtained from chromatin input (1%) and are expressed as the percent input. Deletion of *Ankrd1* stimulated c-Jun binding to the *MMP13* AP-1 site (upper panel), and PMA-stimulated binding was significantly increased in the KO cells (lower panel). In the presence of *Ankrd1* (FLOX), PMA did not significantly affect the binding of Ankrd1 to the AP-1 site. There was no detectable binding of Ankrd1 to the AP-1 site in KO cells (compare upper and lower panels). Error bars indicate the SEM (n = 6). One-way ANOVA was used to determine statistical significance based on at least three independent experiments (**, $P < 0.01$; ****, $P < 0.0001$).

increased secreted MMP-13 protein in the KO cells (**Figure 18C**). These data indicated that the absence of Ankrd1 leads to de-repression of *MMP13* transcription in skin fibroblasts.

In light of our earlier report that Ankrd1 is highly induced by wounding (Shi et al., 2005a), we analyzed *MMP13* expression in normal and wounded skin from FLOX and KO mice. In uninjured KO skin, MMP-13 protein abundance was doubled compared to FLOX (**Figure 19A**). After wounding, both MMP-13 protein and mRNA levels were sharply higher in KO mice than FLOX mice (**Figure 19B and 19C**)**.** Together, these data showed that *Ankrd1* deletion has a significant influence on *MMP13* expression and content *in vivo*.

Removal of Ankrd1 increases binding near the AP-1 site of MMP-13

Based on our *in vitro* findings concerning *MMP13* promoter activity and the MMP-13 phenotype of the KO mouse, we hypothesized that Ankrd1 blocks AP-1-site binding of positive factors by interacting with nucleolin. To test this proposition, we analyzed the binding of factors to the AP-1 site in the absence and presence of Ankrd1 by EMSA. Probes (~60 bp) containing either the wild type or mutated *MMP13* AP-1 binding site were incubated with nuclear extracts from FLOX or KO cells that had been treated for 24h with PMA in DMSO, DMSO alone, or LPS. EMSA showed a marked difference in the gel shift pattern between FLOX and KO nuclear extracts treated with PMA, supporting the concept that the absence of Ankrd1 leads to increased binding of (positive) transcription factors to the AP-1 site of the MMP-13 promoter (**Figure 20A**). EMSA also showed that two mutations in the AP-1 site (5'mut, 3'mut) with markedly

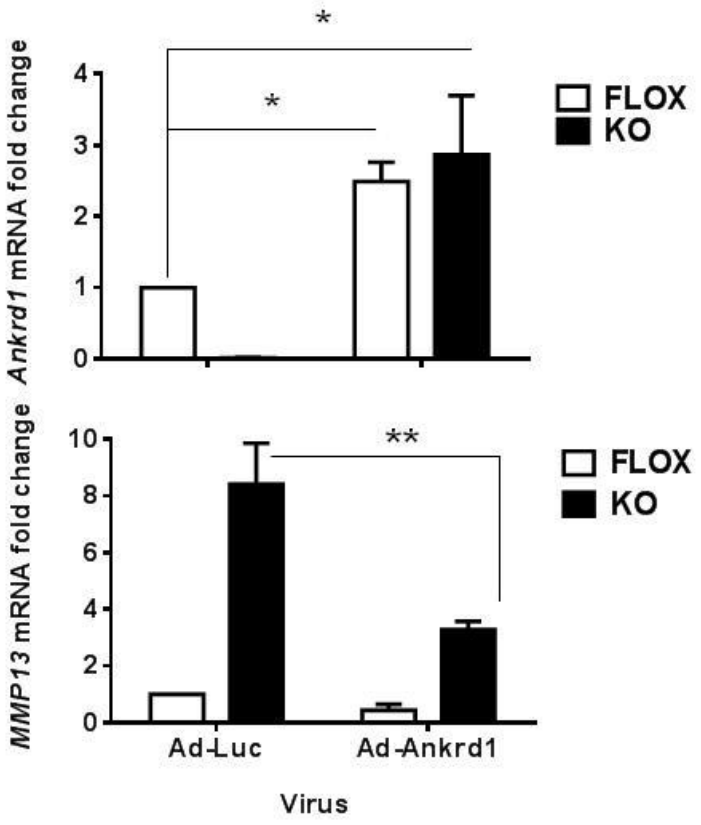


Figure 22: Adenovirus-mediated reconstitution of Ankrd1 in null cells alters *MMP13* transcription.

(Upper panel) *Ankrd1* expression after infection with adenovirus mediating the expression of luciferase as a control (Ad-Luc) or Ankrd1 (Ad-Ankrd1) was assessed in FLOX and KO skin fibroblasts. qRT-PCR confirmed the increased expression of *Ankrd1* in both cell strains after viral infection. The fold change indicates the level of expression of *Ankrd1* in cells relative to FLOX infected with Ad-Luc. Two-way ANOVA with Sidak's multiple-comparison test was used to obtain statistical significance (*, $P < 0.05$). (Lower panel) Overexpression of Ankrd1 in FLOX or KO cells suppresses *MMP13* expression. Adenovirus-mediated overexpression of *Ankrd1* (Ad-Ankrd1) but not luciferase (Ad-Luc) decreased *MMP13* mRNA levels. qRT-PCR analysis of *MMP13* was carried out as described in Materials and Methods. The data represent the results of three independent infection experiments performed in triplicate. Error bars indicate the SEM ($n = 9$). A Student *t* test was used to determine statistical significance (**, $P < 0.01$). The fold change indicates the level of *MMP13* mRNA expression relative to FLOX cells infected with Ad-Luc.

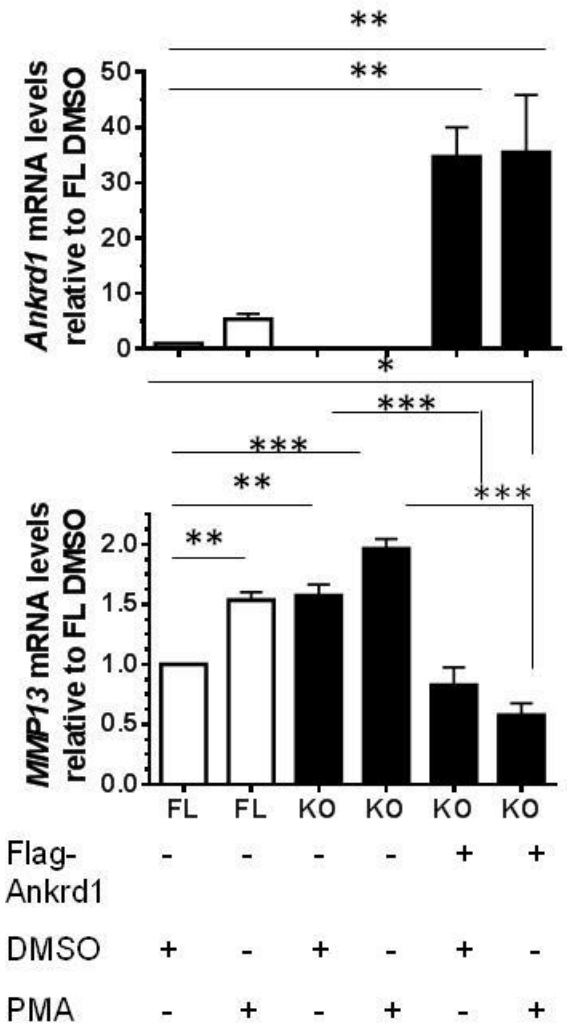


Figure 23: Reconstitution of Ankrd1 by plasmid transfection in KO cells suppresses *MMP13* expression.

For both analyses, Flag-*Ankrd1* or pcDNA 3.1 control plasmid was transfected into FLOX or KO skin fibroblasts, followed by PMA or DMSO treatments. Cells were harvested 24 h after treatment, and total RNA was isolated. (Upper panel) The fold change indicates the level of expression of *Ankrd1* in cells relative to FLOX+DMSO conditions. Two-way ANOVA, followed by Dunnett's multiple-comparison test, was used to determine statistical significance (**, $P < 0.01$). (Lower panel) *MMP13* mRNA levels were elevated by *Ankrd1* deletion (FLOX versus KO) and suppressed by cells that were transfected with Flag-*Ankrd1* plasmid (+) but not control pcDNA3.1 plasmid (−). Overexpression of Flag-*Ankrd1* also masked the response to PMA. The data represent the results of four independent experiments. The fold change indicates the level of expression of *MMP13* in cells relative to FLOX+DMSO. Error bars indicate the SEM ($n = 4$). One-way ANOVA, followed by the Bonferroni multiple-comparison test, was used to determine statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

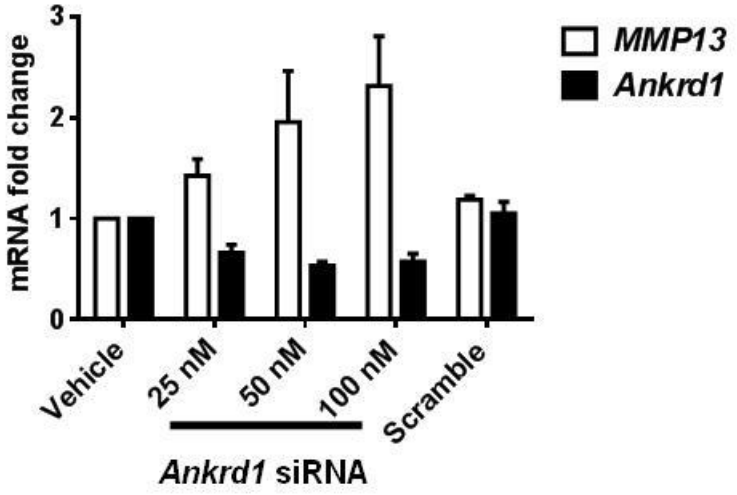


Figure 24: *In vitro* manipulation of *Ankrd1* expression modulates *MMP13* expression.
Knockdown of *Ankrd1* by siRNA dose dependently derepresses *MMP13* levels. FLOX skin fibroblasts were transfected with *Ankrd1* or scrambled siRNA and harvested 48 h after transfection. Expression was normalized to the vehicle control for each transcript. The fold change indicates the level of *MMP13* or *Ankrd1* mRNA expression relative to FLOX cells treated with vehicle. The data represent relative mRNA levels from three independent siRNA experiments, each performed in triplicate. Error bars indicate the SEM (n = 9). One-way ANOVA was used to determine statistical significance. The data for stimulation of *MMP13* or suppression of *Ankrd1* did not reach statistical significance.

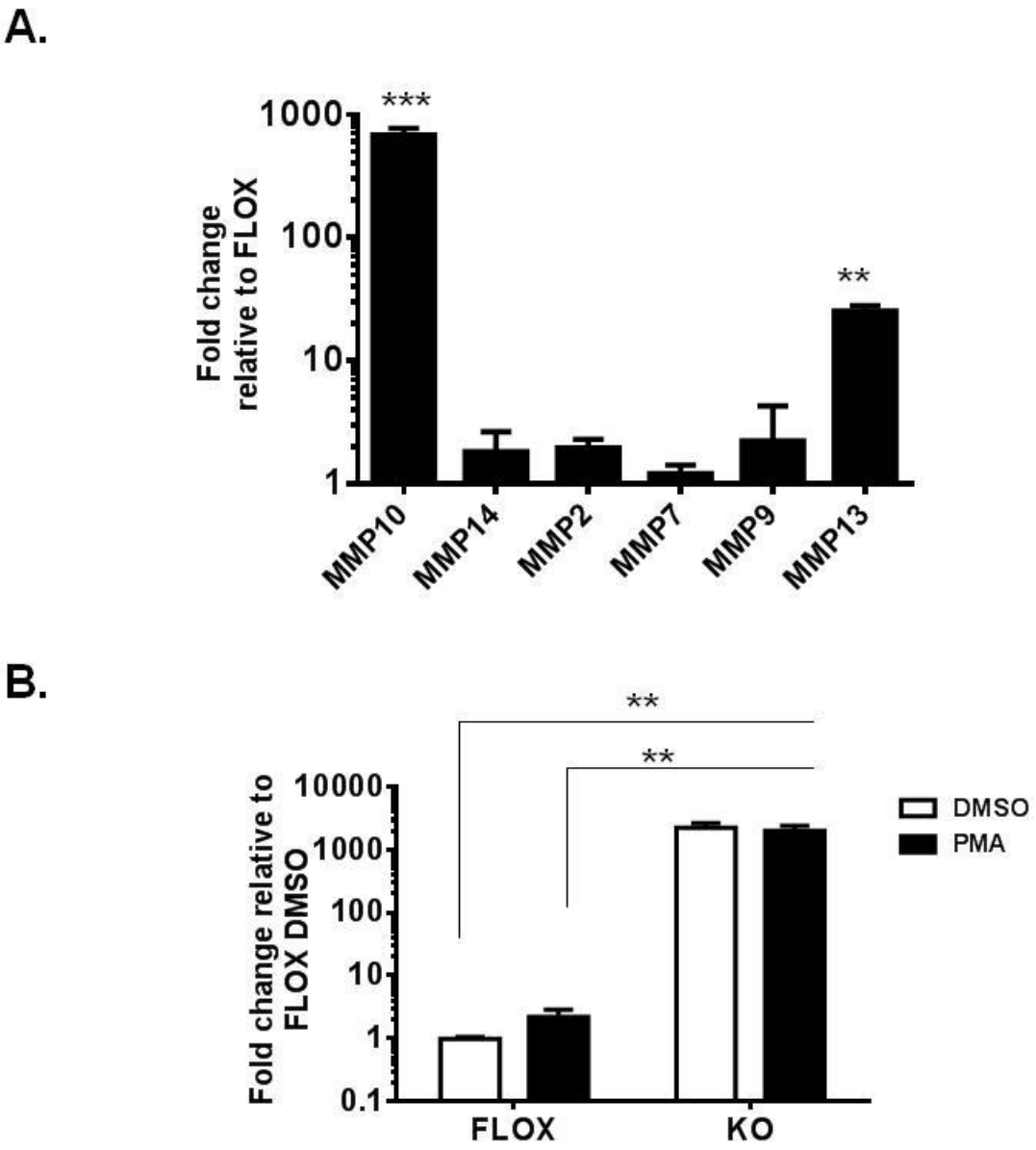


Figure 25: *MMP10* is a target of a Ankrd1 repression.
A. qPCR analysis of matrix metalloproteinases associated with stress responses. *MMP* transcript levels in FLOX and KO skin fibroblasts were quantified by StellArray and expressed on a logarithmic scale as KO relative to FLOX signal. *MMP10* and *MMP13* mRNA levels were significantly increased in the absence of *Ankrd1*. Error bars indicate the SEM (n = 3). A Student t test was used to determine the statistical significance of individual MMP responses (***, $P < 0.001$; **, $P < 0.01$). **B**. Dramatically increased *MMP10* mRNA levels in KO skin fibroblasts mask PMA stimulation. The fold change indicates, on a logarithmic scale, the level of *MMP10* mRNA in cells relative to FLOX cells treated with DMSO. Error bars indicate the SEM (n = 8). A Student t test was used to determine statistical significance (**, $P < 0.01$).

reduced promoter activity (**Figure 20A**) also significantly reduced the binding of transcription factors to the *MMP13* promoter in the presence of PMA. Levels of c-Jun, which is a component of the AP-1 complex, were significantly elevated in KO extracts (**Figure 20B**).

We also assessed the interaction of Ankrd1 with the AP-1 region of the MMP-13 promoter and the effects of *Ankrd1* deletion and PMA stimulation on binding of c-Jun, a positive regulator of MMP-13 transcription (Mengshol, Vincenti, Coon, Barchowsky, & Brinckerhoff, 2000), to the AP-1 site using chromatin immunoprecipitation (ChIP). As a component of AP-1, c-Jun is a potent inducer of transcription of several MMP genes, including *MMP13* (Mak, Turcotte, Popovic, Singh, & Ghert, 2011; Mengshol et al., 2000; Vincenti & Brinckerhoff, 2002). Thus, we analyzed the binding of c-Jun to the *MMP13* AP-1 site in the absence and presence of Ankrd1. This analysis showed that deletion of *Ankrd1* stimulated c-Jun binding to the proximal *MMP13* AP-1 site (**Figure 21**) and that loss of Ankrd1amplified c-Jun binding in cells exposed to PMA. ChIP also showed that endogenous Ankrd1 increased occupancy of the *MMP13* AP-1 site, while this increased occupancy was not affected by AP-1 activation (PMA) (**Figure 21**). These results confirmed that in the absence of Ankrd1, c-Jun expression is increased and it interacts more strongly with the *MMP13* promoter AP-1 site. Collectively, these results suggest that *Ankrd1* removal relieves *MMP13* transcriptional repression and stimulates the binding of positive transcription complexes to the *MMP13* AP-1 site.

In vitro manipulation of Ankrd1 regulates MMP13 expression

Reconstitution of Ankrd1 in *Ankrd1*-deficient skin fibroblasts by infecting cells with an adenovirus expressing Ankrd1 (Ad-Ankrd1) caused significant reduction in

MMP13 mRNA from elevated levels (**Figure 22**). FLOX cells infected with *Ankrd1* had a more moderate reduction (60%). KO cells infected with a control adenovirus expressing luciferase (Ad-Luc) maintained increased MMP-13 transcript levels compared to FLOX cells infected with Ad-Luc. In addition, transient transfection with a Flag-*Ankrd1* plasmid construct counteracted the derepression of *MMP-13* in KO cells, and this effect was further enhanced by the addition of PMA (**Figure 23**). To address the possibility that increased *MMP13* mRNA in the absence of Ankrd1 was an indirect effect of sustained Ankrd1 depletion, we knocked down *Ankrd1* mRNA levels in FLOX cells with siRNA and observed that *Ankrd1* knockdown increased *MMP13* mRNA levels in a dose-dependent manner (**Figure 24**).

Ankrd1 regulates other MMPs

To investigate the impact of *Ankrd1* deletion on additional MMPs, we analyzed a mouse stress response qPCR array (Stellarray, Lonza) with RNA from FLOX and KO skin fibroblasts. The qPCR array confirmed increased *MMP13*, mild overexpression of *MMP2*, *MMP9*, and *MMP14* and a remarkably strong stimulation of *MMP10* (**Figure 25A**) in KO cells. In another experiment, qRT-PCR analysis using RNA from FLOX and KO skin fibroblasts exposed to PMA or DMSO showed a PMA-dependent increase of *MMP10* transcripts in FLOX cells; however, *MMP10* mRNA levels were so dramatically increased in KO cells that regulation by PMA was masked (**Figure 25B**). Because *MMP10* has four AP-1 binding sites in its promoter (Rodriguez et al., 2008), these data suggest that Ankrd1 regulates MMP-10 in a similar, AP-1 dependent fashion to MMP-13.

Discussion

Extracellular matrix remodeling is a key aspect of development, tumorigenesis, and wound healing. Degradation of extracellular matrices is required to remove damaged tissue and to allow cell migration, capillary morphogenesis, and reorganization of the cellular environment (Vaalamo et al., 1997b). Matrix turnover and signaling cascades derived from this process dictate whether tissue repair progresses or becomes chronically impaired. The MMP family contributes to the process of extracellular matrix remodeling by controlled proteolysis in the pericellular environment that is tightly regulated by activation and inhibition. Collagenases are MMPs that selectively degrade native collagenous matrices in the extracellular space (Ravanti, Heino, Lopez-Otin, & Kahari, 1999b; Ravanti et al., 2001) and MMP-13 is the predominant collagenase in the mouse (Balbin et al., 2001). MMP-13 expression has been implicated in both physiological and pathological conditions. It has been shown to be essential for normal generation of granulation tissue in mice (M. Toriseva et al., 2012), and it is expressed in various cancers (Chiang, Wong, Lin, Chang, & Liu, 2006; Decock et al., 2008; Yamagata et al., 2012; Zyada & Shamaa, 2008). MMP-13 plays predominant roles in rheumatoid arthritis and osteoarthritis, and increased expression of MMP-13 induces osteoarthritis in mice (Neuhold et al., 2001). AP-1 is a key regulator of MMP-13 transcription (Burrage, Mix, & Brinckerhoff, 2006). We have provided evidence here that Ankrd1 associates with nucleolin to regulate MMP-13 expression through its

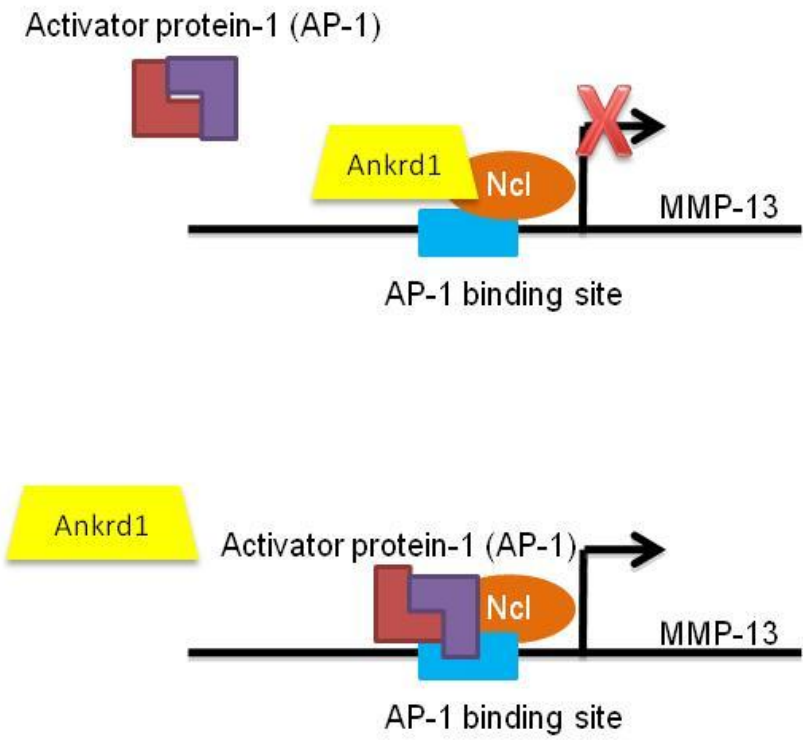


Figure 26: Ankrd1 as a negative coregulator of *MMP13* transcription at the AP-1 site.
(Upper panel) In this model, Ankrd1 association with nucleolin reduces the binding of the activator protein 1 heterodimer to the MMP-13 AP-1 site, resulting in inhibition of MMP-13 expression. (Lower panel) Conversely, reduced levels or the absence of Ankrd1 permits engagement of AP-1 with the transcriptional machinery.

AP-1 binding site. This negative regulation of MMP-13 by Ankrd1 may contribute to limiting potentially pathological levels of MMP-13 and other AP-1 target genes in the context of inflammation and other circumstances with elevated AP-1 levels such as tissue repair and rheumatoid arthritis (Granet, Maslinski, & Miossec, 2004).

In a quiescent state, both *Ankrd1* and *MMP13* mRNA and protein expression are low in non-muscle cells. However, under stress conditions like wounding Ankrd1 increases in skeletal muscle in the subdermis, vessel walls in the dermis, inflammatory cells, and epidermis (Shi et al., 2005a). We hypothesize that this up regulation of Ankrd1 occurs, in part, to limit the expression of *MMP13* and other AP-1 regulated target genes. When *Ankrd1* is absent in the context of tissue damage, we speculate that pathological, unrestrained up regulation of MMP genes affects the tissue repair process. We have observed that *Ankrd1* deletion in mice results in increased necrosis in an ischemic skin flap model that is reversed by *Ankrd1* gene reconstitution, and excisional wounds in KO mice showed reduced signs of skin contraction (S. Samaras, K. Almodóvar-García, N. Wu and J.M. Davidson, submitted for publication) (Chapter II). Dysregulation of MMPs could promote extensive degradation of extracellular matrix during the tissue repair process and result in impaired cell-matrix interaction. We hypothesize that Ankrd1 regulation of *MMP-13* and other targets is important to successful tissue repair. While *MMP10* appears to be strongly affected by *Ankrd1* repression, array data make it clear that strong Ankrd1 transcriptional repression does not extend to several other AP-1 regulated MMPs (*MMP2*, *MMP7*, *MMP9*, and *MMP14*).

Ankrd1 is present in both the cytoplasm and the nucleus, playing two distinct roles, depending on its location. In the cytoplasm of cardiomyocytes, Ankrd1 is involved

in the assembly of sarcomeric protein complex that binds to titin, where this complex may play a role in sarcomere stability and mechanotransduction. Ankrd1 also acts as a mediator of a GATA4 signaling axis that converges to control sarcomere gene expression and maintain sarcomere organization (B. Chen et al., 2012). Ankrd1 can also shuttle from the cytoplasm to the nucleus where it regulates target genes. Here we presented evidence of nuclear Ankrd1 mediating transcriptional modulation of *MMP13* in skin fibroblasts via an AP-1 site. Indeed, over-expression of Ankrd1 in several cell types leads to regulation of hundreds of gene targets identified by microarray analysis (K. Almodóvar-García and J. Davidson, unpublished data). Thus, many functional roles of cytoplasmic and nuclear Ankrd1 remain to be fully described.

In the context of cardiomyogenesis, Ankrd1 has also been shown to interact with YB-1, muscle specific RING finger proteins (MuRFs), myopalladin, and cardiac calsequestrin (Bang et al., 2001b; Miller et al., 2003; Torrado et al., 2005; C. C. Witt et al., 2008; Zou et al., 1997). Ankrd1 interaction with the nuclear factor YB-1 at an HIF-1 site in the myosin light chain 2v (MLC 2v) promoter negatively regulates myosin expression (Zou et al., 1997). YB-1 has also been shown to interact with the AP-1 site within the MMP-13 promoter and its over-expression potently represses AP-1 dependent transactivation (Samuel, Beifuss, & Bernstein, 2007). Interestingly, nucleolin and YB-1 interact with each other and both proteins bind to RNA to form a complex (C. Y. Chen et al., 2000a). While we have no evidence of a transcription complex forming between Ankrd1, YB-1 and nucleolin, we have found that over-expression of YB-1 and Ankrd1 together results in decreased MMP-13 promoter activity (K. Almodóvar-García and J. Davidson, unpublished data). Thus, it is conceivable that Ankrd1, YB-1, and

nucleolin could form a ternary complex to regulate transcription of MMP-13 and other genes with AP-1 binding sites.

Results from ChIP analysis indicate that Ankrd1 and c-Jun bind to the *MMP13* AP-1 site. ChIP studies demonstrated that the absence of Ankrd1 stimulates the binding of c-Jun to the *MMP13* AP-1. We speculate that Ankrd1 associates with nucleolin in the *MMP13* AP-1 site and this interaction prevents the binding of the activator protein 1 heterodimer composed of c-Jun and c-Fos to the AP-1 site, thereby repressing *MMP13* expression. When Ankrd1 is absent, the activator protein 1 could bind to the *MMP13* AP-1 site, perhaps in the presence of nucleolin, and induce *MMP13* expression (**Figure 26**). Interaction between nucleolin and c-Jun has been previously characterized (C. Y. Chen et al., 2000b), and nucleolin/c-Jun complexes play an important role in regulation of genes with Sp1 binding sites (Tsou et al., 2008).

In this study, MMP-13 regulation was analyzed in cells (dermal fibroblasts) derived from *Ankrd1*-deleted mice. Our previous data indicated that the major regulation of the mouse MMP-13 promoter occurs through the AP-1 binding site (Wu et al., 2002). Consistent with this observation, cells transfected with a mutated AP-1 MMP-13 promoter construct had no detectable luciferase reporter activity. Further, we showed by EMSA that point mutations within the AP-1 site affected the binding of nuclear factors to this sequence and that the presence of Ankrd1 reduced binding of transcription factors to the AP-1 site of the MMP-13 promoter. Significantly elevated *MMP13* mRNA and protein levels were observed in the absence of Ankrd1. Reconstitution of *Ankrd1* resulted in decreased, and knockdown of *Ankrd1* increased, *MMP13* mRNA levels. MMP-13 plays an important role during the wound healing process, by coordinating

growth of granulation tissue, inflammation, and angiogenesis, but it also is involved in pathological disorders ranging from chronic wounds to cancer. Since Ankrd1 is also an effector of angiogenic activity (Shi et al., 2005a), a potential inhibitor involved in regulating ECM metabolism, and an important suppressor of *MMP13* and *MMP10* transcription, it may be a novel target for wound healing or cancer therapies.

Chapter IV

INCREASED ANKRD1 EXPRESSION AFTER BURN INJURY IN HUMANS

Introduction

The co-transcription factor, Ankrd1, is strongly induced by acute wounding of mouse skin. Increased Ankrd1 mRNA and protein have been demonstrated in mouse excisional wounds (Shi et al., 2005a). Skeletal muscle, vessel wall, hair follicle, inflammatory cells, and epidermis in the wound area expressed Ankrd1 in response to mouse tissue injury (Shi et al., 2005a). Over-expression of Ankrd1 results in increased microvasculature, robust formation of granulation tissue, and increased perfusion (Samaras et al., 2006b; Shi et al., 2005a). It is still unknown whether a parallel phenomenon occurs in human skin. Thus, we investigated the expression of Ankrd1 in human burns.

Severe burns represent an extreme example of wound repair and are a major public health issue. Burn injuries are among the most devastating forms of trauma. Data from the American Burn Association show that approximately 450,000 cases of burn injuries receive medical treatment in U.S.A. per year (Association, 2013; Ellison, 2013). Moderate to severe burn injuries requiring hospitalization account for approximately 40,000 of these cases, and about 5,000 patients die each year from burn-related complications (Association, 2013; Church, Elsayed, Reid, Winston, & Lindsay, 2006).

Advances in modern medical care have improved outcomes for severely burned patients.

In this study, we analyzed the expression of Ankrd1 by immunohistochemical analysis of healing burn wounds in human skin. We hypothesized that Ankrd1 is highly expressed in burns. Ankrd1 expression was assessed in human burn wounds ranging from Post Burn Day (PBD) 3 to 20, and burn scars. Our findings demonstrate that expression of Ankrd1 is greatly increased in the burn wound.

Materials and Methods

Tissue. Twenty-nine unidentified specimens of varying-age human burn wounds, three human burn scars, and five normal human skin samples were obtained from Lillian B. Nanney, Ph.D. The tissue had been fixed in 4% paraformaldehyde for 24h, paraffin-embedded, and cut into 5-µm sections.

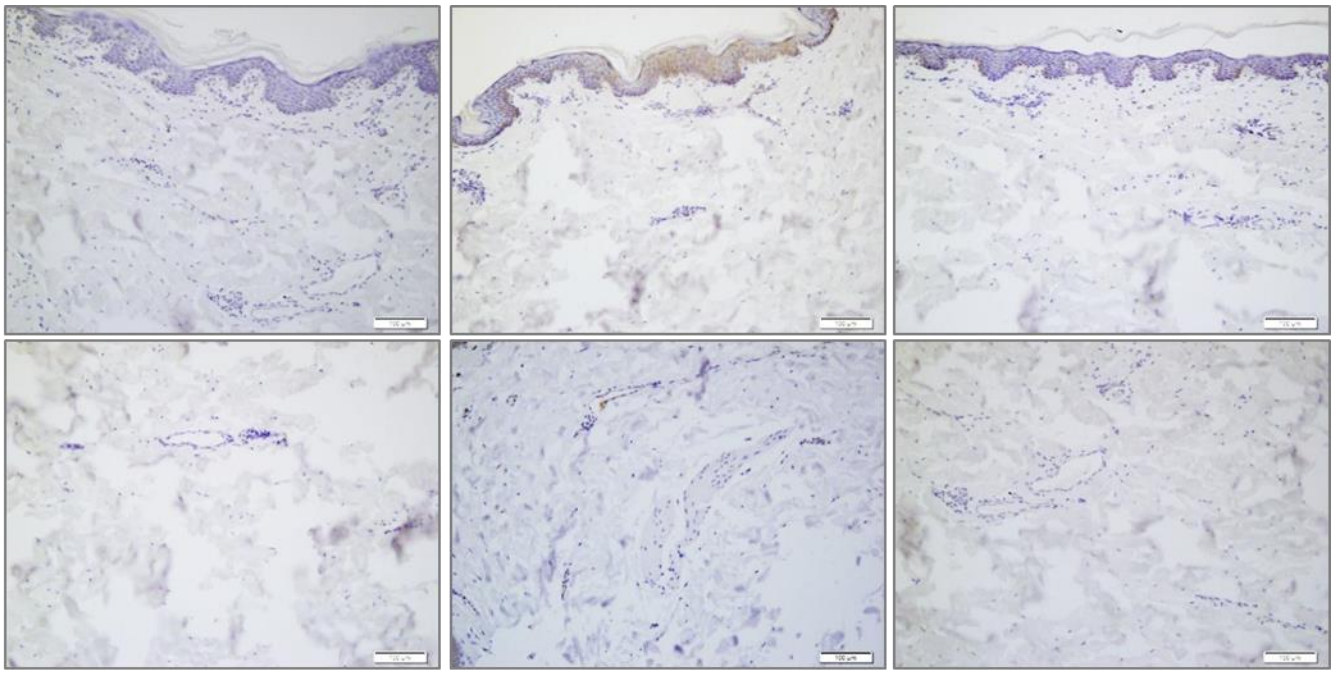
Immunostaining. Sections from normal skin, burn wounds, and burn scar tissue were blocked with 5% porcine serum (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at RT and incubated with anti-Ankrd1 antibody (1:1200) at 4°C overnight. After washing with PBS, slides were incubated with peroxidase-conjugated anti-rabbit IgG (1:250) for 30 minutes at room temperature. Finally, the tissue sections were developed with a DAB kit (Vector Laboratories) and counterstained with hematoxylin. Histological samples were photographed on an Olympus BX50 microscope with Olympus DP71 camera. Digital images were saved using software CellSens Standard 1.6. (Olympus Corporation).

Results

In normal human skin, very faint staining for Ankrd1 was found scattered irregularly throughout the epidermis and dermis (**Figure 27**). Some human normal skin specimens showed little or no Ankrd1 staining (**Figure 27**). In burned skin, intense immunostaining was present in the epidermis and dermis, at different time points, ranging from PBD3 to PBD20 (**Figure 28-30**).

At early time points, PBD3-PBD5, stronger Ankrd1 immunoreactivity was observed, where many different cells stained positively (**Figure 28**). By day 7 and 8, Ankrd1 immunostaining was still very strong in the thickened, hyperproliferative epidermis and underlying mix of dermal fibroblasts and macrophages (**Figure 29 and 30**). On day 11, the basal keratinocytes of the new epidermis stained intensely for Ankrd1, whereas the highly stratified keratinocytes layers of newly formed epidermis stained weakly for Ankrd1 (**Figure 30**). At a later period of wound healing (PBD13-20), staining for Ankrd1 gradually decreased, Ankrd1 positive keratinocytes were most prominent in the basal layers of the epidermis (**Figure 30**). Interestingly, human burn scars showed very faint Ankrd1 immunostaining (**Figure 31**). Intense Ankrd1 staining was observed in blood vessels in injured tissue at all-time points (**Figure 28-30**), while blood vessels in the normal skin were mostly negative for Ankrd1.

Normal skin: 20X



Normal skin: 40X

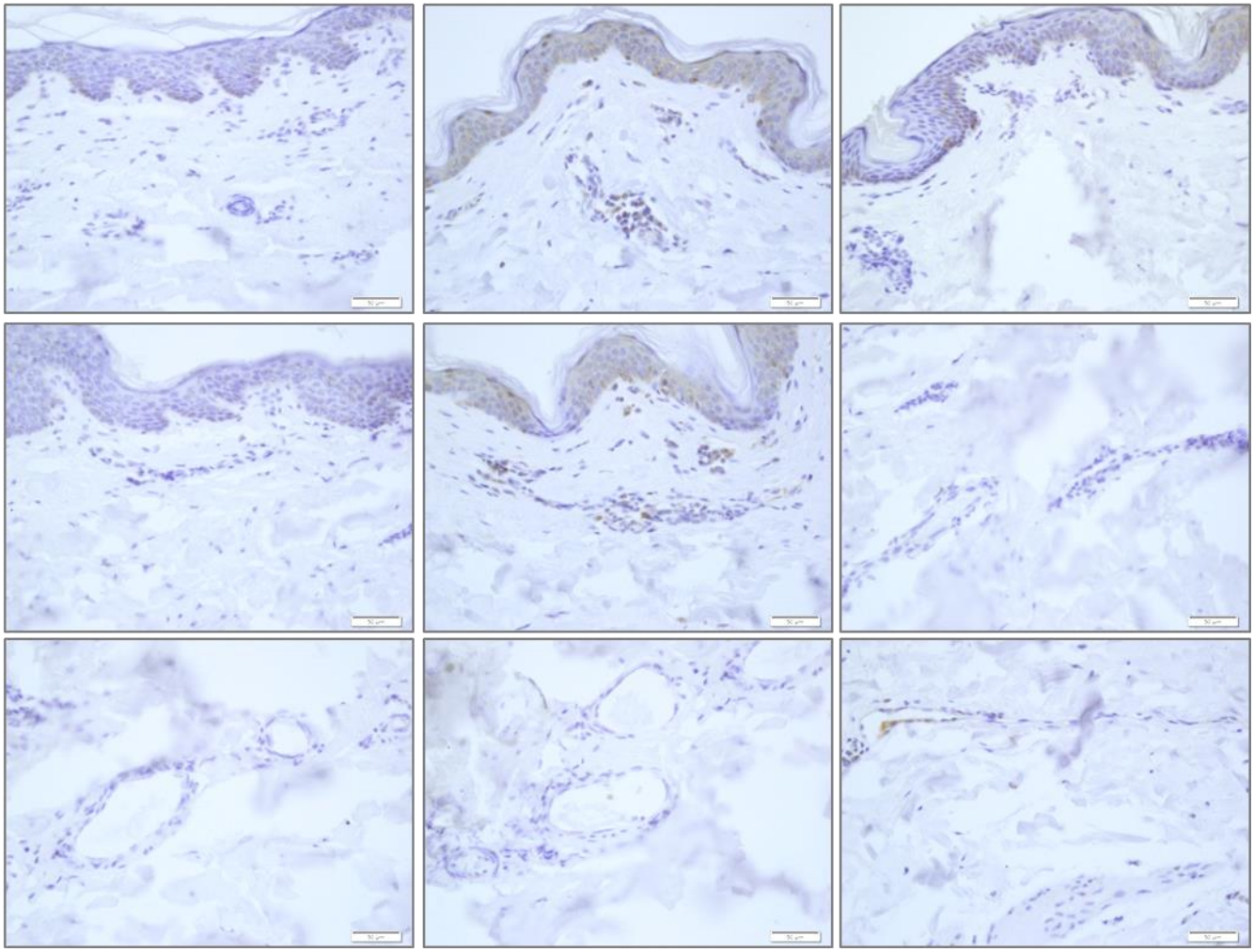


Figure 27: Ankrd1 expression in human normal skin.
Ankrd1 immunostaining in normal skin. A faint Ankrd1 staining is detected in normal skin. Cells of the epidermis and dermis showed very weak or no Ankrd1 expression. Representative images are shown. n=6 20X and 40X images; Scales: 100 µM and 50 µM.

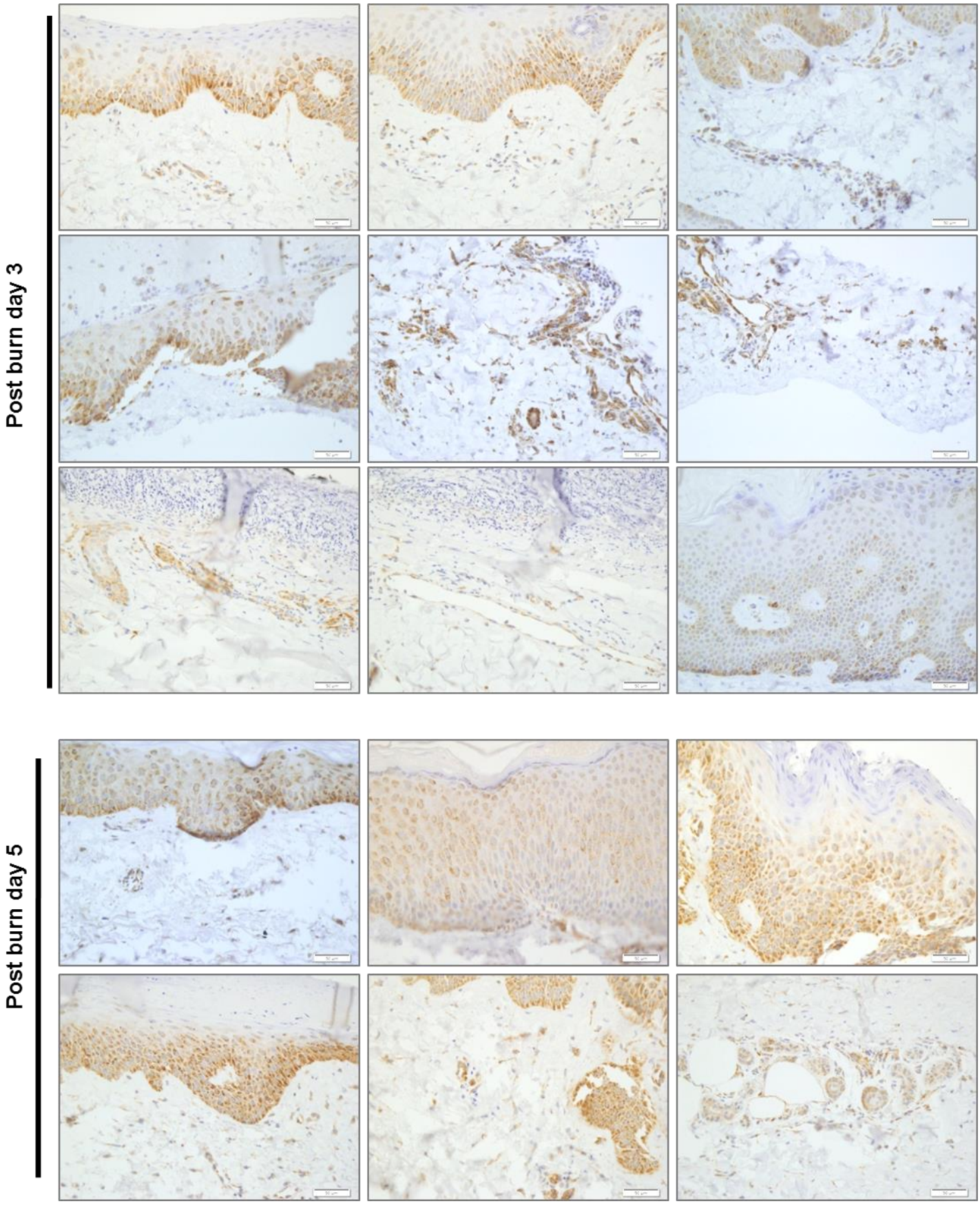

Post burn day 3
Post burn day 5

Figure 28: Ankrd1 immunostaining in human post burn day 3 and day 5 wounds. Early burned tissue strongly stained positively for Ankrd1. Cells of the epidermis and the dermis showed Ankrd1 positive staining. Some samples have a hyperproliferative epidermis that stained very intense for Ankrd1. Representative images are shown. PBD3; n=7, PBD5; n=6. 40X images; Scale: 50 µM.

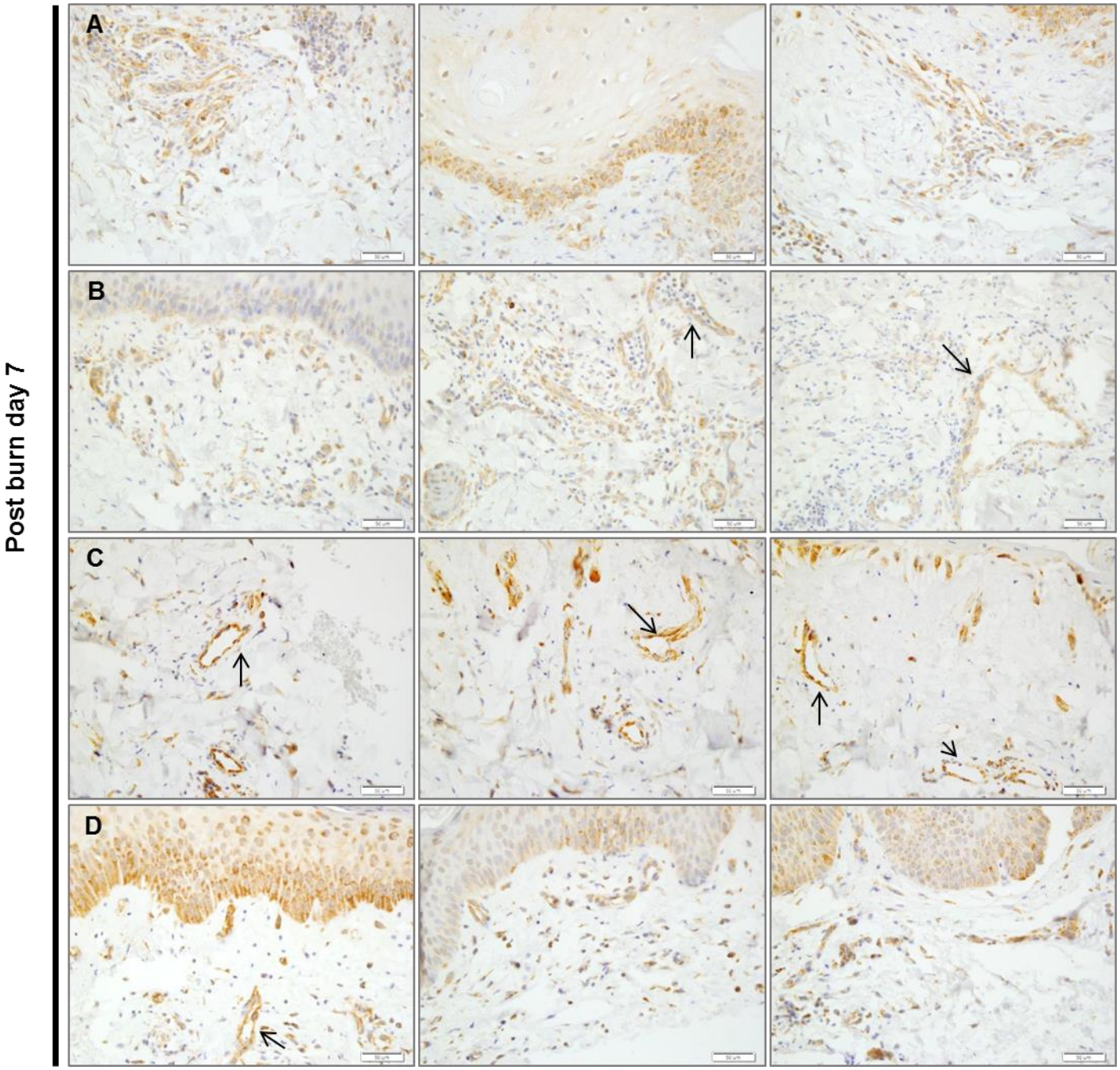


Figure 29: Ankrd1 expression in human post burn day 7 wounds.
Ankrd1 immunostaining in PBD7 wounds. Ankrd1 is highly induced in day 7 burned tissues. Both cells of the epidermis and the dermis showed Ankrd1 positive staining. Blood vessels stained positive for Ankrd1 (arrows). A through D: Three pictures of four burn wound samples are shown. n=4, 40X images; Scale: 50 µM.

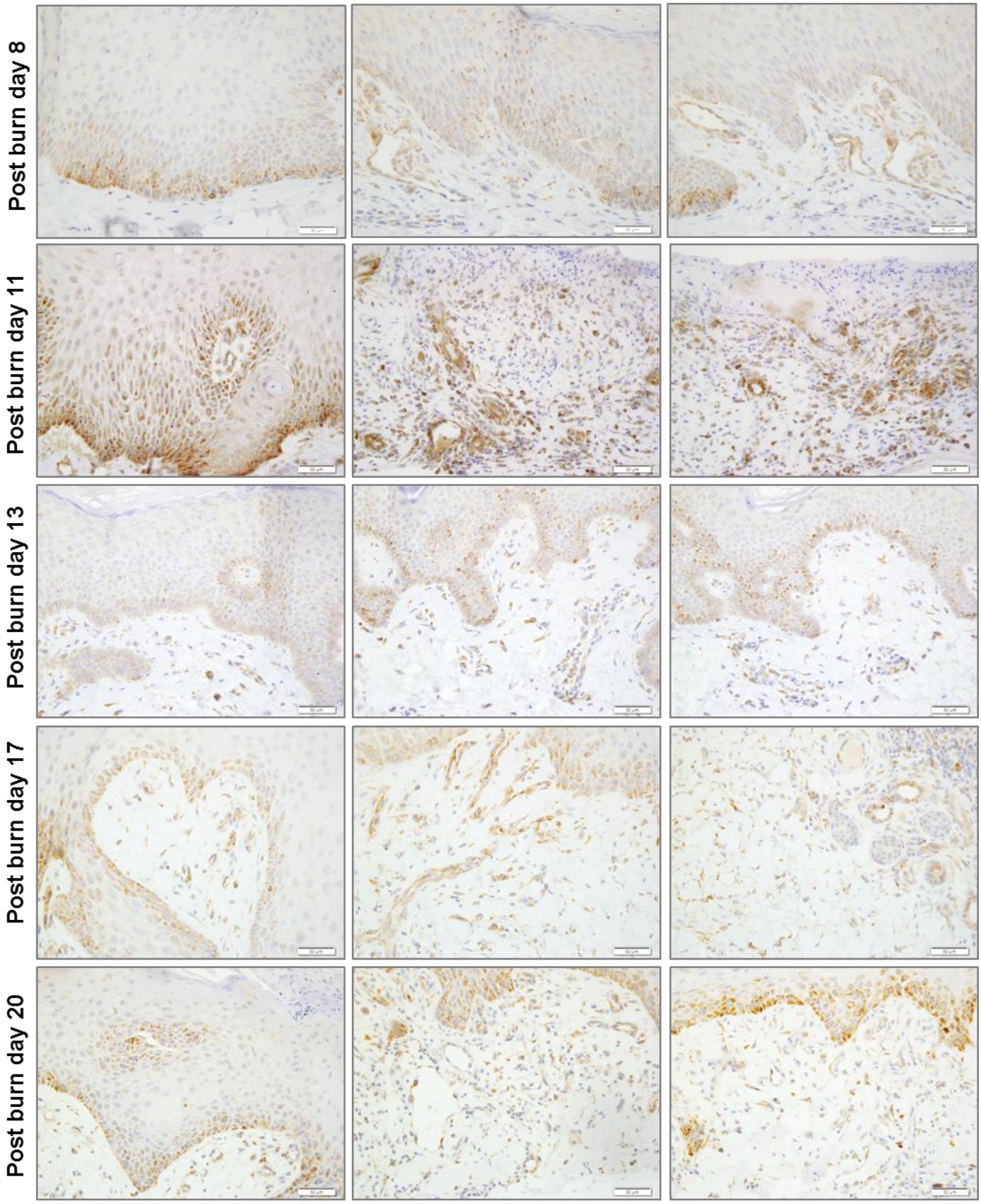

Post burn day 8
Post burn day 11
Post burn day 13
Post burn day 17
Post burn day 20

Figure 30: Ankrd1 expression in human post burn day 8, 11, 13, 17 and 20 wounds.

Ankrd1 immunostaining in PBD7, PBD11, PBD13, PBD17, PBD20 wounds. Ankrd1 is sharply induced up to day 11 after injury. Ankrd1 is detected at a lower level in burns from day 13- 20. One sample of each time point was analyzed. Three pictures of each sample are shown here. 40X images; Scale: 50 µM.

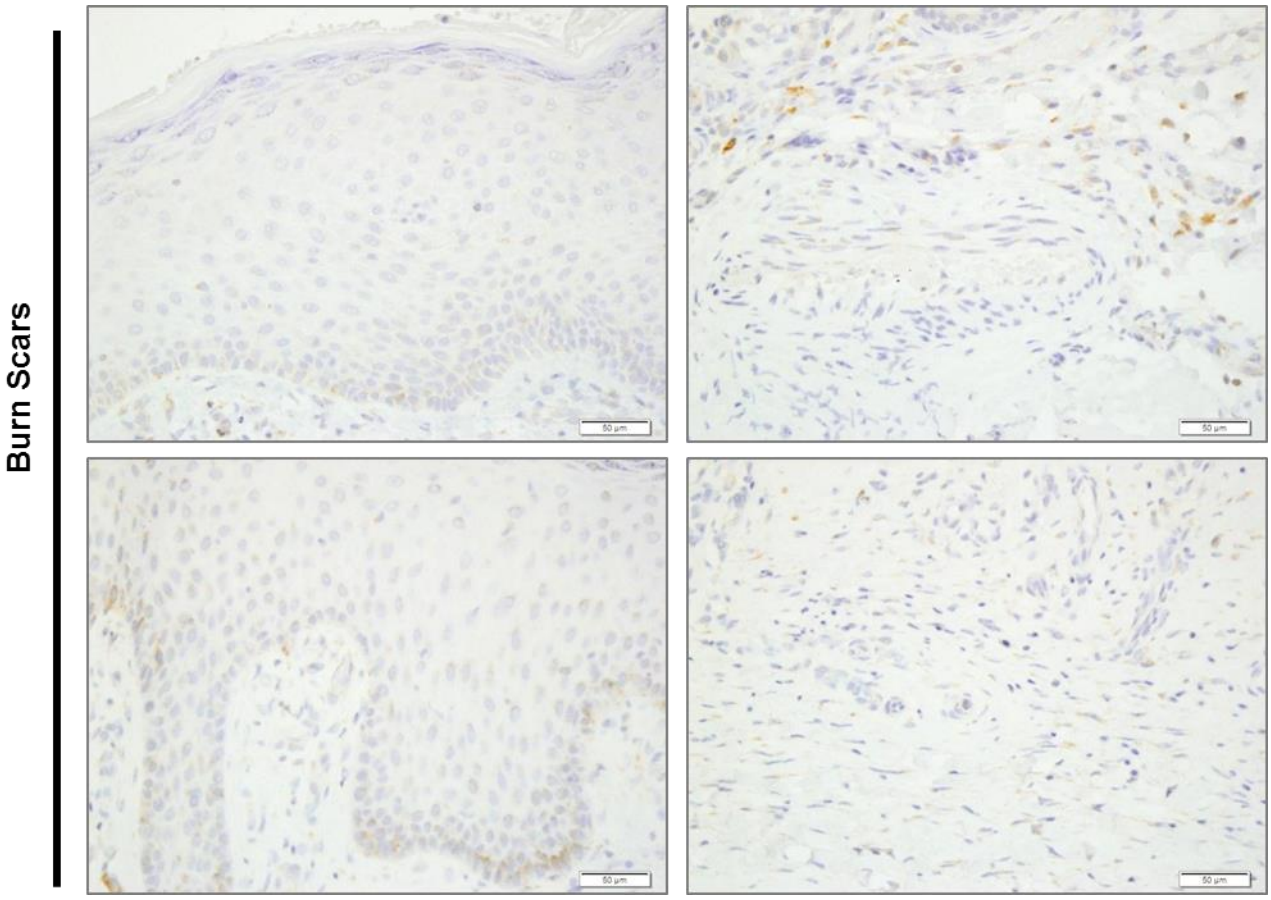


Figure 31: Ankrd1 expression in burn scars.
Ankrd1 positive staining is barely detected in burn scars. Two pictures of two burn scars are shown here. n=3, 40X images; Scale: 50 μM.

In summary, the basal tip of migrating keratinocytes appeared to be stained strongly, while the suprabasal cells at the tip were stained weakly for Ankrd1 at different time In the granulation tissue of the dermis of burned tissue, blood vessels were intensely stained for Ankrd1, while Ankrd1 staining was barely detected in the blood vessels of normal human skin.

Discussion

The present study was carried out to characterize the patterns of expression of Ankrd1 in human skin wounds. Using a specific antiserum to Ankrd1, our study shows that immunoreactive structures are detected faintly in human normal skin but injured tissue stained very intensely after a burn. The low level of Ankrd1 in intact skin agrees with our mouse findings (Shi et al., 2005a). We observed Ankrd1-positive cells in epidermal and dermal cells. Nuclear and cytoplasmic Ankrd1 was observed in positive cells. Keratinocytes in the epidermis were also positive for Ankrd1 in burned tissue that re-epithelialized. Cells in the dermis like fibroblasts and inflammatory cells stained positive for Ankrd1. Interestingly, Ankrd1 expression was observed very frequently in blood vessels in human burn wounds. Similarly, In situ hybridization and immunohistochemistry studies in mice showed increased expression of Ankrd1 protein in the vasculature (Samaras et al., 2006b; Shi et al., 2005a). Shi et al. showed that Ankrd1 significantly increased neovascularization, blood perfusion, and the abundance and organization of migrating vascular endothelial cells (Shi et al., 2005a).

Our data suggest that Ankrd1 is up-regulated within 72 hours following injury and during the prolonged reparative phase. Human burn samples were very variable but in general, Ankrd1 was strongly induced in burn wounds. We observed increased expression of Ankrd1 in cells of the epidermis and dermis. We can speculate that Ankrd1 protein is involved in different stages of the wound healing repair process. Thus, our data show that Ankrd1 is increased in the early burn wound and remains elevated even after epithelialization. Failure to produce Ankrd1 may delay healing and potentially result in chronic non healing wounds. Ankrd1 expression in human wounds has not been previously reported in the literature. Further investigation of the role of Ankrd1 in tissue repair is still needed.

CHAPTER V

SUMMARY

The studies in this dissertation have examined the role of Ankrd1 on selected target genes. Absence of *Ankrd1* results in a wound healing phenotype and impaired cell matrix (collagen) interaction. Fibroblasts and vascular smooth cells isolated from *Ankrd1*$^{-/-}$ mice showed little to no collagen contraction in vitro. Reconstitution of Ankrd1 in null fibroblasts resulted in contraction indicating that Ankrd1 is important for proper cell-collagen interaction. Moreover, KO fibroblasts failed to spread in low-stiffness PA gels coated with collagen. The mechanisms that lead to contraction and cell spread in the presence of Ankrd1 needs to be further evaluated. In addition to impaired cell matrix interaction, we found that Ankrd1 regulates matrix remodeling genes. Ankrd1 association with nucleolin results in negative regulation of MMP-13. MMP-13 is a member of the matrix metalloproteinase family capable of degrading fibrillar collagen. MMP-13 plays an important role during tissue repair because it degrades collagen, which is the main matrix protein synthesized and deposited during the repair process. Future work on Ankrd1 will investigate more profoundly the significance of the regulation of extracellular matrix remodeling molecules by Ankrd1 during wound healing. Finally, Ankrd1 was found to be expressed in human wounds indicating that Ankrd1 is not only

up-regulated after injury in rodents, but also in humans. Future studies will need to investigate the precise roles of Ankrd1 after wounding.

Concluding Discussion

The regeneration of tissue after injury occurs via a progressive cascade of events that requires specialized cell types and cell activities. In an attempt to identify novel wound healing genes, this laboratory generated 5 mm excisional wounds and performed cDNA subtractive hybridization comparing intact skin and day 1 wounds (Shi et al., 2005a). The co-transcription factor, *Ankrd1*, was up-regulated after wounding and overexpression improve wound repair. The overall goal of this thesis project was to determine the intrinsic role of Ankrd1 in tissue repair. In order to understand more about the function of Ankrd1, we focused on identifying Ankrd1 interacting factors, Ankrd1-target genes, and the effects of targeted deletion of Ankrd1.

A yeast two hybrid assay identified many potential *Ankrd1*-interacting factors (**Table 4**). The list of prospective interacting proteins contained several cytoplasmic proteins and a few nuclear factors. Among these, we found that *Ankrd1* associated with nucleolin. Nucleolin is a multifunctional protein that is involved in many pathways including chromatin condensation and remodeling, processing and stabilization of RNA, and activation and repression of transcription (Mongelard & Bouvet, 2007; Tuteja & Tuteja, 1998). One of its important roles is that it binds in vitro and in vivo to the AP-1

site in the promoter sequence of MMP-13 and represses transactivation of the AP-1 sequence (Samuel et al., 2008).

We confirmed the association of *Ankrd1* with nucleolin, and then sought to investigate the involvement of *Ankrd1* in regulation of MMP-13. Deletion of *Ankrd1* relieves *MMP13* transcriptional repression. *Ankrd1* reconstitution in KO fibroblasts decreased *MMP13* mRNA while *Ankrd1* knockdown increased these levels. *MMP13* mRNA and protein were elevated in intact skin and wounds of KO versus FLOX mice. We propose that Ankrd1, in association with factors such as nucleolin, represses *MMP-13* transcription.

Regulation of MMPs plays an important role in tissue remodeling during development and wound healing. In humans, MMP-13 has a key role in bone metabolism, homeostasis, osteoarthritis, and rheumatoid arthritis, tumor invasion and metastasis, and tissue repair (Ala-aho & Kahari, 2005; Hattori et al., 2009a; Takaishi, Kimura, Dalal, Okada, & D'Armiento, 2008; M. Toriseva et al., 2012). Intervention affecting MMP-13 in pathologic tissues has substantial clinical potential. Due to unacceptable side effects, direct MMP inhibitors have failed in many clinical trials (Hayashi, Jin, & Cook, 2007; Kalva, Saranyah, Suganya, Nisha, & Saleena, 2013; Kim et al., 2005; Lauer-Fields et al., 2009). Hence, other targeted treatment alternatives are needed, such as aiming at a higher level, by regulation of MMP-13 at the transcriptional level. From the results of our studies, we found that *Ankrd1* modulates AP-1 and regulates MMP-13 *in vitro* and *in vivo* in the skin. EMSA gel shift patterns suggested that additional transcription factors bind to the *MMP13* AP-1 site in the absence of

Ankrd1, and this concept was reinforced by ChIP analysis, revealed as greater binding of c-Jun to the AP-1 site in extracts from FLOX vs. KO fibroblasts.

MMP-13 is the predominant collagenase in mouse. It has been shown that MMP-13 participates in wound repair during mouse acute wounding, and MMP-13 probably plays an important role in the remodeling of fibrillar collagen (Wu et al., 2002). *Ankrd1* levels increased within 24 hours after injury, and after 14 days the levels decreased back to normal (Shi et al., 2005a). During cutaneous wound healing, MMP-13 expression is low from days 1-15 and maximal at day 18 post-wounding (Wu et al., 2002). It is tempting to suggest that *Ankrd1* expression confers a balanced regulation of MMP-13 gene expression to prevent extracellular matrix remodeling from becoming excessive during the first phases of the wound healing cascade. Thus, we speculate that *Ankrd1* is upregulated immediately after wounding, and it modulates degradation of fibrillar collagen by down regulating MMP-13. Increased collagenase activity and excessive degradation of fibrillar collagens of type I and III may cause formation of chronic non-healing ulcers (Ravanti, Heino, Lopez-Otin, & Kahari, 1999a; Vaalamo et al., 1997a; Weckroth, Vaheri, Lauharanta, Sorsa, & Konttinen, 1996; Wysocki, Staiano-Coico, & Grinnell, 1993). Whether MMP-13 activity changes in the presence or absence of Ankrd1 needs to be evaluated.

MMP-13 is expressed by chondrocytes and synovial cells in human osteoarthritis and rheumatoid arthritis and is thought to play a critical role in cartilage destruction (Mitchell et al., 1996; Reboul, Pelletier, Tardif, Cloutier, & Martel-Pelletier, 1996; Stahle-Backdahl et al., 1997; Wernicke, Seyfert, Gromnica-Ihle, & Stiehl, 2006). Therefore, we

need to further evaluate whether *Ankrd1*-null mice suffer from any joint-related condition.

Ankrd1 has also been shown to negatively regulate MMP-10. *Mmp10* contains two AP-1 sites in its proximal promoter (Benbow & Brinckerhoff, 1997). At the present time, we have not established that *Ankrd1* regulates MMP-10 through its AP-1 sites. PMA is a potent activator of transcription of MMPs acting through AP-1 site (Benbow & Brinckerhoff, 1997). However, in the absence of *Ankrd1*, the MMP-10 mRNA levels were very high; therefore we could not detect any changes after PMA stimulation. In addition, more studies are required to understand the biological significance of Ankrd1 negative regulation of MMP-10. Studies have shown that MMP-10 functions in skeletal development, wound healing, and vascular remodeling; its overexpression is also implicated in lung tumorigenesis and tumor progression (Batra et al., 2012).

We speculate that Ankrd1 may contribute to limiting potentially pathological levels of other AP-1 target genes in conditions with high AP-1 levels such as wound healing. Different lines of evidence have suggested a critical role of the transcription factor AP-1 in skin homeostasis (Angel, Szabowski, & Schorpp-Kistner, 2001; G. Li et al., 2003; Zenz et al., 2003). After skin injury, the release of IL-1 from keratinocytes induces the activity of the AP-1 subunits c-Jun and JunB in fibroblasts leading to a global change in gene expression (Florin et al., 2004). Gene expression profiling identified many AP-1 target genes involved in cutaneous wound healing (Florin et al., 2004). More studies will be necessary to determine whether Ankrd1 is involved in regulation of other AP-1 target genes.

Ankrd1-null mice suffered from two significant wound healing phenotypes: extensive necrosis of ischemic skin flaps that was reversed by adenoviral expression of *Ankrd1* (Samaras et al. Submitted for publication); and delayed excisional wound closure associated with decreased contraction and granulation tissue thickness. Evaluation of MMP-13 and/or MMP-10 involvement in these phenotypes is necessary.

We found that KO fibroblasts failed to contract 3D collagen gels, while reconstitution of Ankrd1 by viral infection stimulated both collagen contraction and actin fiber organization. Interestingly, MMP-13 has been implicated many times in collagen contraction (Barczyk, Lu, Popova, Bolstad, & Gullberg, 2013; Cukierman, Pankov, & Yamada, 2002; Hattori et al., 2009a; M. J. Toriseva et al., 2007), and remodeling of three-dimensional collagen matrices (M. J. Toriseva et al., 2007). Collagen degradation can influence FPCL contraction (Langholz et al., 1995). We have recently reported that KO fibroblasts strongly up regulate both *Mmp10* and *Mmp13* expression *in vitro* and *Mmp13 in vivo* (Chapter III) (Almodovar-Garcia et al., 2014). MMP-13 overexpression was even more strongly induced in both anchored and released FPCL (unpublished observations), a response that has been reported to involve an integrin-dependent pathway (Ravanti et al., 1999a). In contrast to stimulation of FPCL contraction by virally induced overexpression of human MMP-13 (M. J. Toriseva et al., 2007) or by age-related fibroblast overexpression of rat MMP-2 and MMP-9, (Ballas & Davidson, 2001) elevated *Mmp10* and *Mmp13* gene expression in the KO fibroblast appears to have no effect on FPCL reorganization. It is possible that *Mmp-10* and *Mmp-13* levels are sufficiently high at the cell surface of KO cells to prevent interactions with their pericellular environment or that binding and response to ECM is affected by deletion of

Ankrd1. Alternatively, there may be a defect in the synthesis, secretion, activation or activity of these metalloproteinases. Further studies will be needed to define the relative activities of matrix proteinases and receptors *in vivo* and *in vitro*.

Mechanical loading has also been shown to play an important role in the mechanisms of contraction (Grinnell, 2000). In the released model (floating gels), cells-embedded in the collagen matrix are released from the culture dish immediately after polymerization. In the attached model (stressed gels), the cells in the collagen lattice are cultured while anchored to the dish overnight to develop tension. These forces lead to changes in cell morphology and generation of contractile force (Grinnell, 2000). In both conditions, stressed and released gels, we observed that *Ankrd1*-deficient fibroblasts embedded in collagen gels failed to contract.

Future studies will delineate the mechanism or the signaling pathways in *Ankrd1*-deficient cells that is preventing these cells to contract in a traditional collagen assay. The results obtained from the *in vitro* studies might explain the *in vivo* phenotype observed where KO mice contract skin poorly after wounding.

Integrins mediate a molecular dialogue between cells and their environment. Many articles have provided evidence demonstrating that interaction of integrin subunits with matrix molecules such as collagen type I results in reorganization and contraction of a collagen matrix (Barczyk et al., 2013; de Rooij, Kerstens, Danuser, Schwartz, & Waterman-Storer, 2005; Grundstrom, Mosher, Sakai, & Rubin, 2003; Langholz et al., 1995; Magnanti et al., 2001; Miyake et al., 2000; Parekh, Sandulache, Lieb, Dohar, & Hebda, 2007; Schiro et al., 1991; Tian, Lessan, Kahm, Kleidon, & Henke, 2002; Xia, Nho, Kahm, Kleidon, & Henke, 2004). Since we observed impaired collagen type I

contraction in the absence of Ankrd1, we could hypothesize that integrin expression or integrin signaling pathways are affected when *Ankrd1* is deleted. The collagen binding integrins are: α1β1, α2β1, α10β1, α11β1. We studied cell surface expression, mRNA expression and protein expression of the subunits composing these integrins, but our results were inconclusive. Integrin signaling pathways have not been studied yet. Future studies will reveal whether any of these integrins play an important role in cell contraction in *Ankrd1*-null cells.

Another alternative to explain the *Ankrd1*-null contractile phenotype may be through the YAP-TAZ pathway. Recently, Dupont et al. (Dupont et al., 2011) reported that yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) act as nuclear relays of mechanical signals exerted by extracellular matrix rigidity and cell shape. The authors of the paper identified YAP/TAZ as sensors and mediators of mechanical cues instructed by the cellular microenvironment. Interestingly, the same article showed that Ankrd1 is a YAP/TAZ regulated gene. We hypothesize that the nuclear co-transcription factor, Ankrd1, is important for cells to adapt and sense external forces. Therefore, when Ankrd1 is absent, cells cannot respond to the YAP/TAZ signals that controls multiple aspects of cell behavior. causing the cells to enter a “quiescent” or silent state where they cannot react to their environmental cues thus, cells cannot contract soft gels.

Differentiation of fibroblasts into a highly contractile myofibroblast phenotype during wound healing has been shown to generate contractile forces which bring together the edges of an open wound and therefore facilitate wound closure (B. Li & Wang, 2011; Moulin, Auger, Garrel, & Germain, 2000; Moulin et al., 1999). However,

excessive myofibroblast activities, including excessive contraction and over-production of ECM, are also the major cause of tissue fibrosis and scar formation (Hinz et al., 2007; Moulin et al., 2004). It would be tempting to speculate that in the absence of Ankrd1 myofibroblast differentiation is impaired resulting in reduced contraction *in vitro* and *in vivo*.

Increased expression of Ankrd1 in many cell types is observed following wounding in human and mouse tissue, but its effects are not certain. Strong expression of Ankrd1 is observed in human burn wounds. In addition to burn wounds, foot ulcers and pressure ulcers showed significant expression of Ankrd1 (data not shown). As in the mouse, human normal skin showed little to no expression of Ankrd1. In the mouse, overexpression of Ankrd1 in different wound models increases angiogenesis and vasculogenesis (Samaras et al., 2006b; Shi et al., 2005a). Interestingly, Ankrd1 expression was observed very frequently in blood vessels in human wounds, but not in normal human skin. More studies are required to study the role of Ankrd1 in human tissue repair. Moreover, signaling pathways leading to Ankrd1 expression needs to be further studied in different cell types.

The studies in this thesis showed that Ankrd1 regulates matrix-related genes and is required for proper cell-matrix (collagen) interaction *in vitro*. Our lab also observed that overexpression of Ankrd1 in wounds by adenoviral gene transfer produces a remarkable increase in neovascularization (Shi et al., 2005a). Identification of targets of Ankrd1 that mediate Ankrd1-enhanced neovascularization is needed. Thus, expression profiling studies were performed to identify key targets of Ankrd1 in human microvascular endothelial cells (HMVECs). HMVECs were infected with adenovirus

expressing Ankrd1 (ad*Ankrd1*-GFP) or luciferase (ad*Luc*-GFP) virus as a control for 12 hours followed by microarray analysis. 12 hours infection with adAnkrd1-GFP virus affected the expression of a variety of molecules (**Table 6**). Approximately 18,231 genes were classified into inferred functional categories (pathways) based on the Panther (gene expression software program) classification system (Clark et al., 2003; Thomas et al., 2003). Pathways with high number of genes mapped to them are shown in **Table 5**. These pathways were related to cell adhesion, angiogenesis, survival, tissue maintenance and wound repair. Profiling of Ankrd1-dependent gene regulation revealed a compendium of potential angiogenic markers and putative therapeutic targets (**Tables 6 and 7**). Ankrd1 target genes may represent excellent candidates to regulate vascular remodeling during wound repair. Further studies are required to look at these potential Ankrd1 target genes in more detail.

Based on our studies, Ankrd1 is strongly induced in response to injury. Deletion of Ankrd1 impairs contraction, cell spread, and migration *in vitro*. More studies must be conducted in order to understand why *Ankrd1*-null cells failed to contract and spread.. *Ankrd1*-null mice showed reduced wound contraction and thinner granulation tissue formation. At the nuclear level, Ankrd1 is implicated as a transcriptional negative regulator of MMP-13 and possibly, MMP-10. We speculate that Ankrd1 is involved in extracellular matrix remodeling during wound repair. Increased expression of MMPs in KO mice might be implicated in the contraction phenotype (**Figure 32**). We hypothesize that Ankrd1 is involved in a pathway that translates mechanical forces into contractile activities (**Figure 32**). Understanding the role that Ankrd1 plays during tissue repair might lead to the development of a therapy that improves matrix turnover.

Pathways	Number of genes	+/-	p-value
Integrin signaling pathway	187	+	3.19E-09
Cadherin signaling pathway	146	+	4.45E-05
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	149	+	1.36E-03
Angiogenesis	219	+	1.65E-03
De novo purine biosynthesis	43	-	1.44E-03
EGF receptor signaling pathway	154	+	1.76E-03
Inflammation mediated by chemokine and cytokine signaling pathway	308	+	2.56E-03
FGF signaling pathway	138	+	8.01E-03
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	184	+	1.05E-02
VEGF signaling pathway	83	+	1.19E-02
Oxidative stress response	60	+	1.75E-02

Table 5: Ankrd1 over-expression affects the number of genes mapped to pathways categories involved in adhesion, angiogenesis, and survival.

HMVECs were infected with adAnkrd1-GFP or adLuc-GFP. Twelve hours post-infection RNA was isolated and examined by microarray analysis. Approximately 18,231 genes were classified into inferred functional categories (pathways) based on the Panther (gene expression software program) classification system. First column contains the name of the PANTHER classification category. The second column contains the number of genes that map to this particular PANTHER classification category. The third column has either a + or -. A plus sign indicates that for this category, the distribution of values for uploaded list is shifted towards greater values than the overall distribution of all genes that were uploaded. A negative sign indicates that the uploaded list is shifted towards smaller values than the overall list. The fourth column contains the p-value as calculated from the Mann-Whitney U Test (Wilcoxon Rank-Sum test).

Fold Change up-regulated

Molecules	Exp. Value
TNFSF18	↑2.466
IL8	↑1.833
CD274	↑1.732
CXCL1	↑1.582
BLID	↑1.521
ADAMTS6	↑1.509
NUAK1	↑1.497
FST	↑1.484
DKK1	↑1.464
CA2	↑1.454

Fold Change down-regulated

Molecules	Exp. Value
APOLD1	↓-3.157
JUNB	↓-1.781
GADD45B	↓-1.766
THBD	↓-1.746
DUSP1	↓-1.717
CCNE1	↓-1.712
SPEN	↓-1.706
HSPA1B*	↓-1.696
TLE1	↓-1.661
HSPA1A*	↓-1.643

Table 6: **Molecules with significant gene expression changes.**
Expression profiling studies in HMVECs leads to differentially regulation of many gene targets. Potential Ankrd1 target molecules are listed here (top 10 upregulated and downregulated molecules). Overall 12 transcripts were “upregulated” (Cutoff >1.5) and 30 transcripts were “downregulated” (Cutoff <- 1.5).

Potential Ankrd1 target gene	Microarray (Fold-Change (Ankrd1 vs. Luc))	qRT-PCR (Fold-Change (Ankrd1vs. Luc))	Fold-Change(Ankrd1 vs. Luc) (Description)
APOLD1	-3.15705	-4.39433	down
TNFSF18	2.46589	2.81287	up
JUNB	-1.78143	-2.41875	down

Table 7: Summary of gene expression profiling studies.
Overexpression of Ankrd1 for 12 hours in HMVECs leads to differentially regulation of APOLD1, TNSF18, and JUNB. The expression of this three potential Ankrd1 target genes were further studied by qRT-PCR analysis. Data obtained from qRT-PCR analysis were consistent with the microarray results.

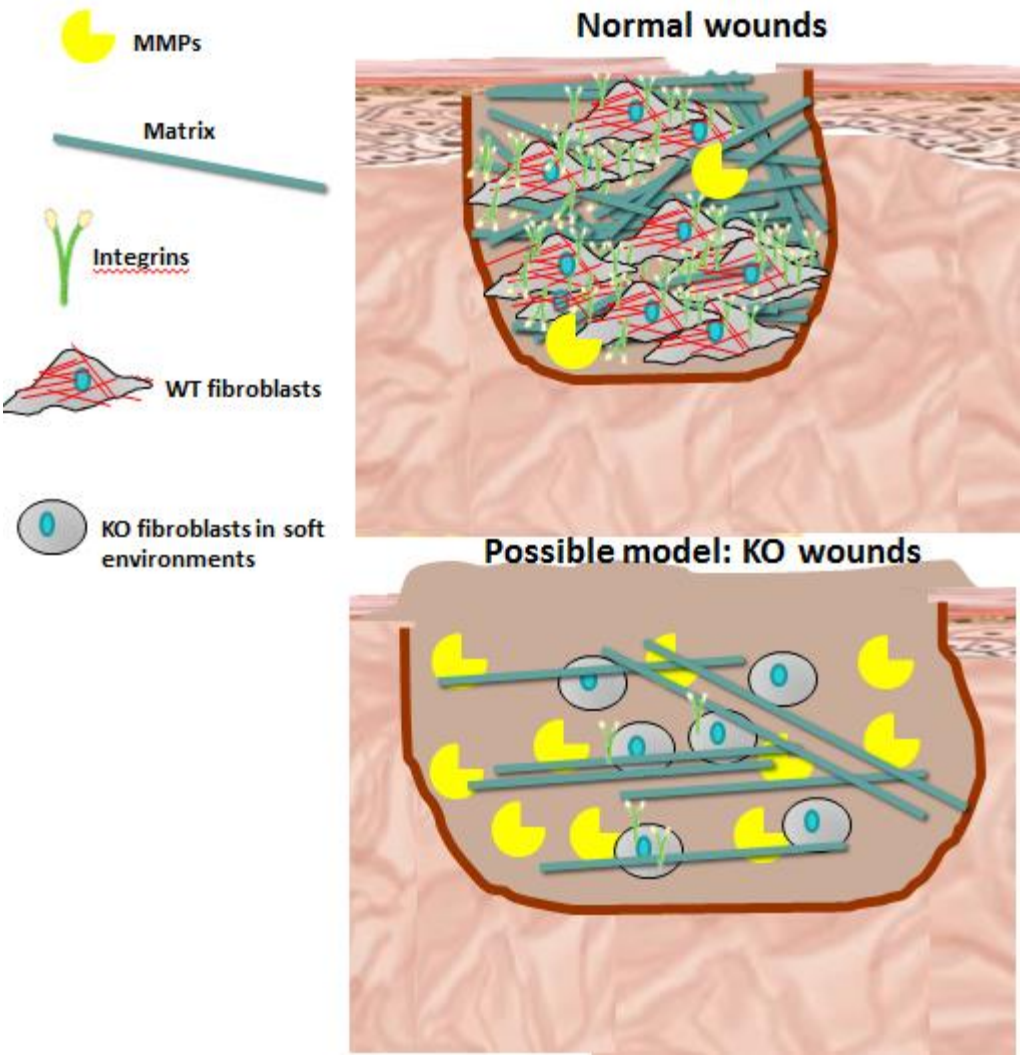


Figure 32: Schematic model depicting wounds in control and KO mice.
We observed that wound repair in control animals was normal, while KO mice healed more slowly, have reduced granulation tissue thickness, and less contraction. We speculate that overexpression of MMPs in KO wounds results in poor formation of granulation tissue. Reduced expression of integrins in KO cells results in impaired matrix interaction and therefore, poor cytoskeleton assembly and reorganization. Thus, KO cells are round and cannot sense their “soft” environment, because they need certain level of stiffness in order to react to external forces.

Future Directions

To address the role of Ankrd1 in tissue repair, our lab created a global knockout mouse. *Ankrd1*$^{-/-}$ mice had at least two significant wound healing phenotypes: extensive necrosis of ischemic skin flaps, and delayed excisional wound closure associated with decreased contraction and granulation tissue thickness. *In vitro* and *in vivo* experiments indicated that Ankrd1 negatively regulates members of the matrix metalloproteinase family including MMP-13 and MMP-10. As part of future work, we will explore the physiological relevance of negative regulation of MMP genes by co-transcription factor Ankrd1. More studies are required in order to determine whether increased levels of members of the MMP family in the absence of Ankrd1 are involved in any of the wound healing phenotypes. Investigating whether the activity of MMP-13 is increased in the absence of Ankrd1 is also required. Additionally, further studies will be needed to determine the mechanisms of regulation of MMP-10 by Ankrd1.

Ankrd1-deficient mice need to be more carefully analyzed to investigate whether they suffer from an arthritic disease. MMP-13 is a major player in rheumatoid arthritis and osteoarthritis. We observed that Ankrd1-null mice are significantly smaller than control mice; hence we suspect that high levels of MMP-13 in these animals might lead to a cartilage disorder.

Cells isolated from the *Ankrd1*$^{-/-}$ mice failed to contract 3D collagen gels, while reconstitution of Ankrd1 by viral infection stimulated both collagen contraction and actin fiber organization suggesting that Ankrd1 is critical for proper interaction of cells with a compliant collagenous matrix *in vitro.* Understanding why the cells that lack Ankrd1 expression cannot contract *in vitro* will delineate whether Ankrd1 plays an important role

in mechanotransduction. Analyses of integrin expression, signaling, trafficking, and focal adhesion formation in KO cells are necessary.

Results from yeast two hybrid studies showed that Ankrd1 associates with numerous cytoplasmic proteins, including α-actinin and tropomyosin. Both, α-actinin and tropomyosin, are involved in structural organization of actin filaments in non-muscle cells (Lazarides, 1976). Further studies are required to confirm association of Ankrd1 with these proteins. In addition, to determine whether formation of protein complexes with cytoplasmic Ankrd1 is important for cell contraction, spreading or migration we need to mutate the nuclear localization signal or nuclear export signal of Ankrd1. Results from these studies will examine the potential effect of cytoplasmic Ankrd1 on regulation of the contractile machinery.

The exact function of Ankrd1 in vivo remains unknown. Immunostaining of human wounds showed significant expression of Ankrd1 in human burn wounds and foot ulcers (data not shown) while normal skin showed little to no staining. Ankrd1 expression was associated with epithelial cells, fibroblasts, and inflammatory cells in human wounds. Analysis of Ankrd1 expression is necessary in more types of human wounds, including normal acute wounds and chronic non-healing wounds. The preliminary studies in human burn wounds emphasize the importance of Ankrd1 in skin repair.

Closing Remarks

Overall, these studies highlight the contributions of Ankrd1 on modulating matrix related molecules and cell-matrix interaction. The work in this thesis provides a foundation for future studies to understand the specific role of Ankrd1 after injury.

REFERENCES

Aihara, Y., Kurabayashi, M., Saito, Y., Ohyama, Y., Tanaka, T., Takeda, S., . . . Nagai, R. (2000). Cardiac ankyrin repeat protein is a novel marker of cardiac hypertrophy: role of M-CAT element within the promoter. *Hypertension, 36*(1), 48-53.

Aihara, Y., Kurabayashi, M., Tanaka, T., Takeda, S. I., Tomaru, K., Sekiguchi, K. I., . . . Nagai, R. (2000). Doxorubicin represses CARP gene transcription through the generation of oxidative stress in neonatal rat cardiac myocytes: possible role of serine/threonine kinase-dependent pathways. *J Mol Cell Cardiol, 32*(8), 1401-1414. doi: 10.1006/jmcc.2000.1173

Ala-aho, R., & Kahari, V. M. (2005). Collagenases in cancer. *Biochimie, 87*(3-4), 273-286. doi: 10.1016/j.biochi.2004.12.009

Almodovar-Garcia, K., Kwon, M., Samaras, S., & Davidson, J. M. (2014). ANKRD1 acts as a transcriptional repressor of MMP-13 via the AP-1 site. *Mol Cell Biol*. doi: 10.1128/MCB.01357-13

Angel, P., Szabowski, A., & Schorpp-Kistner, M. (2001). Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene, 20*(19), 2413-2423. doi: 10.1038/sj.onc.1204380

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., . . . Piccolo, S. (2013). A Mechanical Checkpoint Controls Multicellular Growth through YAP/TAZ Regulation by Actin-Processing Factors. *Cell*. doi: 10.1016/j.cell.2013.07.042

Armstrong, D. G., & Jude, E. B. (2002a). The role of matrix metalloproteinases in wound healing. *Journal of the American Podiatric Medical Association, 92*(1), 12-18.

Armstrong, D. G., & Jude, E. B. (2002b). The role of matrix metalloproteinases in wound healing. *J Am Podiatr Med Assoc, 92*(1), 12-18.

Ashby, W. J., Wikswo, J. P., & Zijlstra, A. (2012). Magnetically attachable stencils and the non-destructive analysis of the contribution made by the underlying matrix to cell migration. *Biomaterials, 33*(33), 8189-8203. doi: 10.1016/j.biomaterials.2012.07.018

Association, A. B. (2013). Burn Incidence and Treatment in the United States: 2013 Fact Sheet. from http://www.ameriburn.org/resources_factsheet.php

Auble, D. T., & Brinckerhoff, C. E. (1991). The AP-1 sequence is necessary but not sufficient for phorbol induction of collagenase in fibroblasts. *Biochemistry, 30*(18), 4629-4635.

Auble, D. T., Sirum-Connolly, K. L., & Brinckerhoff, C. E. (1992). Transcriptional regulation of matrix metalloproteinase genes: role of AP-1 sequences. *Matrix Suppl, 1*, 200.

Avivi, A., Brodsky, L., Nevo, E., & Band, M. R. (2006). Differential expression profiling of the blind subterranean mole rat Spalax ehrenbergi superspecies: bioprospecting for hypoxia tolerance. *Physiol Genomics, 27*(1), 54-64. doi: 10.1152/physiolgenomics.00001.2006

Baker, A. H., Edwards, D. R., & Murphy, G. (2002). Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci, 115*(Pt 19), 3719-3727.

Balbin, M., Fueyo, A., Knauper, V., Lopez, J. M., Alvarez, J., Sanchez, L. M., . . . Lopez-Otin, C. (2001). Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. *J Biol Chem, 276*(13), 10253-10262. doi: 10.1074/jbc.M009586200

Ballas, C. B., & Davidson, J. M. (2001). Delayed wound healing in aged rats is associated with increased collagen gel remodeling and contraction by skin fibroblasts, not with differences in apoptotic or myofibroblast cell populations. *Wound Repair Regen, 9*(3), 223-237.

Bang, M.-L., Gu, Y., Dalton, N. D., Peterson, K. L., Chien, K. R., & Chen, J. (2014). The Muscle Ankyrin Repeat Proteins CARP, Ankrd2, and DARP Are Not Essential for

Normal Cardiac Development and Function at Basal Conditions and in Response to Pressure Overload. *PLoS ONE, 9*(4), e93638. doi: 10.1371/journal.pone.0093638

Bang, M. L., Gu, Y., Dalton, N. D., Peterson, K. L., Chien, K. R., & Chen, J. (2014). The Muscle Ankyrin Repeat Proteins CARP, Ankrd2, and DARP Are Not Essential for Normal Cardiac Development and Function at Basal Conditions and in Response to Pressure Overload. *PLoS ONE, 9*(4), e93638. doi: 10.1371/journal.pone.0093638

Bang, M. L., Mudry, R. E., McElhinny, A. S., Trombitas, K., Geach, A. J., Yamasaki, R., . . . Labeit, S. (2001a). Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. *The Journal of cell biology, 153*(2), 413-427.

Bang, M. L., Mudry, R. E., McElhinny, A. S., Trombitas, K., Geach, A. J., Yamasaki, R., . . . Labeit, S. (2001b). Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. *J Cell Biol, 153*(2), 413-427.

Barash, I. A., Bang, M. L., Mathew, L., Greaser, M. L., Chen, J., & Lieber, R. L. (2007). Structural and regulatory roles of muscle ankyrin repeat protein family in skeletal muscle. *Am J Physiol Cell Physiol, 293*(1), C218-227. doi: 10.1152/ajpcell.00055.2007

Barczyk, M. M., Lu, N., Popova, S. N., Bolstad, A. I., & Gullberg, D. (2013). alpha11beta1 integrin-mediated MMP-13-dependent collagen lattice contraction by fibroblasts: evidence for integrin-coordinated collagen proteolysis. *J Cell Physiol, 228*(5), 1108-1119. doi: 10.1002/jcp.24261

Batra, J., Robinson, J., Soares, A. S., Fields, A. P., Radisky, D. C., & Radisky, E. S. (2012). Matrix metalloproteinase-10 (MMP-10) interaction with tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2: binding studies and crystal structure. *J Biol Chem, 287*(19), 15935-15946. doi: 10.1074/jbc.M112.341156

Belgrano, A., Rakicevic, L., Mittempergher, L., Campanaro, S., Martinelli, V. C., Mouly, V., . . . Faulkner, G. (2011). Multi-tasking role of the mechanosensing protein Ankrd2 in the signaling network of striated muscle. *PLoS ONE, 6*(10), e25519. doi: 10.1371/journal.pone.0025519

Benbow, U., & Brinckerhoff, C. E. (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? *Matrix Biol, 15*(8-9), 519-526.

Berrier, A. L., & Yamada, K. M. (2007). Cell-matrix adhesion. *J Cell Physiol, 213*(3), 565-573. doi: 10.1002/jcp.21237

Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., & Engler, J. A. (1993). Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med, 4*(2), 197-250.

Boengler, K., Pipp, F., Fernandez, B., Ziegelhoeffer, T., Schaper, W., & Deindl, E. (2003). Arteriogenesis is associated with an induction of the cardiac ankyrin repeat protein (carp). *Cardiovasc Res, 59*(3), 573-581.

Bouaouina, M., Harburger, D. S., & Calderwood, D. A. (2012). Talin and signaling through integrins. *Methods Mol Biol, 757*, 325-347. doi: 10.1007/978-1-61779-166-6_20

Brown, P. D. (2000). Ongoing trials with matrix metalloproteinase inhibitors. *Expert Opin Investig Drugs, 9*(9), 2167-2177. doi: 10.1517/13543784.9.9.2167

Bruckner, P. (2010). Suprastructures of extracellular matrices: paradigms of functions controlled by aggregates rather than molecules. *Cell Tissue Res, 339*(1), 7-18. doi: 10.1007/s00441-009-0864-0

Bullard, K. M., Lund, L., Mudgett, J. S., Mellin, T. N., Hunt, T. K., Murphy, B., . . . Banda, M. J. (1999). Impaired wound contraction in stromelysin-1-deficient mice. *Ann Surg, 230*(2), 260-265.

Burgeson, R. E., & Christiano, A. M. (1997). The dermal-epidermal junction. *Curr Opin Cell Biol, 9*(5), 651-658.

Burrage, P. S., Mix, K. S., & Brinckerhoff, C. E. (2006). Matrix metalloproteinases: role in arthritis. *Frontiers in bioscience : a journal and virtual library, 11*, 529-543.

Buxboim, A., Ivanovska, I. L., & Discher, D. E. (2010). Matrix elasticity, cytoskeletal forces and physics of the nucleus: how deeply do cells 'feel' outside and in? *J Cell Sci, 123*(Pt 3), 297-308. doi: 10.1242/jcs.041186

Callewaert, B., Malfait, F., Loeys, B., & De Paepe, A. (2008). Ehlers-Danlos syndromes and Marfan syndrome. *Best Pract Res Clin Rheumatol, 22*(1), 165-189. doi: 10.1016/j.berh.2007.12.005

Carulli, S., Contin, R., De Rosa, L., Pellegrini, G., & De Luca, M. (2013). The long and winding road that leads to a cure for epidermolysis bullosa. *Regen Med, 8*(4), 467-481. doi: 10.2217/rme.13.33

Chang, L. S., Pan, S., Pater, M. M., & Di Mayorca, G. (1985). Differential requirement for SV40 early genes in immortalization and transformation of primary rat and human embryonic cells. *Virology, 146*(2), 246-261.

Chen, B., Zhong, L., Roush, S. F., Pentassuglia, L., Peng, X., Samaras, S., . . . Lim, C. C. (2012). Disruption of a GATA4/Ankrd1 signaling axis in cardiomyocytes leads to sarcomere disarray: implications for anthracycline cardiomyopathy. *PLoS ONE, 7*(4), e35743. doi: 10.1371/journal.pone.0035743

Chen, C. Y., Gherzi, R., Andersen, J. S., Gaietta, G., Jurchott, K., Royer, H. D., . . . Karin, M. (2000a). Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. *Genes Dev, 14*(10), 1236-1248.

Chen, C. Y., Gherzi, R., Andersen, J. S., Gaietta, G., Jurchott, K., Royer, H. D., . . . Karin, M. (2000b). Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. *Genes Dev, 14*(10), 1236-1248.

Chiang, W. C., Wong, Y. K., Lin, S. C., Chang, K. W., & Liu, C. J. (2006). Increase of MMP-13 expression in multi-stage oral carcinogenesis and epigallocatechin-3-gallate suppress MMP-13 expression. *Oral diseases, 12*(1), 27-33. doi: 10.1111/j.1601-0825.2005.01151.x

Chicurel, M. E., Chen, C. S., & Ingber, D. E. (1998). Cellular control lies in the balance of forces. *Curr Opin Cell Biol, 10*(2), 232-239.

Chiquet, M., Gelman, L., Lutz, R., & Maier, S. (2009). From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim Biophys Acta, 1793*(5), 911-920. doi: 10.1016/j.bbamcr.2009.01.012

Chu, W., Burns, D. K., Swerlick, R. A., & Presky, D. H. (1995a). Identification and characterization of a novel cytokine-inducible nuclear protein from human endothelial cells. *J Biol Chem, 270*(17), 10236-10245.

Chu, W., Burns, D. K., Swerlick, R. A., & Presky, D. H. (1995b). Identification and characterization of a novel cytokine-inducible nuclear protein from human endothelial cells. *The Journal of biological chemistry, 270*(17), 10236-10245.

Church, D., Elsayed, S., Reid, O., Winston, B., & Lindsay, R. (2006). Burn wound infections. *Clin Microbiol Rev, 19*(2), 403-434. doi: 10.1128/CMR.19.2.403-434.2006

Clark, A. G., Glanowski, S., Nielsen, R., Thomas, P. D., Kejariwal, A., Todd, M. A., . . . Cargill, M. (2003). Inferring nonneutral evolution from human-chimp-mouse orthologous gene trios. *Science, 302*(5652), 1960-1963. doi: 10.1126/science.1088821

Cooper, T. W., & Bauer, E. A. (1984). Epidermolysis bullosa: a review. *Pediatr Dermatol, 1*(3), 181-188.

Coulombe, P. A., Kerns, M. L., & Fuchs, E. (2009). Epidermolysis bullosa simplex: a paradigm for disorders of tissue fragility. *J Clin Invest, 119*(7), 1784-1793. doi: 10.1172/JCI38177

Cox, E. A., Sastry, S. K., & Huttenlocher, A. (2001). Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol Biol Cell, 12*(2), 265-277.

Cox, T. R., & Erler, J. T. (2011). Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis Model Mech, 4*(2), 165-178. doi: 10.1242/dmm.004077

Cukierman, E., Pankov, R., & Yamada, K. M. (2002). Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol, 14*(5), 633-639.

Davidson JM, Y. F., Opalenik SR. (2013). Splinting strategies to overcome confounding wound contraction in experimental animal models. *Advanced Wound Care*(2), 142-148.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M. A., & Waterman-Storer, C. M. (2005). Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol, 171*(1), 153-164. doi: 10.1083/jcb.200506152

Decock, J., Hendrickx, W., Vanleeuw, U., Van Belle, V., Van Huffel, S., Christiaens, M. R., . . . Paridaens, R. (2008). Plasma MMP1 and MMP8 expression in breast cancer: protective role of MMP8 against lymph node metastasis. *BMC cancer, 8*, 77. doi: 10.1186/1471-2407-8-77

Dunsmore, S. E., Saarialho-Kere, U. K., Roby, J. D., Wilson, C. L., Matrisian, L. M., Welgus, H. G., & Parks, W. C. (1998). Matrilysin expression and function in airway epithelium. *J Clin Invest, 102*(7), 1321-1331. doi: 10.1172/JCI1516

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., . . . Piccolo, S. (2011). Role of YAP/TAZ in mechanotransduction. *Nature, 474*(7350), 179-183. doi: 10.1038/nature10137

Eble, J. A. (2001). The molecular basis of integrin-extracellular matrix interactions. *Osteoarthritis Cartilage, 9 Suppl A*, S131-140.

Eckes, B., & Krieg, T. (2004). Regulation of connective tissue homeostasis in the skin by mechanical forces. *Clin Exp Rheumatol, 22*(3 Suppl 33), S73-76.

Ellison, D. L. (2013). Burns. *Crit Care Nurs Clin North Am, 25*(2), 273-285. doi: 10.1016/j.ccell.2013.02.003

Ezzell, R. M., Goldmann, W. H., Wang, N., Parashurama, N., & Ingber, D. E. (1997). Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. *Exp Cell Res, 231*(1), 14-26. doi: 10.1006/excr.1996.3451

Florin, L., Hummerich, L., Dittrich, B. T., Kokocinski, F., Wrobel, G., Gack, S., . . . Angel, P. (2004). Identification of novel AP-1 target genes in fibroblasts regulated during cutaneous wound healing. *Oncogene, 23*(42), 7005-7017. doi: 10.1038/sj.onc.1207938

Geback, T., Schulz, M. M., Koumoutsakos, P., & Detmar, M. (2009). TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. *Biotechniques, 46*(4), 265-274. doi: 10.2144/000113083

Gill, S. E., & Parks, W. C. (2008). Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol, 40*(6-7), 1334-1347. doi: 10.1016/j.biocel.2007.10.024

Gillitzer, R., & Goebeler, M. (2001). Chemokines in cutaneous wound healing. *J Leukoc Biol, 69*(4), 513-521.

Granet, C., Maslinski, W., & Miossec, P. (2004). Increased AP-1 and NF-kappaB activation and recruitment with the combination of the proinflammatory cytokines IL-1beta, tumor necrosis factor alpha and IL-17 in rheumatoid synoviocytes. *Arthritis Res Ther, 6*(3), R190-198. doi: 10.1186/ar1159

Grinnell, F. (2000). Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. *Trends Cell Biol, 10*(9), 362-365.

Grundstrom, G., Mosher, D. F., Sakai, T., & Rubin, K. (2003). Integrin alphavbeta3 mediates platelet-derived growth factor-BB-stimulated collagen gel contraction in cells expressing signaling deficient integrin alpha2beta1. *Exp Cell Res, 291*(2), 463-473.

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature, 453*(7193), 314-321. doi: 10.1038/nature07039

Halder, G., Dupont, S., & Piccolo, S. (2012). Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat Rev Mol Cell Biol, 13*(9), 591-600. doi: 10.1038/nrm3416

Halper, J., & Kjaer, M. (2014). Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. *Adv Exp Med Biol, 802*, 31-47. doi: 10.1007/978-94-007-7893-1_3

Hamill, K. J., Kligys, K., Hopkinson, S. B., & Jones, J. C. (2009). Laminin deposition in the extracellular matrix: a complex picture emerges. *J Cell Sci, 122*(Pt 24), 4409-4417. doi: 10.1242/jcs.041095

Hartenstein, B., Dittrich, B. T., Stickens, D., Heyer, B., Vu, T. H., Teurich, S., . . . Angel, P. (2006). Epidermal development and wound healing in matrix metalloproteinase 13-deficient mice. *J Invest Dermatol, 126*(2), 486-496. doi: 10.1038/sj.jid.5700084

Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'Armiento, J., & Okada, Y. (2009a). MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol, 175*(2), 533-546. doi: 10.2353/ajpath.2009.081080

Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'Armiento, J., & Okada, Y. (2009b). MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol, 175*(2), 533-546. doi: 10.2353/ajpath.2009.081080

Hayashi, R., Jin, X., & Cook, G. R. (2007). Synthesis and evaluation of novel heterocyclic MMP inhibitors. *Bioorg Med Chem Lett, 17*(24), 6864-6870. doi: 10.1016/j.bmcl.2007.10.020

Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., & Gabbiani, G. (2007). The myofibroblast: one function, multiple origins. *Am J Pathol, 170*(6), 1807-1816. doi: 10.2353/ajpath.2007.070112

Hussain, S. H., Limthongkul, B., & Humphreys, T. R. (2013). The biomechanical properties of the skin. *Dermatol Surg, 39*(2), 193-203. doi: 10.1111/dsu.12095

Hynes, R. (1990). Structure of Fibronectins *Fibronectins* (pp. 113-175): Springer New York.

Ikeda, K., Emoto, N., Matsuo, M., & Yokoyama, M. (2003). Molecular identification and characterization of a novel nuclear protein whose expression is up-regulated in insulin-resistant animals. *J Biol Chem, 278*(6), 3514-3520. doi: 10.1074/jbc.M204563200

Jaalouk, D. E., & Lammerding, J. (2009). Mechanotransduction gone awry. *Nat Rev Mol Cell Biol, 10*(1), 63-73. doi: 10.1038/nrm2597

Jeyaseelan, R., Poizat, C., Baker, R. K., Abdishoo, S., Isterabadi, L. B., Lyons, G. E., & Kedes, L. (1997a). A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *J Biol Chem, 272*(36), 22800-22808.

Jeyaseelan, R., Poizat, C., Baker, R. K., Abdishoo, S., Isterabadi, L. B., Lyons, G. E., & Kedes, L. (1997b). A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *The Journal of biological chemistry, 272*(36), 22800-22808.

Jormsjo, S., Ye, S., Moritz, J., Walter, D. H., Dimmeler, S., Zeiher, A. M., . . . Eriksson, P. (2000). Allele-specific regulation of matrix metalloproteinase-12 gene activity is associated with coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease. *Circ Res, 86*(9), 998-1003.

Kalva, S., Saranyah, K., Suganya, P. R., Nisha, M., & Saleena, L. M. (2013). Potent inhibitors precise to S1' loop of MMP-13, a crucial target for osteoarthritis. *J Mol Graph Model, 44*, 297-310. doi: 10.1016/j.jmgm.2013.06.005

Kanitakis, J. (2002). Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol, 12*(4), 390-399; quiz 400-391.

Kim, S. H., Pudzianowski, A. T., Leavitt, K. J., Barbosa, J., McDonnell, P. A., Metzler, W. J., . . . Pitts, W. (2005). Structure-based design of potent and selective inhibitors of collagenase-3 (MMP-13). *Bioorg Med Chem Lett, 15*(4), 1101-1106. doi: 10.1016/j.bmcl.2004.12.016

Kojic, S., Nestorovic, A., Rakicevic, L., Belgrano, A., Stankovic, M., Divac, A., & Faulkner, G. (2010). A novel role for cardiac ankyrin repeat protein Ankrd1/CARP

as a co-activator of the p53 tumor suppressor protein. *Arch Biochem Biophys, 502*(1), 60-67. doi: 10.1016/j.abb.2010.06.029

Kojic, S., Radojkovic, D., & Faulkner, G. (2011). Muscle ankyrin repeat proteins: their role in striated muscle function in health and disease. *Critical Reviews in Clinical Laboratory Sciences, 48*(5-6), 269-294. doi: doi:10.3109/10408363.2011.643857

Kojic, S., Radojkovic, D., & Faulkner, G. (2011). Muscle ankyrin repeat proteins: their role in striated muscle function in health and disease. *Crit Rev Clin Lab Sci, 48*(5-6), 269-294. doi: 10.3109/10408363.2011.643857

Konttinen, Y. T., Salo, T., Hanemaaijer, R., Valleala, H., Sorsa, T., Sutinen, M., . . . Lopez-Otin, C. (1999). Collagenase-3 (MMP-13) and its activators in rheumatoid arthritis: localization in the pannus-hard tissue junction and inhibition by alendronate. *Matrix Biol, 18*(4), 401-412.

Korpos, E., Wu, C., & Sorokin, L. (2009). Multiple roles of the extracellular matrix in inflammation. *Curr Pharm Des, 15*(12), 1349-1357.

Krieg, T., & Aumailley, M. (2011). The extracellular matrix of the dermis: flexible structures with dynamic functions. *Exp Dermatol, 20*(8), 689-695. doi: 10.1111/j.1600-0625.2011.01313.x

Krieg, T., & LeRoy, E. C. (1998). Diseases of the extracellular matrix. *J Mol Med (Berl), 76*(3-4), 224-225.

Kuo, H., Chen, J., Ruiz-Lozano, P., Zou, Y., Nemer, M., & Chien, K. R. (1999). Control of segmental expression of the cardiac-restricted ankyrin repeat protein gene by distinct regulatory pathways in murine cardiogenesis. *Development, 126*(19), 4223-4234.

Labbe, E., Lock, L., Letamendia, A., Gorska, A. E., Gryfe, R., Gallinger, S., . . . Attisano, L. (2007). Transcriptional cooperation between the transforming growth factor-beta and Wnt pathways in mammary and intestinal tumorigenesis. *Cancer Res, 67*(1), 75-84. doi: 10.1158/0008-5472.CAN-06-2559

Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., & Eckes, B. (1995). Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins. *J Cell Biol, 131*(6 Pt 2), 1903-1915.

Lauer-Fields, J. L., Minond, D., Chase, P. S., Baillargeon, P. E., Saldanha, S. A., Stawikowska, R., . . . Fields, G. B. (2009). High throughput screening of potentially selective MMP-13 exosite inhibitors utilizing a triple-helical FRET substrate. *Bioorg Med Chem, 17*(3), 990-1005. doi: 10.1016/j.bmc.2008.03.004

Lazarides, E. (1976). Actin, alpha-actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. *J Cell Biol, 68*(2), 202-219.

Li, B., & Wang, J. H. (2011). Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability, 20*(4), 108-120. doi: 10.1016/j.jtv.2009.11.004

Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J. M., Pogliano, K., . . . Johnson, R. S. (2003). c-Jun is essential for organization of the epidermal leading edge. *Dev Cell, 4*(6), 865-877.

MacPherson, M., & Fagerholm, S. C. (2010). Filamin and filamin-binding proteins in integrin-regulation and adhesion. Focus on: "FilaminA is required for vimentin-mediated cell adhesion and spreading". *Am J Physiol Cell Physiol, 298*(2), C206-208. doi: 10.1152/ajpcell.00505.2009

Magnanti, M., Gismondi, A., Gandini, O., Rossi, F. M., Michetti, P. M., Santiemma, V., & Morrone, S. (2001). Integrin pattern and effect on contraction in cultured testicular peritubular myoid cells. *Am J Reprod Immunol, 45*(1), 21-27.

Mak, I. W., Turcotte, R. E., Popovic, S., Singh, G., & Ghert, M. (2011). AP-1 as a Regulator of MMP-13 in the Stromal Cell of Giant Cell Tumor of Bone. *Biochem Res Int, 2011*, 164197. doi: 10.1155/2011/164197

Mariani, T. J., Sandefur, S., Roby, J. D., & Pierce, R. A. (1998). Collagenase-3 induction in rat lung fibroblasts requires the combined effects of tumor necrosis factor-alpha and 12-lipoxygenase metabolites: a model of macrophage-induced,

fibroblast-driven extracellular matrix remodeling during inflammatory lung injury. *Mol Biol Cell, 9*(6), 1411-1424.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science, 276*(5309), 75-81.

Martins, V. L., Caley, M., & O'Toole, E. A. (2013). Matrix metalloproteinases and epidermal wound repair. *Cell Tissue Res, 351*(2), 255-268. doi: 10.1007/s00441-012-1410-z

Matsuura, K., Uesugi, N., Hijiya, N., Uchida, T., & Moriyama, M. (2007). Upregulated expression of cardiac ankyrin-repeated protein in renal podocytes is associated with proteinuria severity in lupus nephritis. *Hum Pathol, 38*(3), 410-419. doi: 10.1016/j.humpath.2006.09.006

McNaughton, V., & Brazil, K. (1995). Wound and skin team. Impact on pressure ulcer prevalence in chronic care. *J Gerontol Nurs, 21*(2), 45-49.

Medina, A., Scott, P. G., Ghahary, A., & Tredget, E. E. (2005). Pathophysiology of chronic nonhealing wounds. *J Burn Care Rehabil, 26*(4), 306-319.

Mengshol, J. A., Vincenti, M. P., Coon, C. I., Barchowsky, A., & Brinckerhoff, C. E. (2000). Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum, 43*(4), 801-811. doi: 10.1002/1529-0131(200004)43:4<801::AID-ANR10>3.0.CO;2-4

Midwood, K. S., Williams, L. V., & Schwarzbauer, J. E. (2004). Tissue repair and the dynamics of the extracellular matrix. *Int J Biochem Cell Biol, 36*(6), 1031-1037. doi: 10.1016/j.biocel.2003.12.003

Mikhailov, A. T., & Torrado, M. (2006). The cardiac ankyrin repeat domain 1 protein: do you know enough about its dimerization properties? *J Muscle Res Cell Motil, 27*(3-4), 203-204; author reply 251-202. doi: 10.1007/s10974-006-9061-x

Mikhailov, A. T., & Torrado, M. (2008). The enigmatic role of the ankyrin repeat domain 1 gene in heart development and disease. *Int J Dev Biol, 52*(7), 811-821. doi: 10.1387/ijdb.082655am

Miller, M. K., Bang, M. L., Witt, C. C., Labeit, D., Trombitas, C., Watanabe, K., . . . Labeit, S. (2003). The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J Mol Biol, 333*(5), 951-964.

Mitchell, P. G., Magna, H. A., Reeves, L. M., Lopresti-Morrow, L. L., Yocum, S. A., Rosner, P. J., . . . Hambor, J. E. (1996). Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J Clin Invest, 97*(3), 761-768. doi: 10.1172/JCI118475

Mittelstadt, M. L., & Patel, R. C. (2012). AP-1 Mediated Transcriptional Repression of Matrix Metalloproteinase-9 by Recruitment of Histone Deacetylase 1 in Response to Interferon beta. *PLoS ONE, 7*(8), e42152. doi: 10.1371/journal.pone.0042152

PONE-D-12-08983 [pii]

Miyake, K., Kimura, S., Nakanishi, M., Hisada, A., Hasegawa, M., Nagao, S., & Abe, Y. (2000). Transforming growth factor-beta1 stimulates contraction of human glioblastoma cell-mediated collagen lattice through enhanced alpha2 integrin expression. *J Neuropathol Exp Neurol, 59*(1), 18-28.

Mohamed, J. S., & Boriek, A. M. (2012). Loss of desmin triggers mechanosensitivity and up-regulation of Ankrd1 expression through Akt-NF-kappaB signaling pathway in smooth muscle cells. *FASEB J, 26*(2), 757-765. doi: 10.1096/fj.10-160291

Mongelard, F., & Bouvet, P. (2007). Nucleolin: a multiFACeTed protein. *Trends Cell Biol, 17*(2), 80-86. doi: 10.1016/j.tcb.2006.11.010

Moore, B. A., Aznavoorian, S., Engler, J. A., & Windsor, L. J. (2000). Induction of collagenase-3 (MMP-13) in rheumatoid arthritis synovial fibroblasts. *Biochim Biophys Acta, 1502*(2), 307-318.

Moore, K. (1999). Cell biology of chronic wounds: the role of inflammation. *J Wound Care, 8*(7), 345-348.

Mosavi, L. K., Cammett, T. J., Desrosiers, D. C., & Peng, Z. Y. (2004). The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci, 13*(6), 1435-1448. doi: 10.1110/ps.03554604

Mott, J. D., & Werb, Z. (2004). Regulation of matrix biology by matrix metalloproteinases. *Curr Opin Cell Biol, 16*(5), 558-564. doi: 10.1016/j.ceb.2004.07.010

Moulik, M., Vatta, M., Witt, S. H., Arola, A. M., Murphy, R. T., McKenna, W. J., . . . Towbin, J. A. (2009). ANKRD1, the gene encoding cardiac ankyrin repeat protein, is a novel dilated cardiomyopathy gene. *J Am Coll Cardiol, 54*(4), 325-333. doi: 10.1016/j.jacc.2009.02.076

Moulin, V., Auger, F. A., Garrel, D., & Germain, L. (2000). Role of wound healing myofibroblasts on re-epithelialization of human skin. *Burns, 26*(1), 3-12.

Moulin, V., Garrel, D., Auger, F. A., O'Connor-McCourt, M., Castilloux, G., & Germain, L. (1999). What's new in human wound-healing myofibroblasts? *Curr Top Pathol, 93*, 123-133.

Moulin, V., Larochelle, S., Langlois, C., Thibault, I., Lopez-Valle, C. A., & Roy, M. (2004). Normal skin wound and hypertrophic scar myofibroblasts have differential responses to apoptotic inductors. *J Cell Physiol, 198*(3), 350-358. doi: 10.1002/jcp.10415

Nagueh, S. F., Shah, G., Wu, Y., Torre-Amione, G., King, N. M., Lahmers, S., . . . Granzier, H. L. (2004). Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. *Circulation, 110*(2), 155-162. doi: 10.1161/01.CIR.0000135591.37759.AF

Nakada, C., Tsukamoto, Y., Oka, A., Nonaka, I., Takeda, S., Sato, K., . . . Moriyama, M. (2003). Cardiac-restricted ankyrin-repeated protein is differentially induced in duchenne and congenital muscular dystrophy. *Lab Invest, 83*(5), 711-719.

Neuhold, L. A., Killar, L., Zhao, W., Sung, M. L., Warner, L., Kulik, J., . . . DeGennaro, L. J. (2001). Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J Clin Invest, 107*(1), 35-44. doi: 10.1172/JCI10564

Ngo, P., Ramalingam, P., Phillips, J. A., & Furuta, G. T. (2006). Collagen gel contraction assay. *Methods Mol Biol, 341*, 103-109. doi: 10.1385/1-59745-113-4:103

Nizet, J. L., & Pierard, G. E. (2009). [Biomechanical properties of skin during tissue expansion for breast-reconstructive surgery]. *Ann Chir Plast Esthet, 54*(1), 45-50. doi: 10.1016/j.anplas.2008.05.002

Normand, J., & Karasek, M. A. (1995). A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In Vitro Cell Dev Biol Anim, 31*(6), 447-455. doi: 10.1007/BF02634257

Ogawa, R., Akaishi, S., & Izumi, M. (2009). Histologic analysis of keloids and hypertrophic scars. *Ann Plast Surg, 62*(1), 104-105. doi: 10.1097/SAP.0b013e3181855172

Olsen, C. E., Liguori, A. E., Zong, Y., Lantz, R. C., Burgess, J. L., & Boitano, S. (2008). Arsenic upregulates MMP-9 and inhibits wound repair in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 295*(2), L293-302. doi: 10.1152/ajplung.00134.2007

Paller, M. S. (1997). Integrins and repair after acute renal injury. *Kidney Int Suppl, 61*, S52-55.

Parekh, A., Sandulache, V. C., Lieb, A. S., Dohar, J. E., & Hebda, P. A. (2007). Differential regulation of free-floating collagen gel contraction by human fetal and adult dermal fibroblasts in response to prostaglandin E2 mediated by an EP2/cAMP-dependent mechanism. *Wound Repair Regen, 15*(3), 390-398. doi: 10.1111/j.1524-475X.2007.00241.x

Piccolo, S., Cordenonsi, M., & Dupont, S. (2013). Molecular Pathways: YAP and TAZ Take Center Stage in Organ Growth and Tumorigenesis. *Clin Cancer Res*. doi: 10.1158/1078-0432.CCR-12-3172

Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., & Parks, W. C. (1997). The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol, 137*(6), 1445-1457.

Pilcher, B. K., Wang, M., Qin, X. J., Parks, W. C., Senior, R. M., & Welgus, H. G. (1999). Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. *Annals of the New York Academy of Sciences, 878*, 12-24.

Pozzi, A., & Zent, R. (2003). Integrins: sensors of extracellular matrix and modulators of cell function. *Nephron Exp Nephrol, 94*(3), e77-84. doi: 72025

Price, L. S., Leng, J., Schwartz, M. A., & Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell, 9*(7), 1863-1871.

Ravanti, L., Heino, J., Lopez-Otin, C., & Kahari, V. M. (1999a). Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. *J Biol Chem, 274*(4), 2446-2455.

Ravanti, L., Heino, J., Lopez-Otin, C., & Kahari, V. M. (1999b). Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. *The Journal of biological chemistry, 274*(4), 2446-2455.

Ravanti, L., & Kahari, V. M. (2000). Matrix metalloproteinases in wound repair (review). *International journal of molecular medicine, 6*(4), 391-407.

Ravanti, L., Toriseva, M., Penttinen, R., Crombleholme, T., Foschi, M., Han, J., & Kahari, V. M. (2001). Expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts is induced by transforming growth factor beta via p38 mitogen-activated protein kinase. *FASEB J, 15*(6), 1098-1100.

Ray, J. L., Leach, R., Herbert, J. M., & Benson, M. (2001). Isolation of vascular smooth muscle cells from a single murine aorta. *Methods in cell science : an official journal of the Society for In Vitro Biology, 23*(4), 185-188.

Reboul, P., Pelletier, J. P., Tardif, G., Cloutier, J. M., & Martel-Pelletier, J. (1996). The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. *J Clin Invest, 97*(9), 2011-2019. doi: 10.1172/JCI118636

Ricard-Blum, S., & Ruggiero, F. (2005). The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol Biol (Paris), 53*(7), 430-442. doi: 10.1016/j.patbio.2004.12.024

Rock, K., & Fischer, J. W. (2011). [Role of the extracellular matrix in extrinsic skin aging]. *Hautarzt, 62*(8), 591-597. doi: 10.1007/s00105-011-2133-x

Rodriguez, J. A., Orbe, J., Martinez de Lizarrondo, S., Calvayrac, O., Rodriguez, C., Martinez-Gonzalez, J., & Paramo, J. A. (2008). Metalloproteinases and atherothrombosis: MMP-10 mediates vascular remodeling promoted by inflammatory stimuli. *Frontiers in bioscience : a journal and virtual library, 13*, 2916-2921.

Saaristo, A., Tammela, T., Farkkila, A., Karkkainen, M., Suominen, E., Yla-Herttuala, S., & Alitalo, K. (2006). Vascular endothelial growth factor-C accelerates diabetic wound healing. *Am J Pathol, 169*(3), 1080-1087. doi: S0002-9440(10)62782-4 [pii]

10.2353/ajpath.2006.051251

Samaras, S. E., Shi, Y., & Davidson, J. M. (2006a). CARP: fishing for novel mechanisms of neovascularization. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research, 11*(1), 124-131.

Samaras, S. E., Shi, Y., & Davidson, J. M. (2006b). CARP: fishing for novel mechanisms of neovascularization. *J Investig Dermatol Symp Proc, 11*(1), 124-131.

Samuel, S., Beifuss, K. K., & Bernstein, L. R. (2007). YB-1 binds to the MMP-13 promoter sequence and represses MMP-13 transactivation via the AP-1 site. *Biochim Biophys Acta, 1769*(9-10), 525-531. doi: 10.1016/j.bbaexp.2007.07.003

Samuel, S., Twizere, J. C., Beifuss, K. K., & Bernstein, L. R. (2008). Nucleolin binds specifically to an AP-1 DNA sequence and represses AP1-dependent transactivation of the matrix metalloproteinase-13 gene. *Mol Carcinog, 47*(1), 34-46. doi: 10.1002/mc.20358

Schiro, J. A., Chan, B. M., Roswit, W. T., Kassner, P. D., Pentland, A. P., Hemler, M. E., . . . Kupper, T. S. (1991). Integrin alpha 2 beta 1 (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell, 67*(2), 403-410.

Schultz, G. S., & Wysocki, A. (2009). Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen, 17*(2), 153-162. doi: 10.1111/j.1524-475X.2009.00466.x

Schwarzbauer, J. E., & Sechler, J. L. (1999). Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr Opin Cell Biol, 11*(5), 622-627.

Sedgwick, S. G., & Smerdon, S. J. (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci, 24*(8), 311-316.

Shi, Y., Reitmaier, B., Regenbogen, J., Slowey, R. M., Opalenik, S. R., Wolf, E., . . . Davidson, J. M. (2005a). CARP, a cardiac ankyrin repeat protein, is up-regulated during wound healing and induces angiogenesis in experimental granulation tissue. *Am J Pathol, 166*(1), 303-312. doi: 10.1016/S0002-9440(10)62254-7

Shi, Y., Reitmaier, B., Regenbogen, J., Slowey, R. M., Opalenik, S. R., Wolf, E., . . . Davidson, J. M. (2005b). CARP, a cardiac ankyrin repeat protein, is up-regulated during wound healing and induces angiogenesis in experimental granulation tissue. *The American journal of pathology, 166*(1), 303-312. doi: 10.1016/S0002-9440(10)62254-7

Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *N Engl J Med, 341*(10), 738-746. doi: 10.1056/NEJM199909023411006

Singh, P., Carraher, C., & Schwarzbauer, J. E. (2010). Assembly of fibronectin extracellular matrix. *Annu Rev Cell Dev Biol, 26*, 397-419. doi: 10.1146/annurev-cellbio-100109-104020

Sorokin, L. (2010). The impact of the extracellular matrix on inflammation. *Nat Rev Immunol, 10*(10), 712-723. doi: 10.1038/nri2852

Stahle-Backdahl, M., Sandstedt, B., Bruce, K., Lindahl, A., Jimenez, M. G., Vega, J. A., & Lopez-Otin, C. (1997). Collagenase-3 (MMP-13) is expressed during human fetal ossification and re-expressed in postnatal bone remodeling and in rheumatoid arthritis. *Lab Invest, 76*(5), 717-728.

Takaishi, H., Kimura, T., Dalal, S., Okada, Y., & D'Armiento, J. (2008). Joint diseases and matrix metalloproteinases: a role for MMP-13. *Curr Pharm Biotechnol, 9*(1), 47-54.

Takino, T., Sato, H., & Seiki, M. (1995). [Molecular biology of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), and the regulation of these genes in tumor tissues]. *Nihon Rinsho, 53*(7), 1791-1797.

Thomas, P. D., Kejariwal, A., Campbell, M. J., Mi, H., Diemer, K., Guo, N., . . . Doremieux, O. (2003). PANTHER: a browsable database of gene products organized by biological function, using curated protein family and subfamily classification. *Nucleic Acids Res, 31*(1), 334-341.

Tian, B., Lessan, K., Kahm, J., Kleidon, J., & Henke, C. (2002). beta 1 integrin regulates fibroblast viability during collagen matrix contraction through a phosphatidylinositol 3-kinase/Akt/protein kinase B signaling pathway. *J Biol Chem, 277*(27), 24667-24675. doi: 10.1074/jbc.M203565200

Toriseva, M., Laato, M., Carpen, O., Ruohonen, S. T., Savontaus, E., Inada, M., . . . Kahari, V. M. (2012). MMP-13 regulates growth of wound granulation tissue and modulates gene expression signatures involved in inflammation, proteolysis, and cell viability. *PLoS ONE, 7*(8), e42596. doi: 10.1371/journal.pone.0042596

Toriseva, M. J., Ala-aho, R., Karvinen, J., Baker, A. H., Marjomaki, V. S., Heino, J., & Kahari, V. M. (2007). Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts. *J Invest Dermatol, 127*(1), 49-59. doi: 10.1038/sj.jid.5700500

Torrado, M., Nespereira, B., Lopez, E., Centeno, A., Castro-Beiras, A., & Mikhailov, A. T. (2005). ANKRD1 specifically binds CASQ2 in heart extracts and both proteins

are co-enriched in piglet cardiac Purkinje cells. *J Mol Cell Cardiol, 38*(2), 353-365.

Tse, J. R., & Engler, A. J. (2010). Preparation of hydrogel substrates with tunable mechanical properties. *Curr Protoc Cell Biol, Chapter 10*, Unit 10 16. doi: 10.1002/0471143030.cb1016s47

Tsou, J. H., Chang, K. Y., Wang, W. C., Tseng, J. T., Su, W. C., Hung, L. Y., . . . Chen, B. K. (2008). Nucleolin regulates c-Jun/Sp1-dependent transcriptional activation of cPLA2alpha in phorbol ester-treated non-small cell lung cancer A549 cells. *Nucleic Acids Res, 36*(1), 217-227. doi: 10.1093/nar/gkm1027

Tuteja, R., & Tuteja, N. (1998). Nucleolin: a multifunctional major nucleolar phosphoprotein. *Crit Rev Biochem Mol Biol, 33*(6), 407-436. doi: 10.1080/10409239891204260

Vaalamo, M., Mattila, L., Johansson, N., Kariniemi, A. L., Karjalainen-Lindsberg, M. L., Kahari, V. M., & Saarialho-Kere, U. (1997a). Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. *J Invest Dermatol, 109*(1), 96-101.

Vaalamo, M., Mattila, L., Johansson, N., Kariniemi, A. L., Karjalainen-Lindsberg, M. L., Kahari, V. M., & Saarialho-Kere, U. (1997b). Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. *The Journal of investigative dermatology, 109*(1), 96-101.

van Lunteren, E., Moyer, M., & Leahy, P. (2006). Gene expression profiling of diaphragm muscle in alpha2-laminin (merosin)-deficient dy/dy dystrophic mice. *Physiol Genomics, 25*(1), 85-95. doi: 10.1152/physiolgenomics.00226.2005

Vincenti, M. P., & Brinckerhoff, C. E. (2002). Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res, 4*(3), 157-164.

Vu, T. H., & Werb, Z. (2000). Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev, 14*(17), 2123-2133.

Wagenseil, J. E., & Mecham, R. P. (2009). Vascular extracellular matrix and arterial mechanics. *Physiol Rev, 89*(3), 957-989. doi: 10.1152/physrev.00041.2008

Weckroth, M., Vaheri, A., Lauharanta, J., Sorsa, T., & Konttinen, Y. T. (1996). Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol, 106*(5), 1119-1124.

Wernicke, D., Seyfert, C., Gromnica-Ihle, E., & Stiehl, P. (2006). The expression of collagenase 3 (MMP-13) mRNA in the synovial tissue is associated with histopathologic type II synovitis in rheumatoid arthritis. *Autoimmunity, 39*(4), 307-313. doi: 10.1080/08916930600807709

Witt, C. C., Witt, S. H., Lerche, S., Labeit, D., Back, W., & Labeit, S. (2008). Cooperative control of striated muscle mass and metabolism by MuRF1 and MuRF2. *EMBO J, 27*(2), 350-360. doi: 10.1038/sj.emboj.7601952

Witt, S. H., Labeit, D., Granzier, H., Labeit, S., & Witt, C. C. (2005). Dimerization of the cardiac ankyrin protein CARP: implications for MARP titin-based signaling. *J Muscle Res Cell Motil, 26*(6-8), 401-408. doi: 10.1007/s10974-005-9022-9

Wu, N., Opalenik, S., Liu, J., Jansen, E. D., Giro, M. G., & Davidson, J. M. (2002). Real-time visualization of MMP-13 promoter activity in transgenic mice. *Matrix Biol, 21*(2), 149-161.

Wysocki, A. B. (1999). Skin anatomy, physiology, and pathophysiology. *Nurs Clin North Am, 34*(4), 777-797, v.

Wysocki, A. B., Staiano-Coico, L., & Grinnell, F. (1993). Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol, 101*(1), 64-68.

Xia, H., Nho, R. S., Kahm, J., Kleidon, J., & Henke, C. A. (2004). Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J Biol Chem, 279*(31), 33024-33034. doi: 10.1074/jbc.M313265200

Xu, Y., Swerlick, R. A., Sepp, N., Bosse, D., Ades, E. W., & Lawley, T. J. (1994). Characterization of expression and modulation of cell adhesion molecules on an immortalized human dermal microvascular endothelial cell line (HMEC-1). *J Invest Dermatol, 102*(6), 833-837.

Yamagata, K., Li, X., Ikegaki, S., Oneyama, C., Okada, M., Nishita, M., & Minami, Y. (2012). Dissection of Wnt5a-Ror2 signaling leading to matrix metalloproteinase (MMP-13) expression. *J Biol Chem, 287*(2), 1588-1599. doi: 10.1074/jbc.M111.315127

Yan, C., & Boyd, D. D. (2007). Regulation of matrix metalloproteinase gene expression. *J Cell Physiol, 211*(1), 19-26. doi: 10.1002/jcp.20948

Yu F, O. S., Samson, PC, Schaffer DK, Wikswo JP, Davidson JM. (2010). *A novel PDMS device to prevent contraction in rodent excisional wounds*. Abstract. Wound Repair Regen.

Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., . . . Wagner, E. F. (2003). c-Jun regulates eyelid closure and skin tumor development through EGFR signaling. *Dev Cell, 4*(6), 879-889.

Zhou, Z., Cornelius, C. P., Eichner, M., & Bornemann, A. (2006). Reinnervation-induced alterations in rat skeletal muscle. *Neurobiol Dis, 23*(3), 595-602. doi: 10.1016/j.nbd.2006.05.012

Zolk, O., Frohme, M., Maurer, A., Kluxen, F. W., Hentsch, B., Zubakov, D., . . . Eschenhagen, T. (2002). Cardiac ankyrin repeat protein, a negative regulator of cardiac gene expression, is augmented in human heart failure. *Biochem Biophys Res Commun, 293*(5), 1377-1382. doi: 10.1016/S0006-291X(02)00387-X

Zolk, O., Marx, M., Jackel, E., El-Armouche, A., & Eschenhagen, T. (2003). Beta-adrenergic stimulation induces cardiac ankyrin repeat protein expression: involvement of protein kinase A and calmodulin-dependent kinase. *Cardiovasc Res, 59*(3), 563-572.

Zou, Y., Evans, S., Chen, J., Kuo, H. C., Harvey, R. P., & Chien, K. R. (1997). CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway. *Development, 124*(4), 793-804.

Zyada, M. M., & Shamaa, A. A. (2008). Is collagenase-3 (MMP-13) expression in chondrosarcoma of the jaws a true marker for tumor aggressiveness? *Diagnostic pathology, 3*, 26. doi: 10.1186/1746-1596-3-26