Elucidating interactions between the dermal fibroblast phenotype, inflammatory signals and extra- cellular matrix components

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Elucidating interactions between the dermal fibroblast phenotype, inflammatory mediators and extra- cellular matrix components

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The study of dermal wound healing has long been used to elucidate the cellular and molecular processes guiding the connective tissue response to injury. Of particular interest are the mechanisms by which soluble mediators, including inflammatory signals, guide fibroblast activity within the wound bed. This thesis addresses the role of prostaglandin E2 (PGE2) in the regulation of fibroblast activities relevant to restoration of tissue structure and function. Although PGE2 has been previously shown to play an important role in various wound healing steps, its precise contribution to the overall outcome of dermal repair is unclear. Using three well defined human dermal fibroblast phenotypes this study demonstrates that while PGE2 signaling during dermal repair triggers pro-inflammatory cascades, its effects on fibroblast activities are putatively anti-fibrotic. Specifically, exogenous PGE2 decreases the migratory and contractile potential of dermal fibroblasts through destabilization of the actin cytoskeleton and inhibits endogenous collagen synthesis. While PGE2 effects on fibroblast activity are largely conserved across phenotypes, fetal fibroblasts maintain a quantitatively diminished response to PGE2-induced alterations of cytoskeletal dynamics.

Upon further analysis, this effect was shown to be representative of a larger intrinsic fibroblast phenotype. Fetal dermal fibroblasts were shown to maintain elevated rates of migration and contraction, as part of a generalized hyperactive dynamic state. Surprisingly, this phenotype was found to be sufficiently robust so as to persist despite changes in substrate and environmental constraints. In light of this finding, one additional approach was used to ascertain the robustness of the fetal fibroblast. Transplantation of fetal dermal fibroblasts into an adult wound environment was used to assess whether the intrinsic fetal fibroblast phenotype can survive the multitude of events comprising adult wound healing. While results are preliminary, this approach does present a useful tool for future studies aimed at elucidating the precise fetal fibroblast phenotype and its contribution to overall wound healing response.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

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1.0 ABSTRACTS, BOOK CHAPTERS AND PEER REVIEWED ARTICLES

Abstracts

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2.0 INTRODUCTION

Abnormal wound healing, characterized by improper fibroblast activity in the wound bed, is responsible for multiple pathologies, ranging from diabetic ulcers, to fibrosis and scarring. Fibroblasts are the primary cell type responsible for deposition/organization of the extracellular matrix (ECM) replacing injured tissue. This process is largely controlled by soluble signals including inflammatory mediators secreted by infiltrating immune cells. Early in the repair process, the emphasis is on rapid collagen secretion. In the latter phases of wound healing, collagen deposition decreases, and the focus shifts to re-organization of the secondary matrix. This shift is accompanied by a switch in the inflammatory mediator profile toward anti-inflammatory, anti-fibrotic mediators, which down-regulates fibroblast activity in the wound bed until homeostasis is achieved. Despite eventual resolution of the repair process, in post-natal wound healing, the original ECM structure is not completely regained, the ECM composition is not regenerated and as such wound healing results in the formation of scar/fibrosis. In contrast to post-natal wound healing, fetal wound healing is a scarless, regenerative process. This phenomenon has been associated with intrinsic fetal fibroblast properties, a diminished inflammatory response to injury and the deposition of specific extra-cellular matrix components. While fetal wound healing has yet to be reproduced in an adult tissue using exogenous manipulation, it offers an important tool for the study of wound healing in general, and of the relationship between inflammation and fibroplasia in particular.[1,2,3] Using this tool, a series of studies was designed under the umbrella of a generalized hypothesis: wound healing outcomes are determined by the multi-faceted interactions between the intrinsic fibroblast phenotype, inflammatory mediators and extra-cellular matrix components. To test this hypothesis, the interactions of distinct fibroblast phenotypes with a specific inflammatory mediator (prostaglandin E2) and varying environmental constraints were analyzed with regard to cell activities relevant to the wound healing response.

Prostaglandin E2 (PGE2), a ubiquitous inflammatory mediator, has been shown to regulate fibroblast proliferation, migration and extracellular matrix (ECM) synthesis and re- modeling. Abnormalities in PGE2 signaling have been linked to the development of fibrosis in a variety of tissues. However, the precise role of PGE2 during dermal wound healing remains unclear. This is a result of several phenomena: 1) PGE2 effects on fibrosis are tissue specific (with most research to date focused on areas other than skin), 2) PGE2 effects on in vitro fibroblast activity do not correlate with in vivo findings which associate elevated PGE2 levels with increased fibroplasia, 3) PGE2 effects on dermal inflammation cannot be separated from direct PGE2 effects on fibroblast activity under in vivo conditions. This study was designed to clarify the role of PGE2 in dermal wound healing using a combination of in vivo and in vitro approaches. Specifically, analysis focused on the characterization of PGE2 signaling in

dermal fibroblasts of various phenotypes (fetal, normal/adult, keloid) to elucidate three aspects of this pathway: 1) fibroblast synthesis and degradation of PGE2, 2) PGE2 receptor expression in fibroblasts and 3) fibroblast responses to exogenous PGE2. This constitutes the first comprehensive investigation of PGE2 signaling in fetal, adult, and keloid fibroblasts, and as such serves to clarify the putative effects of PGE2 during dermal tissue repair.

The approach detailed above has been previously employed to study the effects of various soluble mediators on the activity of fibroblasts of different phenotypes (fetal, adult, etc). Unfortunately, this analytical paradigm suffers from the drawback of excessive reduction. Wound healing has been shown to be a complex process, displaying a carefully choreographed series of events, coordinated by multiple soluble mediators. In recent years, it has become increasingly obvious that simple reductionist approaches to the study of wound healing have insufficient predictive capacity for the larger repair process. The current investigation utilized an analytical approach which relies on increasingly complex extrinsic conditions to better understand and characterize differences in the intrinsic fibroblast phenotype. This approach involves challenging fibroblasts with increasing exogenous constraints, in order to determine loss or preservation of the intrinsic phenotype. Such an approach, when combined with more traditional investigative means (as described above) holds promise for providing a better understanding of the dynamic processes underlying dermal wound healing. The specific aims of the series of studies detailed in this manuscript are summarized below.

Specific Aim 1: To characterize the regulation of PGE2 signaling in dermal fibroblasts with respect to: 1) fibroblast synthesis of PGE2, 2) fibroblast degradation of PGE2, and 3) PGE2 receptor expression in fibroblasts. Endogenous PGE2 production and degradation by fetal, normal/adult, and keloid fibroblasts in response to primary inflammatory cues (IL-1β) were examined with respect to the following parameters: expression of cycloxygenase-2 (COX-2), microsomal PGE2 synthase (mPGES) and 15 prostaglandin dehydrogenase (15-PGDH) and secretion of PGE2. PGE2 receptor expression in these three cell types were ascertained and putative regulatory triggers were examined.

Hypothesis 1.1: IL-1β up-regulates production, and inhibits degradation of endogenous dermal fibroblast PGE2.

Hypothesis 1.2: Endogenous dermal fibroblast production of PGE2 is enhanced in keloid fibroblasts and diminished in fetal fibroblasts compared to their adult/normal counterparts.

Specific Aim 2: To characterize the effects of PGE2 on fibroblast migration, collagen lattice contraction and collagen production. The effects of PGE2 on the migratory, contractile and synthetic properties of fetal, adult/normal and keloid fibroblasts were characterized using in vitro methodology. This analysis offers a needed insight into the role of PGE2 in regulation of dermal wound healing and scar formation.

Hypothesis 2.1: PGE2 effects on fibroblast migration, contraction and ECM synthesis are reduced in keloid fibroblasts and enhanced in fetal fibroblasts, when compared to normal/adult dermal fibroblasts.

Specific Aim 3: To develop an analytical approach addressing the impact of changing environmental constraints on the fibroblast phenotype. Fetal and adult dermal fibroblast interactions with ECM components

were analyzed in the context of increasing exogenous constraints such as: 1) transition from inert to bioactive substrates, 2), transition from a 2- to a 3- dimensional construct and 3) transplantation of fetal fibroblasts into immuno- competent adult dermis. Interactions of fibroblasts with these substrates were analyzed with respect to: 1) effects of environment on fibroblast activity (grafting, invasion, survival) and 2) effects of fibroblast activity on extracellular matrix re- modeling.

Hypothesis 3.1: Fetal fibroblasts display unique properties with respect to interactions with 2- or 3- dimensional, inert or bioactive substrates.

Hypothesis 3.2: Fetal fibroblasts transplanted into adult wounds can accelerate dermal wound healing and alter overall ECM organization in the wound bed.

The studies detailed below, reveal that there exists a complex interaction between the fibroblast phenotype, inflammatory mediators (PGE2) and exogenous constraints. Specifically, PGE2 was found to have profound, and putatively anti-fibrotic effects on fibroblast activities, which are partially conserved across fibroblast phenotypes. In general, the intrinsic fibroblast phenotype was found to be relatively robust and to survive significant changes in environmental conditions.

2.1 WOUND HEALING OVERVIEW

Wound healing is a complex process with the goal of restoration of pre- injury tissue structure and function. Like other complex physical processes, wound healing can result in a variety of outcomes characterized by the degree to which the initial structure and function of the tissue is recapitulated. Exuberant wound healing (i.e. keloid formation) characterized by excessive scar formation and incomplete regain of tissue function [1] represents one end of the spectrum. In contrast, fetal wound healing represents a more regenerative model of healing. Although it has long been recognized that the quality of postnatal wound healing is age-dependent to a certain extent, the repair process at the fetal level is fundamentally different from the postnatal process. Healed fetal wounds are often clinically and histologically indistinguishable from unwounded tissue.[2] Normal adult wound healing falls between these two extremes, producing an acceptable level of scar. Several differences in the cascade of cellular and molecular events are thought to be responsible for these varied wound healing outcomes. Two key processes that appear to be dramatically altered in these wound healing phenotypes are: 1) inflammation and 2) extracellular matrix (ECM) synthesis and remodeling.

The normal wound healing response is a time-dependent process which is composed of early, intermediate and late stages: hemostasis, inflammation, fibroplasia, cellular proliferation, and remodeling (Figure 1).[1,3,4] Hemostasis is achieved by a combination of vasoconstriction and platelet aggregation yielding the prevention of excessive fluid (blood) loss. Fibrin clot formation plugs the initial tissue gap and serves as a host to the initial wave of inflammatory cells which consists primarily of macrophages and neutrophils. These cells clear endogenous cell debris, necrotic tissue, protein fragments and invasive pathogens, clearing the wound bed for subsequent tissue

repair. The activity of inflammatory cells within the initial wound bed matrix is highly organized via a complex network of soluble mediators which includes growth factors, cytokines, chemokines and arachidonic acid derivatives. These mediators further aid in the coordination of inflammation with subsequent wound healing steps such as re- epithelialization, ECM synthesis and deposition.

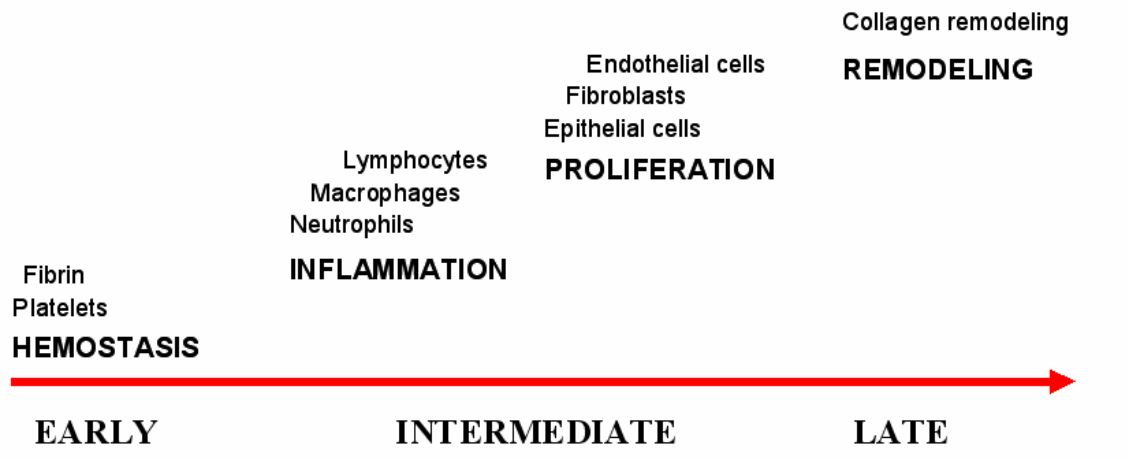


Figure 1: Wound healing is composed of multiple sequential, overlapping stages

The initial fibrin matrix infiltrate is subsequently replaced by a provisional matrix of granulation tissue, rich in new blood vessels, proteoglycans and hyaluronic acid. Bulk tissue repair coincides with wound re-epithelialization, a process driven by keratinocyte migration, proliferation and differentiation. Collagen is the primary component of normal dermis and is also important in tissue repair. Collagen deposition in particular, and ECM rebuilding in general, within the wound bed is a function of fibroblast activity. During wound healing, fibroblasts transition from an invasive, proliferative phenotype to primarily a synthetic one, during which collagen production is the primary goal. This transition is highlighted by the appearance in the wound bed of myofibroblasts, a distinct fibroblast subtype whose predominant characteristics include increased collagen synthesis, elevated migratory and contractile rates. Over weeks and months wound fibroblasts remodel the secreted collagen fibrils into the ultimate repaired connective tissue, or scar. The tensile strength of the wound gradually increases during the remodeling phase presumably as a result of increased cross-linking of the collagen fibers. Despite this extensive remodeling the scar never regains the strength of uninjured tissue and is associated with a permanent loss of specialized function.[4,5]

The most important aspect of normal wound healing is the precise coordination of multiple processes involving various cell types achieved primarily via soluble mediator cross- talk. This coordination ensures that hemostasis is sufficient, inflammation is not excessive, and subsequent ECM rebuilding occurs in a timely, if not completely regenerative, manner. Changes in signaling pathways that disturb the normal sequence of events can have disastrous consequences for the larger wound healing response and lead to a range of pathological conditions. One common aberrant dermal wound healing response is chronic ulcer formation. Whether the result of prolonged

physical tissue compression or systemic disease processes such as diabetes, ulcers are generally associated with significant mortality and morbidity. Insufficient wound healing associated with ulcer formation, which fails to restore dermal integrity, is primarily thought to result from impaired tissue vascularity and innervation and not an intrinsically altered dermal cell phenotype. [6] In contrast, other dermal wound pathologies, such as hypertrophic scarring and keloid formation are thought to be a result of localized tissue abnormalities exacerbated by additional systemic problems. [7,8,9]

In contrast to adult wound healing, fetal dermal wound healing within certain gestational limits (up to 24 weeks gestation in humans), presents a regenerative (scarless) alternative, which restores the initial tissue structure and function. The factors responsible for this wound healing phenotype have only been partially elucidated to date, and are discussed in more detail below.

2.2 FETAL WOUND HEALING

Fetal wound healing has been shown to represent a distinct repair phenotype, which completely recapitulates the initial tissue structure and function. In contrast to adult healing, fetal wounds heal without scar formation, and look indistinguishable from non- injured tissue. Numerous studies have attempted to precisely characterize the fetal wound healing phenotype. This phenotype appears to be dictated by quantitative and qualitative alterations in the various phases of normal wound healing with an emphasis on alterations in the inflammatory and fibroplastic phases of wound healing. [2]

The inflammatory response to injury in the fetus is greatly reduced. There is minimal acute inflammatory infiltrate in response to fetal dermal tissue damage [10] which contains macrophages, [11] but generally lacks lymphocytes. [12] Neutrophil activity within the fetal wound is also hindered by intrinsically reduced phagocytic activity. Systemically, fetal endogenous immunoglobulins are absent, attributed to the immature fetal immune system. As mentioned above, inflammatory cell activity within the wound bed is coordinated via cytokines, chemokines, growth factors and other soluble mediators. These soluble mediators can have either pro- or anti-inflammatory activity. The precise ratio of pro- to anti- inflammatory activity in the wound bed dictates the final degree of inflammatory cell activity. During fetal wound healing, this ratio is tilted heavily in favor of anti-inflammatory cytokines. [13,14,15] Fetal wounds contain diminished levels of important pro- inflammatory chemokines such as interleukin (IL) -8 which can stimulate chemotaxis of neutrophils and keratinocytes. It has been reported that fetal fibroblasts express lower IL-8 mRNA levels and secrete less IL-8 protein. In contrast, the expression of anti-inflammatory mediators such as IL-10 is thought to play an important role in scarless fetal wound healing. Unlike normal fetal skin wounds which have minimal inflammation and reticular collagen patterns, fetal wounds in the skin of IL-10 knockout mice display significant inflammatory cell infiltrates accompanied by scar formation.

Perhaps the most important distinction between fetal and adult wound healing lies within the area of fibroblast activity: proliferation, migration/invasion and ECM synthesis. Fetal fibroblast proliferation and DNA

synthesis are sensitive to stimulation by growth factors such as transforming growth factor (TGF)-β, with fibroblasts derived from early gestation organisms being more sensitive to such regulation. [16] In general, growth rates of dermal fibroblasts have been shown to decrease with the increasing age of the cell donor. [17] Fetal fibroblasts differ from their adult counterparts with respect to both intrinsic migratory properties, and their response to exogenous cues. Using collagen gel invasion assays meant to mimic wound healing processes, Schor et al [18] have demonstrated that fetal fibroblast invasion represents a phenotype intermediate between adult and transformed fibroblasts. This phenotype may be dependent on intrinsic fibroblast properties and autocrine signaling loops which result in the production of motility enhancing factors. [19,20] It may also be dependent on the presence of ECM associated proteins, like fibronectin, which can affect invasion without altering adhesion, proliferation or cell morphology. [21]

The primary function of fibroblasts within the wound bed is to replace the damaged tissue with new ECM components. During adult wound healing, fibroblast ECM synthesis and deposition are poorly coordinated, often excessive and fails to achieve the structural and functional characteristics of uninjured tissue. Fetal fibroblasts have a robust synthetic and secretory phenotype with respect to multiple collagen subtypes and other extracellular matrix components.[22] Collagen and proteoglycan synthetic rates decrease during aging down to 10-25% of fetal rates. A distinct characteristic of the fetal ECM is the primacy of hyaluronic acid (HA), a large, hydrated, glycosaminoglycan, which has been shown to regulate cell proliferation, migration and maintenance of dedifferentiated phenotypes. During fetal wound healing, increased HA levels are thought to regulate inflammatory cell infiltration and decrease fibroblast invasion and collagen deposition within the wound bed. [23] Fetal fibroblasts express increased density of the HA cell surface receptor indicating a significant regulatory role for HA during fetal repair. Dermal HA is produced via the activity of intracellular synthase isoforms. Its production is highly regulated by soluble mediators including cytokines such as IL-1β, via regulation of HA synthesizing enzymes (HAS). [24] Cytokine regulation of HAS activity in fetal fibroblasts differs from that which occurs within their adult counterparts.

The various aspects of fibroblast activity within the wound bed (invasion, proliferation, ECM synthesis) are closely coordinated. Specifically, HA production by fetal fibroblasts appears to correlate with their migratory phenotype. [25] While high adult fibroblast cell density can diminish HA synthetic capability, fetal fibroblasts maintain maximal synthesis even at confluence, a phenomenon which appears to be linked to intrinsically higher rates of fetal fibroblast invasion of collagen gels. Fibroblast invasion and HA synthesis are linked, and co- regulated by extrinsic signals including growth factors (TGF-β1, epithelial growth factor (EGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF)). [26]

The latter stages of wound healing consist primarily of ECM re-modeling, a function of myofibroblast driven wound contraction and approximation of the wound margins and biochemical re- arrangement of collagen fibers via enzymatic activity. Wound contraction does not appear to contribute to wound closure in fetal dermal wounds.[2,27] Substantial evidence indicates that impaired contraction in fetal wounds is a result of intrinsic fetal fibroblast properties.[28] When stimulated by TGF-β, fetal fibroblast contraction is inhibited, while adult fibroblast contraction is stimulated, in a manner which correlates with an increase in α smooth muscle actin (SMA) and select integrin isoforms in adult but not fetal fibroblasts. In general, increasing gestational age results in greater fibroblast

contractile ability.[29] This represents an intrinsic phenotypic difference and does not reflect differential TGF-β receptor expression in the two fibroblast types.[30] Differences in contractile rates may also result from differential interactions of fetal fibroblasts with collagen fibers in the wound bed. In general, these interactions occur via dedicated membrane receptors, called integrins. Integrin expression can be regulated with respect to both overall protein levels and specific isoform ratios. Moulin et al [31] suggested that fetal cells express lower α1 and α3, but higher α2 integrin isoform levels. In general, integrin expression and contractile capacity is more transient in adult fibroblast cultures than in their fetal counterparts.

In summary, two important distinguishing characteristics of fetal wound healing are decreased inflammation, and reduced fibroplastic activity in the dermal wound bed. There exists a general consensus in wound healing research that these two phenomena are interlinked to some degree. As such, diminished inflammation during wound healing is associated with scarless wound healing. On the other hand, altered inflammatory states have been linked to the development of dermal fibrosis, such as in the case of keloid formation. The effects of altered inflammatory and fibroplastic activity in the wound bed may be not only additive, but also synergistic. As such, small intrinsic alterations in either process may result in more dramatic global alterations in the larger wound healing response.

2.3 KELOID FORMATION

Keloids represent a specific abnormal dermal wound healing response in which scar formation is excessive and spreads outside the boundaries of the original tissue injury. Keloids form with varying time courses, and can persist for years. Most often keloids occur in patients between the ages of 10 and 30, with a higher prevalence among darkly pigmented individuals, localized to the chest, shoulders, upper arms, back and head and neck areas. Successful treatments for keloids remain elusive. Current therapeutic modalities include various combinations of surgical and laser excision, steroids and immunomodulators.[32] Recurrence rates following treatment vary, but remain unacceptably high, ranging between 45-100% following surgical excision. More recent approaches have combined surgical excision with post- operative radiotherapy [33] in order to improve clinical outcomes. Utilization of chemotherapeutic agents such as 5-fluorouracil [34] can improve the wound healing dynamics associated with keloid formation. Such treatments, along with interferon or imiquimod, rely upon a recent understanding that keloid formation contains an altered inflammatory/immune component. However, the precise contribution of this component to the larger aberrant wound healing phenotype remains poorly characterized and understood. Unlike in fetal wound healing, a role for inflammation in this altered wound healing response remains unclear. While excessive inflammation is generally associated with increased scar formation and poor wound healing outcomes, [35] the contribution of inflammation to keloid formation is as yet unclear.[13,14,15]

Several studies have demonstrated an association between keloid formation and aberrant inflammatory/immune activity. McCauley et al demonstrated that patients with keloids had increased

concentrations of interleukin (IL)-6, tumor necrosis factor (TNF) α and interferon (IFN)-β in peripheral blood mononuclear cells, along with altered circulating levels of immuno-globulin (IgG, IgM, IgA) and complement (C3, C4) levels.[7,9,36] These systemic changes are associated with local tissue level alterations specific to keloid fibroblasts. Keloid fibroblast production of the pro-inflammatory cytokine IL-6 is higher than their normal counterparts under both IFN-γ stimulated and basal conditions. Keloid fibroblast expression of cyclooxygenase (COX) -2, the inducible synthetic enzyme responsible for PGE2 production may be impaired under certain stimulatory conditions,[37] although basal COX-2 expression and PGE2 secretion appear to mimic those of normal dermal fibroblasts.[38]

Wound healing processes which may be most relevant during keloid formation are underscored by the observed keloid phenotype: excessive collagen deposition within the wound bed. Collagen production following dermal injury is primarily a fibroblast function, and as such, keloid fibroblasts are thought to be a crucial component of this altered phenotype. It is important to note, however, that although fibroblasts produce ECM components and are responsible for their subsequent re-modeling, both processes are highly regulated by soluble mediators. Keloid formation has been described as the formation of benign tumors, and keloid fibroblasts appear to possess reduced growth factor dependence, a characteristic associated with tumor cells. Whereas TGF-β1 reduces dermal fibroblast proliferation in response to EGF stimulation, it enhances the effects of EGF in keloid fibroblasts. Keloid fibroblast growth *in vitro* occurs under high cell density and reduced serum concentrations compared to their normal counterparts, indicating a generalized diminished requirement for growth factors. [39] Although phorbol esters have been shown to inhibit normal dermal fibroblast proliferation, keloid fibroblast proliferation is refractory to inhibition. While keloid fibroblasts have enhanced sensitivity to specific growth factors such as TGF-β1, this phenomenon does not appear to be generalizable to other important dermal growth factor signaling pathways such as epithelial growth factor (EGF). Satish et al [40] demonstrated that although keloid fibroblasts express normal levels of EGFR (receptor), they also display diminished EGFR activation and activation of down- stream enzymes such as PLCγ, likely due to the internalization of EGFR in response to EGF. As a result, the EGF induced migratory phenotype differs in keloid fibroblasts compared to normal dermal fibroblasts.

In addition to enhanced proliferative capacity, keloid fibroblasts possess an altered synthetic profile with respect to various ECM components including glycosaminoglycans and collagens. This profile can be further enhanced by soluble factors provided by keloid keratinocytes.[41] The addition of keloid keratinocytes *in vitro* enhances normal dermal fibroblast gene expression and synthesis of collagen I and III. Dermal-epidermal communication is also thought to be responsible for changes in hyaluronic acid (HA) production during keloid formation. Keloid fibroblasts have been shown to produce less endogenous HA. [42] At the tissue level, HA presence in the dermal component of keloids is decreased, while the epidermal component was increased, with HA present in the spinous and granular layers of the epidermis. This is potentially an important observation in that *increased* HA synthesis is associated with scarless fetal wound healing.

Keloid formation is an example of aberrant dermal wound healing in which the fibroplastic response to injury is greatly enhanced, partially due to intrinsic fibroblast abnormalities further exacerbated by exogenous signals. This general mechanism is consistent with that which is thought responsible for fetal wound healing. These

intrinsic phenotypic differences are further influenced by extrinsic cues, including inflammatory mediators. A role for inflammation in wound healing has been demonstrated, and a general link between increased inflammation and abnormal scar formation postulated (see below).

2.4 INFLAMMATION DURING DERMAL WOUND HEALING

Inflammation is one of the most important early events in dermal wound healing. Its function is to prevent or reduce pathogen invasion, assist in wound debridement, and stimulate tissue repair. Macrophages, neutrophils, dendritic cells and lymphocytes are dedicated inflammatory/ immune cells which make up the cellular component of inflammation, with macrophages and neutrophils contributing to the early inflammatory response to injury. Inflammatory cell activity in the dermal wound bed is time dependent, peaking around 3 days post injury, and resolving within the first week. Subsequent wound healing steps, such as re-epithelialization, fibroblast invasion and ECM deposition and re- modeling, depend on a timely resolution of the inflammatory phase. Therefore, when inflammation is excessive, with respect to either magnitude or time, the entire wound healing sequence may be altered.

The activity of inflammatory cells in the wound bed is coordinated via a wide array of soluble mediators consisting of cytokines, chemokines, growth factors and lipid mediators. These mediators serve multiple purposes and can be described as: primary vs secondary and pro- vs anti-inflammatory. Primary inflammatory mediators are triggered by factors which are foreign to the wound bed itself, such as bacterial lipopolysaccharide (LPS), complement and clotting proteins, etc. A well characterized primary inflammatory mediator is interleukin (IL) -1β. This cytokine is up-regulated early in the wound healing response and has been linked to activation of a wide variety of secondary inflammatory cascades, such as IL-6 (cytokine), IL-8 (chemokine) and COX-2 (cyclooxygenase-2). [43] COX-2 activity is responsible for production of a variety of lipid mediators, including prostacyclins, prostaglandins and thromboxanes. [44,45] Of these, the primary mediator in the dermis is prostaglandin E2 (PGE2). [46,47] The production of both primary and secondary inflammatory mediators occurs in a variety of cell types, including macrophages, neutrophils, keratinocytes and fibroblasts.[14,48,49] Some inflammatory mediators have been shown to have an anti- inflammatory potential. Of these, the best characterized is interleukin (IL) -10 which has been shown to inhibit leukocyte, macrophage and neutrophil infiltration of the wound bed, while at the same time inhibiting the expression of chemokines such as monocyte chemo-attractant protein-1 and macrophage inflammatory protein-1α and pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα.[50]

Although immune cells are the primary component of the inflammatory response to injury, several other cell types participate in the inflammatory response including keratinocytes and fibroblasts. It has been demonstrated that, in the lung, epithelial cells express COX-2 and secrete substantial amounts of endogenous PGE2. Exposure of epithelial cells to LPS or IL-1β can enhance endogenous PGE2 production which can subsequently increase fibroblast proliferation. [51] Additional keratinocyte production of primary inflammatory mediators such as IL-1 has

been shown to increase endogenous fibroblast production of secondary inflammatory mediators such as IL-6. This is part of a complex paracrine interplay, by which IL-1β secreted by keratinocytes up-regulates keratinocyte growth factor production by fibroblasts which then feeds back to signal keratinocytes. Additional evidence indicates that another important inflammatory mediator, interferon (IFN)-γ participates in a keratinocyte based autocrine loop.[52]

Fibroblasts from a variety of tissues have been shown to secrete inflammatory mediators belonging to each of the traditional classes: cytokines, chemokines and lipid mediators.[13,14,15,53] The participation of fibroblasts in the inflammatory response raises an interesting issue regarding the role of inflammation in altered wound healing states. While macrophages and neutrophils have a limited life span within the wound bed, fibroblasts persist into the latter stages of wound healing. As such, endogenous fibroblast production of inflammatory mediators past the normal timeline, may contribute to excessive inflammatory stimulation and altered wound healing outcomes.

Dermal fibroblasts express COX-2 and produce endogenous PGE2; stimulation with exogenous IL-1β results in up-regulation of both processes. [54] Fibroblasts also produce an important inflammatory cytokine, IL-6. Production of IL-6 by normal human fibroblasts appears to be age dependent, with the ability to produce both basal and induced amounts decreasing with progressing age.[55] Interestingly, IL-6 production during fetal wound healing is also decreased compared to the adult wound healing response; diminished fetal fibroblast production of IL-6 indicates a link between the fibroblast phenotype and tissue level of this important inflammatory mediator. There appears to be substantial interplay between these two secondary inflammatory mediators. Specifically, it has been demonstrated that blocking PGE2 during carrageenan- induced inflammation, results in decreased IL-6 levels, and generalized anti-inflammatory effects similar to non-steroidal anti-inflammatory drugs.[56] It is thought that PGE2 may represent an intermediate component of inflammatory pathways activated in response to IL-1β production. Pharmacological blockade of COX-2, results in decreased PGE2 production and impaired subsequent fibroblast production of IL-6.[57]

Dermal fibroblasts also retain the ability to secrete IL-8, an important chemokine secreted under a variety of stimulated conditions, including pathological conditions (psoriasis), ultraviolet light, or exposure to tumor necrosis factor (TNF)α.[58,59,60] Interestingly, dermal fibroblasts not only respond to IL-1β, but also produce endogenous IL-1β. Exogenous and endogenous IL-1β levels appear to be interlinked through feedback loops, along with the expression of IL-1 receptors and a variety of growth factors.[61]

The link between the inflammatory response to injury and the ultimate outcome of wound healing remains somewhat unclear. An inflammatory response is essential to wound healing. However, excessive inflammation is thought to contribute to scar formation. Evidence for this interaction comes primarily from altered wound healing phenotypes. Specifically, fetal wound healing, which has been shown to be scarless, contains a minimal inflammatory component.[2,13,14,35] Any increase in the fetal inflammatory phase, via pharmacological manipulation, has been shown to correlate with increased scar formation. In addition, the removal of anti-inflammatory mediators such as IL-10, are associated with increased scarring. In general, keloid formation has been linked to local and systemic abnormalities in the immune/inflammatory response to injury.[7,8,9] Increased cyclooxygenase-2 expression and PGE2 synthesis in fibroblasts contribute to the development of scleroderma another fibrotic pathologic condition of the dermis. [62]

The link between an aberrant inflammatory response and fibrosis/hypertrophic scarring has been established in other tissues as well. A role for IL-1β and PGE2 in the development of arthritic conditions has been described.[43,63] In the airway, abnormal PGE2 and TNFα signaling contributes to the development of pulmonary fibrosis.[53,64]

As described above, inflammation is a complex process involving multiple cell types and soluble mediators. Therefore, it is impossible to address all aspects of inflammation as they relate to the larger dermal wound healing response. To date, much attention has been focused on primary cytokines such as IL-1β, pro-inflammatory secondary cytokines such as IL-6 and chemokines such as IL-8. In contrast, few recent studies have addressed the role of lipid mediators, particularly prostaglandins, in dermal wound healing. This is unfortunate for two reasons. First, there is increasing evidence that PGE2 plays a crucial role in the development of tissue fibrosis in multiple tissue types including skin. Second, there exists a wide pharmacological armamentarium available for manipulation of endogenous tissue PGE2 levels which hold potential for possible clinical applications.

2.5 PROSTAGLANDIN E2

Multiple studies to date have demonstrated a wide variety of PGE2 activities that are relevant to both tissue homeostasis and repair including vascularity, epithelialization, ECM synthesis and re- modeling, inflammatory cell proliferation and chemotaxis.[51,81,82,83,84,85] These activities suggest a putative role in the regulation of tissue repair at large, and dermal wound healing specifically.

Prostaglandin E2 is a lipid based soluble mediator synthesized from arachidonic acid, a component of the cellular membrane released via phospholipase A2 activity (Figure 2). Arachidonic acid is then modified enzymatically converted by cyclooxygenases into an intermediate molecule, prostaglandin H2.

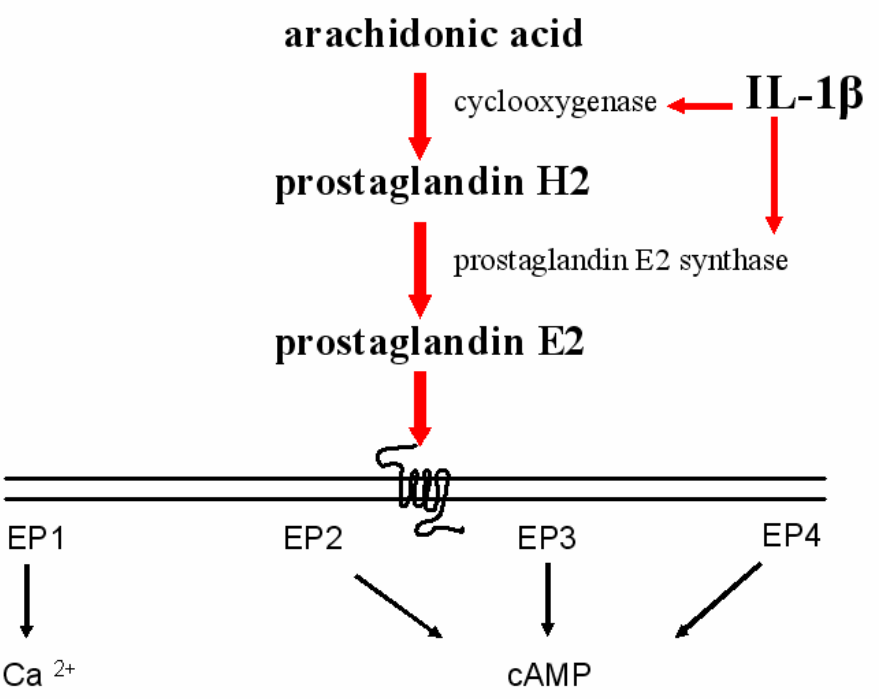


Figure 2: Prostaglandin E2 synthesis and signaling.

Cyclooxygenase activity falls into two general categories: constitutive and inducible. Constitutive COX activity is centered primarily on COX-1, an enzyme which is constitutively expressed in a variety of dermal cells. In contrast, the inducible isoform, COX-2, is up-regulated only following stimulation by specific extracellular cues, including primary inflammatory mediators such as IL-1β and TNFα. Following modification by COX enzymes, PGH2 can be modified into a variety of derivatives, including prostacyclins, prostaglandins and thromboxanes via subsequent enzymatic activity. Conversion of PGH2 into PGE2 occurs via the activity of PGE2 synthases (PGES). Multiple synthase isoforms have been identified: cytosolic PGES identical to p23, a heat shock protein 90 associated protein which is ubiquitously expressed and mPGES, an inducible enzyme.[65] Its regulatory mechanisms are still being investigated, but mPGES appears to be induced by the same mechanisms which regulate COX-2, with IL-1β representing a demonstrable inducer via activation of NF-kb transcriptional regulator.[66] Both synthase isoforms are important in dictating the final cellular output of endogenous PGE2. [67]

Once synthesized, soluble PGE2 exerts its effects on cellular behavior via E prostanoid receptors (EP1, EP2, EP3, EP4). Prostaglandin receptors belong to the general category of G- coupled protein receptors (7 trans-membrane domain receptors which are integrated within the cellular envelope and allow for transduction of extracellular signals by coupling to G protein which can subsequently activate multiple intracellular signaling pathways). The four PGE2 receptors have been shown to exhibit coupling to multiple G proteins, including Gs, Gi and Gq and activate two secondary messenger cascades: calcium and cyclic-adenosine monophosphate (cAMP) signaling.[44,45] In general EP1 signaling has been linked to calcium activation, while EP2, 3 and 4 signaling is coupled to changes in intracellular cAMP. However, the precise signaling mechanisms are complicated by the fact

that while EP2 and EP4 result in up-regulated cAMP, EP3 signaling is dependent on multiple splicing variants, some of which can up-regulate, down-regulate or have no effect of cAMP. Overall, the precise effects of PGE2 on subsequent cell activity are largely determined by the membrane expression pattern of these four receptor subtypes. The pharmacology of the specific EP receptor subtypes has been elucidated over the last few years with specific agonists and antagonists identified for each of the EP receptor subtypes.[44,45,68,69]

PGE2 tissue levels are dictated by its synthesis and degradation. Inactivation of PGE2 occurs primarily inside the cell, following carrier-mediated transport, which is required due to poor PGE2 diffusion through the cell membrane.[70] The major carriers are PGT (transporter, mactrin) and multispecific organic anion transporter (MOAT). Intracellular degradation occurs through multiple reactions, starting with enzymatic oxidation of the 15 hydroxyl group which causes loss of biological activity. This response is catalyzed by the activity of 15-hydroxy prostaglandin dehydrogenase (PGDH) and carbonyl reductase (CR). CR activity reduces PGE2 into PGF2a, a less potent inflammatory mediator. Both PGE2 transporters and degradative enzymes can be controlled by extracellular mediators, and are regulated during systemic inflammatory states such as lipopolyssacharide (LPS) induced fever.

PGE2 signaling is highly regulated at all levels of the pathway. Synthesis of PGE2 following specific triggering events is regulated at the level of both cyclooxygenases and synthases. Specifically, COX-2 and mPGES mRNA and protein levels can be induced by pro-inflammatory stimulators such as IL-1α and β, TNFα and LPS. [71] It remains unclear whether there is absolute coordination of mPGES and COX-2 expression. It is possible that the inducible and constitutive forms of COX and PGES may coordinate their activity in multiple combinations.[72] More recently, evidence has been provided that PGE2 degradation is also regulated. Specifically, the expression of 15-PGDH transcript and protein can be induced by TGF-β1.[73] More generally, 15-PGDH expression is down- regulated during the development of colorectal cancer, a process associated with increased tissue levels of PGE2 which are thought to contribute to pathogenesis.[74] There exists evidence that regulation of PGE2 synthesis and degradation is in fact coordinated. COX-2 inhibitors such as indomethacin, dexamethasone and glucocorticoids have been shown to be potent enhancers of 15-PGDH expression and activity.[75,76]

Additional regulation of PGE2 signaling can occur at the level of receptor expression. It has been demonstrated that PGE2 receptor expression is regulated during a variety of cell and tissue conditions, though precise mechanisms remain unclear. EP receptor function is complicated by several factors: four receptor subtypes, multiple splice variants for EP3, varied cellular expression of subtypes and intracellular localization of receptors. Interestingly, while EP receptors are generally thought to be localized to the cell envelope, studies have demonstrated that EP3 and EP4 can exhibit a perinuclear localization pattern which is consistent with retained function and the ability to transcriptionally control various genes.[77] An additional complication is the fact that EP4 can change its localization pattern in response to PGE2 itself.[78] Specifically, EP4 undergoes ligand induced desensitization accomplished by agonist- induced internalization of the receptor, which appears to be dependent on the carboxyl terminal portion of the receptor.

The expression profile of the four PGE2 receptors has been shown to change during specific physiologic and pathologic processes, including estrous uterine cycling and wound healing. EP2 receptor levels are closely regulated during the estrous cycle with maximal expression in all uterine cell types during latter stages of the estrous

cycle and pregnancy.[79] This is significant because PGE2 activity is thought to be an important regulator of uterine ECM dynamics during pregnancy. This indicates that EP regulation may play an important role in regulating PGE2 dynamics. At the cell level, IL-1β has been shown to stimulate expression of EP2 and EP4 transcripts in a concentration and time dependent manner in granulosa-luteal cells. [80] Harizi et al demonstrated that LPS can regulate the expression of EP receptors in dendritic cells. During intestinal inflammation, PGE2 receptor expression is regulated.[81] Specifically, EP4 expression in T lymphocytes increases, along with the expression profiles for COX-1 and COX-2 enzymes. Recently, it has been shown that PGE2 receptor expression is altered following dermal injury. Interestingly, EP expression appears to be differentially regulated during fetal, neonatal and adult dermal wound healing, raising interesting questions regarding the role of this regulatory mechanism during the larger tissue repair response. However, to date, the signaling mechanisms which regulate this differential EP expression during wound healing remain unclear in two respects: 1) which dermal cell type is responsible for this differential expression and 2) is EP expression regulated by LPS, growth factors, cytokines or chemokines?

A role has been suggested for PGE2 regulation of the larger wound healing response. Specifically, PGE2 is thought to direct immune cell infiltration and collagen deposition in the wound bed.[46,82,83]Additionally, PGE2 may play an important role in regulating keratinocyte activity with EP2 and EP4 agonists capable of reversing indomethacin induced keratinocyte growth inhibition through a cAMP based mechanism, while EP3 activity enhanced the inhibition. [84]

As discussed above, inflammation is an early and important wound healing event. The role of PGE2 in the inflammatory phase of wound healing is thought to be important, though the precise nature of its involvement remains somewhat unclear. PGE2 appears to be a master regulator of the inflammatory response to injury with both pro- and anti-inflammatory activities. Its anti-inflammatory role includes increasing dendritic cell secretion of IL-10 (an anti-inflammatory cytokine) [85] inhibition of interleukin-2 (IL-2) production and reduction of T cell proliferation.[86] This can in summary have an immunosuppressive effect through COX-2 dependent PGE2 activation which blocks IL-2 dependent proliferation of CTLL-2 T cells primarily through EP4 signaling.[87] PGE2 signaling decreases LPS induced macrophage secretion of cytokines and chemokines.[88] At the same time, PGE2 can down-regulate inducible nitric oxide synthase (iNOS) [89] and reduce NO production. The pro-inflammatory activity of PGE2 seems to be largely centered around IL-1 regulation of production of IL-6, generally considered an important pro-inflammatory cytokine.[90] IL-6 may have a larger role in skin homeostasis, by promoting maturation and migration of Langerhans cells into regional lymph nodes, enhancing the expression of co- stimulatory molecules and their potentiation of T cells. These effects are thought to be dependent on EP4 signaling, as indicated by the fact that EP4 knockouts display impaired hypersensitivity to antigens.[91]

PGE2 also plays an important role in modulating fibroblast activity in the wound bed. Prostaglandin E2 signaling modulates changes in fibroblast adhesion, migration and chemotaxis largely through a cAMP dependent mechanism.[92,93,94] It has been shown to modulate ECM synthesis) via two mechanisms.[95,96] First, PGE2 can directly regulate production of ECM components such as collagen and glycosaminoglycans. Karlinski et al [97] reported that lung fibroblast production of sulfated glycosaminoglycans increased with PGE2 in a dose dependent manner, likely a result of increased activity of glycosaminglycan synthetase enzymes. This effect may be achieved via activation of

intracellular cAMP.[98,99,100] Second, PGE2 can regulate the expression of enzymes which are essential for the proper re-arrangement of ECM components, such as metalloproteinases (MMP) and lysyl oxidase. Specifically, MMP1, MMP3, and MMP13 mRNA levels were increased by TNFα, but endogenous production of PGE2 may decrease the activity of MMP13 activity (counteraction) but not the others. At the tissue level, application of PGE2 to the cervix results in tissue wide changes, specifically, time- dependent enzymatic collagen degradation by collagenases and elastase, while GAG levels are increased. [101] In cases where PGE2 signaling is altered, fibrosis/scarring is exacerbated as in the case of scleroderma and pulmonary fibrosis.''

Although a role has been established for PGE2 signaling in hypertrophic/fibrotic scarring, to date there is little data regarding the role of PGE2 regulation of fetal wound healing. Morykwas et al suggested that PGE2 may be important to the minimal inflammatory response associated with fetal wound healing.[102] More recent data indicate that reduced PGE2 levels may be important to the scarless fetal wound healing phenotype, and any exogenous increases can reverse this phenotype. Our laboratory has shown that the EP4 receptor is differentially expressed in healing fetal as compared to adult tissue.[103]

There is tremendous difficulty in precisely characterizing the role of PGE2 in dermal wound healing for several reasons. First, the majority of PGE2 research has been conducted in three tissue types: lower airway, oral mucosa and uterine/cervical tissue. Unfortunately, it is not only inaccurate to extrapolate from these tissue types to the skin, it is inappropriate. For example, wound healing in the oral mucosa is demonstrably different from dermal repair.[104] Specifically, there are lower levels of macrophages, neutrophils and T-cells, along with lower levels of pro-inflammatory mediators such as IL-6 and TGF-β1 in the oral mucosa. PGE2 activity in the lower airway is anti-fibrotic, while PGE2 activity in the dermis appears to be pro-fibrotic.[35] Second, PGE2 has been demonstrated to have a multitude of effects on various cell types and cell processes. As such, the manipulation of tissue levels of PGE2 can cause a wide variety of specific effects. It is difficult to specifically attribute tissue levels effects to a particular signaling pathway. More specifically, PGE2 may control the final outcome of wound healing in one of two ways. Increased PGE2 in the wound bed could stimulate subsequent inflammatory mediator activity such as IL-6 production, and translate into maintained infiltration of the wound bed by macrophages, neutrophils and lymphocytes.

It is also possible that PGE2 regulates wound healing by direct activity on keratinocytes and fibroblasts as detailed above. Specifically, effects on fibroblast invasion, contraction and ECM synthesis may alter the degree of scar formation. Unfortunately, it is difficult to correlate in vitro effects correlate with in vivo findings indicating a pro-fibrotic role. Fibroblast responses associated with PGE2 would be considered anti-fibrotic. Specifically, inhibition of migration, contraction and collagen synthesis should all decrease fibroplastic activity in the wound bed and result in reduced scar formation. The best illustration of this paradox is offered by Kolodsick et al.[105] Using well established standards which identify the transition of fibroblast to myofibroblasts the investigators showed that this entire phenotype, induced by TGF-β1 was reversed by PGE2 through a cAMP dependent mechanism likely through EP2. Additional work in vivo has further demonstrated that PGE2 can retain anti- fibrotic activity under normal dermal conditions.[106] Specifically, when IL-1β and PGE2 are delivered into sponges implanted into the dermis of immuno competent animals, collagen production is significantly decreased, with respect to protein levels, but

without mRNA level changes. In contrast blockade of PGE2 by indomethacin resulted in increased collagen production.

While the exact role of PGE2 in dermal repair remains unclear, a possible explanation exists for its seemingly paradoxical effects on various cell types and processes. PGE2 may act as a pro-inflammatory and anti-fibrotic agent. As such, the ultimate contribution of PGE2 signaling toward wound healing outcomes may be dictated by precise balance between these two activities. This hypothesis will be addressed in more detail throughout this dissertation.

2.6 EXTRACELLULAR MATRIX

The ultimate goal of the wound healing process is to reconstitute the pre-wound tissue structure. Of the ECM proteins, collagen type I is the predominant component of the dermal matrix, interspersed with elastin and surrounded by a variety of hydrated proteoglycans and glycosaminoglycans. Collagen provides the dermis with structural integrity and tensile strength, while other ECM components provide for flexibility and compliance. The precise make-up of the dermal matrix dictates the functional parameters of the skin. It is also an important component of the overall wound healing outcome. During tissue repair, collagen synthesis, deposition and remodeling is coordinated with other ECM components in a manner that is reparative, but not regenerative. [1,2,3]

A notable difference between adult, fetal and hypertrophic wound healing relates to the composition of the extracellular matrix (ECM), synthesized primarily by fibroblasts. The ECM provides scaffolding for the adhesion and migration of cells within the healing wound. The healed adult wound matrix consists almost entirely of excessive, disorganized collagen type I along with some fibrin, fibronectin and proteoglycans.[3,11] Collagen secretion and poor ECM organization are exacerbated in fibrotic wound healing. Keloid formation in the skin represents a well characterized aberrant wound healing response, where fibroblasts continue to secrete extracellular matrix components even after the structural integrity is restored. Lim et al have shown that keloid fibroblasts have an intrinsically abnormal collagen secretory phenotype. Keloid fibroblasts fail to properly regulate the production of collagen types I and III which translates into increased tissue deposition of collagen I and III.[50,107] Production of glycosaminoglycans (GAGs), specifically hyaluronic acid is also aberrant in keloids, with decreased fibroblast synthesis of hyaluronic acid.[42]

In contrast to normal and hypertrophic scarring, fetal wound healing results in complete regeneration of the original ECM. Collagen deposition in the fetal wound exhibits a highly organized reticular pattern.[108] Hyaluronic acid (HA) rather than collagen, is the predominant fetal ECM component.[109] It has been suggested that the HA-rich fetal wound allow for more orderly deposition of collagen. HA is thought to play a regulatory role in the fetal wound by inhibiting fibroplasia, collagen deposition, and neovascularization.[110] Lovvorn et al [111] discussed collagen fiber distribution within fetal dermal wounds. Their analysis indicates that fetal wounds express high rates of collagen I,

III and V deposition, but in a reticular pattern resembling normal skin. Collagen type I is the predominant structural component in both fetal and adult wounds. Increasing gestational age results in increased rates of collagen I cross-linking and corresponded with the transition to scarring.

There is a close association between matrix component synthesis and organization and TGF-β1 signaling. Decorin, a small, leucine rich proteoglycan is known to regulate the activity of TGF-β1 and increases following tissue injury. In later gestational age fetal wounds, decorin up-regulation occurs, but to a much lesser degree than in adult wounds. In early gestation, scarless fetal wounds, however, decorin is actually down- regulated.[112] Similarly, changes in MMPs also correlate with this transition. [113] Later gestational age skin expresses higher levels of MMP-1 (collagenase-1), MMP-3 (stromelysisn-1) and MMP-9 (gelatinase-B), while membrane associated (MT) MMPs and tissue inhibitors of MMPs (TIMPs) remain constant.

While a multitude of ECM related events have been shown to be differentially regulated during fetal and adult wound healing, the precise reparative phenotype remains unclear. One possibility is that fetal wounds may achieve a better synthetic balance. Frantz et al [114] reported that adult wound protein synthesis was skewed toward collagen, while fetal wounds had elevated levels of collagen *and* non- collagen protein synthesis, without excessive collagen deposition and scar formation. As such multiple intrinsic fibroblast properties and altered extrinsic signals may combine in the fetal wound healing response to achieve a synthetic profile that very much resembles the original developmental process.

2.7 SUMMARY

The study of wound healing is the study of subtleties. It is the study of how minute changes in the amount and release timing of specific soluble mediators (growth factors, cytokines, chemokines, lipid mediators) exacerbate small intrinsic differences in cell phenotypes resulting in substantial alterations in the larger wound healing response. As such, wound healing research applies reductionist approaches to a process which is particularly complex. The studies detailed below employed standard in vitro and in vivo approaches to highlight the role inflammation plays during dermal repair. Specifically, they focused on the role of an important inflammatory mediator, PGE2, in determining the final outcome of the larger wound healing process. This research sought to characterize the regulation of PGE2 signaling in fibroblasts derived from tissue types associated with different wound healing phenotypes (fetal, normal/adult, keloid) with respect to: 1) synthesis, 2) degradation, 3) receptor expression and 4) cellular responses to PGE2. It focused on: key PGE2 regulators (IL-1β), inducible enzymatic isoforms (COX-2, mPGES, 15-PGDH), receptor expression (EP1-EP4) and fibroblast activities relevant to wound healing (migration, contraction, collagen synthesis). By addressing these issues, the following questions were answered:

1) Is endogenous PGE2 production dependent on the phenotype of dermal fibroblasts? How does PGE2 synthesis correlate with fibroblast phenotype? How does PGE2 synthesis correlate with the synthesis of other inflammatory mediators such as IL-6 and IL-8?

2) Do primary inflammatory triggers (IL-1β) regulate all aspects of the PGE2 pathway in dermal fibroblasts? Is this regulation coordinated toward a particular global effect?

3) Does exogenous PGE2 exhibit pro- or anti- fibrotic effects on dermal fibroblasts? Are fetal and keloid fibroblasts more or less sensitive to exogenous PGE2 than their normal/adult counterparts?

In answering these questions, some clarity was gleaned with respect to the role of PGE2 in regulating dermal scar formation. Specifically, it was possible to demonstrate that PGE2 effects on dermal fibroblast activity are indeed anti-fibrotic, regardless of cell phenotype, and therefore postulate that pro-fibrotic effects observed under in vivo conditions may be related to the inflammatory and not fibroplastic effects of this mediator. Furthermore, a link was established between endogenous fibroblast PGE2 production and its role in regulating fibroblast activity in the larger context of cell phenotype.

The results of the preliminary analysis were placed in the larger wound healing context, by analyzing the effects of increasingly complex environmental constraints on the intrinsic phenotypic differences between these fibroblast types. Specifically, ECM components, in both 2- and 3- dimensional arrangements were used in conjunction with transplantation of fetal fibroblasts into immunocompetent adult skin. This was meant to be an approach which moved away from standard reductionist techniques to a more complex investigation of how a specific fibroblast phenotype (fetal) behaves in a larger tissue wound healing response (healing adult skin).

3.0 PART I: PROSTAGLANDIN E2 SIGNALING IN DERMAL FIBROBLASTS

Inflammation, one of the earliest events in wound healing, is thought to significantly regulate the outcome of dermal repair. Primary inflammatory mediators (e.g., IL-1β), released immediately following injury activate a series of secondary signaling cascades in inflammatory, epithelial and mesenchymal cells. Production of PGE2 by these cell types has putative effects on both inflammation and fibroblasia. PGE2 stimulates secretion of pro-inflammatory cytokines and chemokines and increases macrophage and neutrophil chemotaxis. Maintainance of the larger inflammatory response can trigger fibroplastic events via dual function mediators such as TGF-β1. In addition to perpetuating inflammation, PGE2 can directly impact fibroplasia, by regulating fibroblast proliferation, migration, contraction, ECM synthesis and remodeling. The studies described below were designed to further our understanding of this signaling cascade and its importance during dermal repair, by using well defined fibroblast phenotypes: fetal, normal adult and fibrotic (keloid). Specifically, they focus on: 1) IL-1β regulation of PGE2 signaling in dermal fibroblasts and 2) PGE2 regulation of fibroblast migration, contraction and collagen synthesis and remodeling. These outcome measures were made on the basis of both theoretical and practical constraints. It should be noted that macrophages and neutrophils are reduced in both number and activity during fetal wound healing and as such represent a poor target for investigation. In addition, the time course of keloid formation precludes the analysis of associated inflammatory events. From a practical perspective, studies of PGE2's effects on dermal inflammation would require extensive and expensive in vivo experimentation. In contrast, fetal, normal adult and keloid dermal fibroblasts have been well characterized and can maintain a specific phenotype under in vitro conditions. As such this analysis will utilize these intrinsic phenotypes to better understand both the regulation and effects of PGE2 signaling during dermal repair.

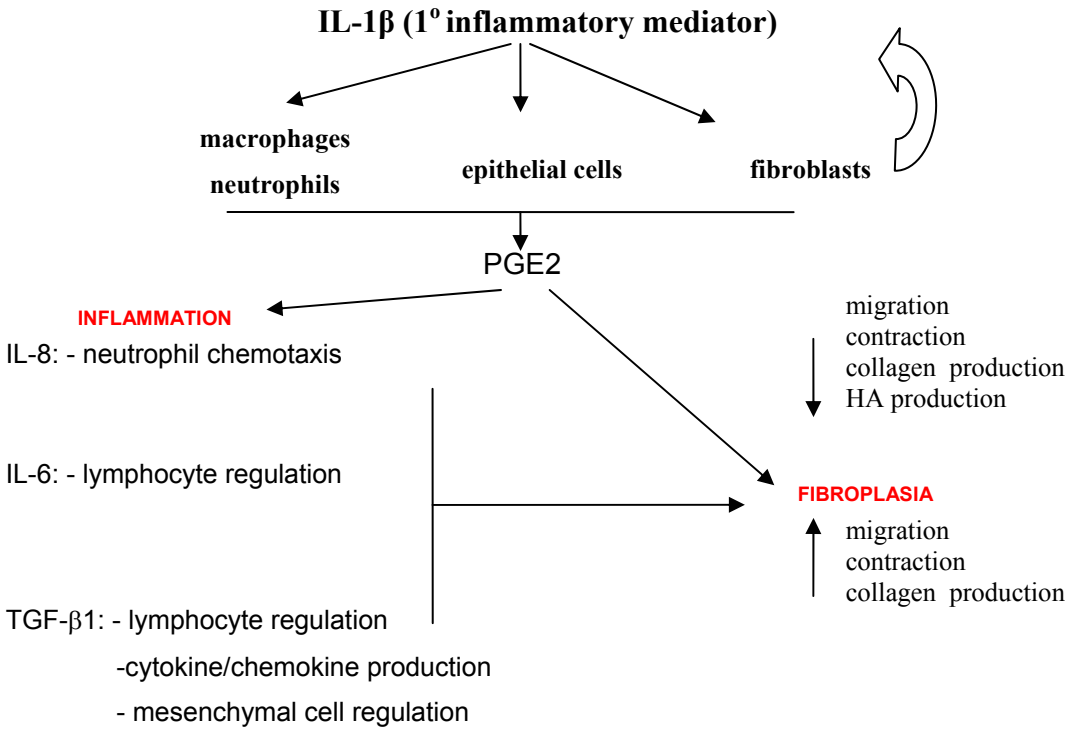


Figure 3. IL-1β and PGE2 signaling during wound healing

3.1 REGULATION OF INTERLEUKIN-1B AND PROSTAGLANDINE 2 SIGNALING IN FETAL AND ADULT HEALING DERMAL TISSUE AND FIBROBLASTS.

3.1.1 Introduction

Inflammation is an important component of wound healing. Following injury, inflammatory cells invade the wound and secrete a variety of soluble mediators that modulate the response of resident fibroblasts and epithelial cells. An important early inflammatory mediator associated with dermal wound healing is interleukin (IL) -1β, a cytokine secreted by a variety of cells including epithelial cells, macrophages and neutrophils. IL-1β secretion within the wound bed can stimulate subsequent production of secondary inflammatory mediators such as IL -6, IL -8 and prostaglandin E2 (PGE2). These mediators are part of a well integrated inflammatory response that coordinates subsequent activity in the wound bed.

Although inflammation is a necessary component of wound healing, excessive inflammation has been linked to abnormal wound healing outcomes, specifically fibrosis and scar formation. On the other hand, diminished inflammatory reaction to injury, as in the case of fetal wound healing, is associated with a regenerative wound healing process which lacks scar formation/ fibrosis. Inflammatory cell infiltration (macrophages, neutrophils) of the fetal wound bed is minimal, and results in decreased production of soluble inflammatory mediators in the wound bed. Previous studies have indicated that the link between inflammation and fibroblast activity in the wound bed may be partially responsible for the final outcome of wound healing, and the degree of scarring.[13,14,15] It is apparent that both intrinsic fetal fibroblast responses to inflammatory signals as well as the overall tissue levels of inflammatory cytokines and chemokines are responsible for the minimal negative impact of inflammation on fetal wound healing. To date however, it remains unclear, what the precise role of fetal fibroblasts is in the larger inflammatory response to injury. This role gains increased importance, given that fetal wound healing contains fewer dedicated inflammatory cells such as macrophages and neutrophils.

PGE2 is an important inflammatory mediator, synthesized from arachidonic acid in a multi-step process involving cyclooxygenases (COX) and synthases (PGES) and degraded by multiple enzymes including dehydrogenases. Once secreted, PGE2 can modulate the activity of keratinocytes**,** dendritic cells**,** and fibroblasts via 4 E prostanoid (EP) receptors coupled to either intracellular calcium or cAMP signaling cascades.[44,77,78,45,84,85,105] The expression profile of the PGE2 receptors is cell dependent and can be regulated by exogenous cues such as lipopolysaccharide. EP receptor expression at the tissue level has been shown to change subsequent to dermal injury. Changes in receptor levels differ between fetal and adult dermal wound healing and may be coordinated with changes in expression of enzymes responsible for PGE2 production. During adult dermal wound healing, COX-2 expression and PGE2 secretion increase, though the precise contribution of PGE2 toward the final outcome of dermal wound healing remains unclear. While PGE2 appears to have some pro-fibrotic effects**,** other studies conflict as to the importance of endogenous PGE2 and other cyclooxygenase products to dermal repair.[35,46,47,83,82] In the lower airway mucosa, PGE2 appears to have an anti-fibrotic effect [115,116,117] which may result from the demonstrated ability of PGE2 to down- regulate fibroblast motility and collagen synthesis.[53,64,94,105]

PGE2 synthesis is highly regulated by exogenous cues, IL-1β, at both the cyclo-oxygenase (COX-2) and synthase (mPGES-1) [118] levels.[64] PGE2 breakdown in the tissue is thought to occur primarily via the activity of degradative enzymes, particularly 15-hydroxyprostaglandin dehydrogenase whose expression is regulated by exogenous cues, including transforming growth factor (TGF)-β1 and steroids.[73,76] While the most physiologically significant stimuli remain unknown, PGE2 receptor expression has also been shown to be amenable to modulation during in vivo dermal wound healing. In summary, it appears that all three aspects of PGE2 signaling: 1) production, 2) degradation and 3) reception, are subject to regulation. The precise integration of these regulatory mechanisms remains unclear at both the tissue and cell level. Furthermore, the precise integration between the PGE2 pathway and secondary interleukin pathways is yet to be determined. While fibroblasts can both produce and respond to secondary inflammatory mediators such as PGE2, IL-6 and IL-8 throughout the wound healing process the interplay between these pathways is unclear.[14,90]

This study was designed to determine how the PGE2 pathway is regulated during fetal and adult wound healing. Specifically, it was meant to address two questions. First, is PGE2 secretion, degradation and reception differentially regulated during fetal and adult wound healing? Second, do fetal fibroblasts express an altered PGE2 pathway compared to their adult counterparts, and does this correlate with an overall attenuated inflammatory phenotype? The answers to these questions should allow us to determine how the PGE2 pathway is regulated during different wound healing phenotypes, and how much of this regulation occurs at the level of the dermal fibroblasts. PGE2 production is hypothesized to be diminished during fetal dermal wound healing reflective not of an altered fetal fibroblast phenotype, but rather of a broader diminished inflammatory response to injury.

3.1.2 Materials and methods

Animals: All animal protocols and procedures were compliant with institutional guidelines and subject to prior approval. Adult pregnant and non-pregnant *Pasteurella*-free New Zealand white rabbits were obtained from an approved supplier (Hazelton Research Products, Denver, Pa). Fetal rabbits at gestational day 21 to 23 (term is 31d) were used for the model of fetal wound healing. Adult rabbits (>6 mo) were used for adult wound healing experiments. Rabbit surgery was performed as previously described. Cutaneous wounds were excised with minimal margins at 12h post injury, rapidly harvested and flash-frozen in liquid nitrogen. Tissue from like wounds was pooled in order to increase the total amount of mRNA and processed. Total RNA was extracted by means of TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the instructions of the manufacturer. The RNA was digested with DNase I (MessageClean Kit; GenHunter Co, Nashville, TN) to reduce chromosomal DNA contamination. mRNA was reverse transcribed into cDNA and expression analysis for selected genes was conducted as detailed below.

Chemicals: Prostaglandin E2, (Cayman Chemical, Ann Arbor, MI), IL-1β, TNFα, IFNγ (Sigma Aldrich, St.Louis, MO).

Cell culture: Dermal fibroblasts were obtained from human tissues produced during standard medical procedures, with institutional review and approval for work with human-derived materials. For this series of experiments, 6

human fetal dermal fibroblast cultures of 15, 16, 18, 21 and 22 weeks of gestation and six human adult dermal fibroblast cultures were obtained from frozen stocks in our laboratory. Frozen cells were thawed and cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY) and 10U/ml penicillin, 10U/ml streptomycin sulfate and 0.025µg/ml of Amphotericin B (Antibiotic-Antimycotic; Life Technologies, Rockville, MD) in a humidified atmosphere containing 5%CO_2 at 37ºC. Culture medium was changed twice per week and cells were subcultured as they became confluent. All cells were used prior to passage 10 (approximately 15 total population doublings).

mRNA analysis: Confluent cell cultures were lysed and mRNA was isolated using standard techniques and reverse transcribed into cDNA. Rabbit (rbt) and human (hu) specific primers were designed for the following genes

Gene	Gene Bank	5' sequence	3' sequence
18S (rb)	AY150553	AAGCCATGCATGTCTAAGTACGCA	CAAGTAGGAGAGGAGCGAGCGACC
IL-1β (rb)	M26295	CGGCAGG TCTT GTCAGTCGTT	TGCAGAGGACGGGTTCTTCTT
PTGS2 (rb)	U97696	CCATGGGTGTGAAAGGCAAGA	TGGGTGAAGTGCTGGGCAAAG
IL-1β (hu)	NM_000576	TCTCTTCAGCCAATCTTCATT	TGTTT AGGGCCATCAGCTTCA
PTGS2 (hu)	NM_000963	ATGATTGCC CGACTCCCTT	TGGCCCTCGCTTATGATCTGTCTT
PTGER1 (hu)	NM_000955	CGCCTCGTCCGCCTCGTCCATC	GCCAGCGCCACCAACACCAGCATT
PTGER2 (hu)	NM_000956	ACCTGCCTCATCAGCCCAGTGGTA	AAGGTCATGGCGAAAGCGAAGTAG
PTGER3 (hu)	NM_000957	CACCCGCCTCAACCACTCCTA	GCCCACGAAACCAGTGAGCAGCAT
PTGER4 (hu)	NM_000958	CCTGTCCGGCCTCAGCATCATCTG	CCGCGAG CTACCGAGACCCATGTT

A real-time PCR protocol was followed as previously described. Briefly, the reverse transcription reaction included 500 ng of DNA-free total RNA pooled from each group, random primers, and SuperScript II (Gibco BRL, Gaithersburg, MD), and was incubated at 25^0C for 10 min, 48^0C for 30 min, and 95^0C for 5 min in a PE 9600 thermocycler (Applied Biosystems, Foster City, CA). SYBR Green PCR reagents (Applied Biosystems, Foster City, CA) were used for PCR amplification. The PCR reaction (in triplicate) included 5 µL of 10X SYBR PCR Buffer, 6 µL of 25 mM MgCl2, 4 µL of each dNTPs (blended with 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP), 2.5 µL of each gene-specific primer (5 µM), 0.5 µL of AmpErase UNG (0.5 unit), 0.25 µl of AmpliTaq Gold (1.25 units) and 5 µL of cDNA in a final volume of 50 µL. The conditions for TaqMan PCR were as follows: 50^0C for 2 min, 95^0C for 12 min, and 40 cycles at 95^0C for 15 sec, and 60^0C for 1 min in an ABI PRISM 7700 Sequence Detection system (Applied Biosystems, Foster City, CA). The 7700 Sequence Detection Software (Applied Biosystems, Foster City, CA) was used for instrument control, automated data collection, and data

analysis. Relative quantification (fold difference) of the expression levels of each transcript for each group was calculated using the 2-ΔΔCt method.

Protein analysis: Cells were allowed to reach confluency under standard cell culture conditions, lysed on ice in 62.5mM tris, ph 6.6, 10% glycerol, 1%SDS, 0.5mM PMSF, 2ug/ml aprotinin, 10ug/ml leupeptin and 5ug/ml pepstatin, homogenized and boiled for 5 min. Cell debris was removed by centrifugation at 9000g for 30 minutes. Protein concentration in the supernatant was determined using the bicinchoninic acid assay, using 1mg/ml bovine serum albumin (Sigma Chemical Co) as a standard. Aliquots of protein were electrophoresed on SDS-PAGE gels using a BioRad mini-gel system, and transferred onto PVDF (a modified nitrocellulose) membranes. Membranes were blocked with 5% milk in PBS with 0.1% Tween-20 for one hour at room temperature. Membranes were then probed with primary antibodies: polyclonal rabbit anti-human EP1, EP2, EP3 or EP4, anti-cyclo-oxygenase-2 (COX-2), anti-15 hydroxy prostaglandin dehydrogenase, monoclonal mouse anti-microsomal PGE2 synthase 1 (mPGES1), (Cayman Chemical, Ann Arbor, MI), followed by a one hour incubation with horseradish peroxidase conjugated goat anti-rabbit, or goat anti-mouse IgG (Pierce, Rockford IL) in 1% milk in PBS/Tween. Protein bands were visualized using a chemiluminescent kit (Pierce, Rockford, IL). In order to standardize for protein loading, β-actin was identified using a monoclonal primary antibody (Sigma, StLouis MO) and secondary goat anti-mouse (Jackson ImmunoResearch, West Grove PA).

Inflammatory mediator secretion: Fetal and adult fibroblast production and secretion of IL-6, IL-8 and PGE2 was assayed using human specific commercially available enzyme linked immunoassays (R&D Systems, Minneapolis MN). The amount of each secreted inflammatory mediator was quantified and standardized to the total of amount of protein present within the cell layer.

3.1.3 Results

in vivo analysis of IL-1β and PGE2 signaling during fetal and adult wound healing

COX-2 and IL-1β expression are differentially regulated during fetal and adult dermal wound healing. Using a well established rabbit dermal incisional wound model we analyzed the gene expression profiles of IL-1β and COX-2. By quantitative molecular analysis, data indicated that IL-1β and COX-2 mRNA levels increased 12 hours post-injury in both fetal and adult wounds (Figure 4). The up-regulation of both molecules in adult wounds was higher than in their fetal counterparts. Specifically, adult IL-1β levels increased approximately 15 fold, and COX-2 levels increase approximately 7 fold over basal levels, In contrast, fetal IL-1β levels increase 12 fold, and COX-2 levels increase 3 fold over basal levels. In order to maximize the amount of wound mRNA, like wound tissue was pooled and analyzed as a single entity. As such, statistical analysis for mRNA levels is not appropriate.

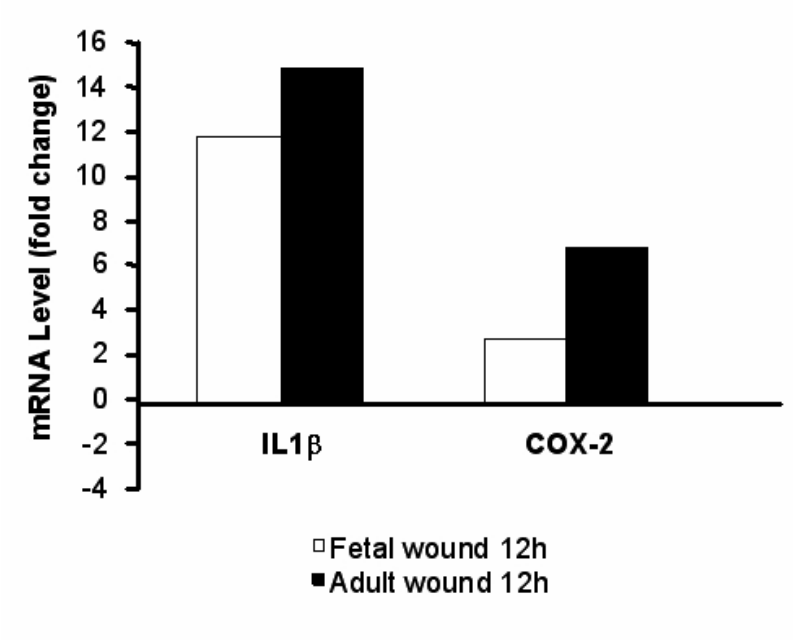


Figure 4. COX-2 and IL-1β expression are differentially regulated during fetal and adult dermal wound healing.

Wound tissue from adult and fetal wounds was harvested at 12 hours post injury, like tissue was pooled, mRNA isolated and reverse transcribed into cDNA. Quantitative PCR (Taqman) was used to analyze changes in mRNA levels for COX-2, IL-1β and EP1-4 using rabbit specific primers. IL-1β and COX-2 mRNA levels increased 12 hours post dermal injury in both fetal and adult wounds. The up-regulation of both molecules in adult wounds was higher than in their fetal counterparts. Data are expressed as fold change from unwounded skin levels.

in vitro analysis of IL-1β regulation of PGE2 signaling in fetal and adult fibroblasts

IL-1β up-regulates dermal fibroblast production of IL-6 and IL-8. Confluent monolayers of human fetal and adult fibroblasts were treated with IL-1β at a dose of 1ng/ml for 24 hours. Secreted levels of IL-6 and IL-8 were measured quantitatively using commercial ELISAs (Figure 5). Treatment of adult fibroblasts with IL-1β resulted in a significant increase in secreted IL-6 ($p= 0.036$) and IL-8 ($p= 0.019$) levels. In contrast fetal fibroblasts showed unchanged endogenous production of IL-6 and IL-8 in response to IL-1β stimulation. Adult fibroblasts produce more IL-6 and IL-8 than fetal fibroblasts under basal conditions, though these differences reach statistical significance only in the case of IL-6 ($p=0.048$), and not IL-8 ($p= 0.15$). In the case of fetal fibroblasts, treatment with IL-1β (1ng/ml) results in an increase in IL-6 and IL-8 levels, but this increase did not reach statistical significance (IL-6 $p= 0.146$, IL-8 $p= 0.154$).

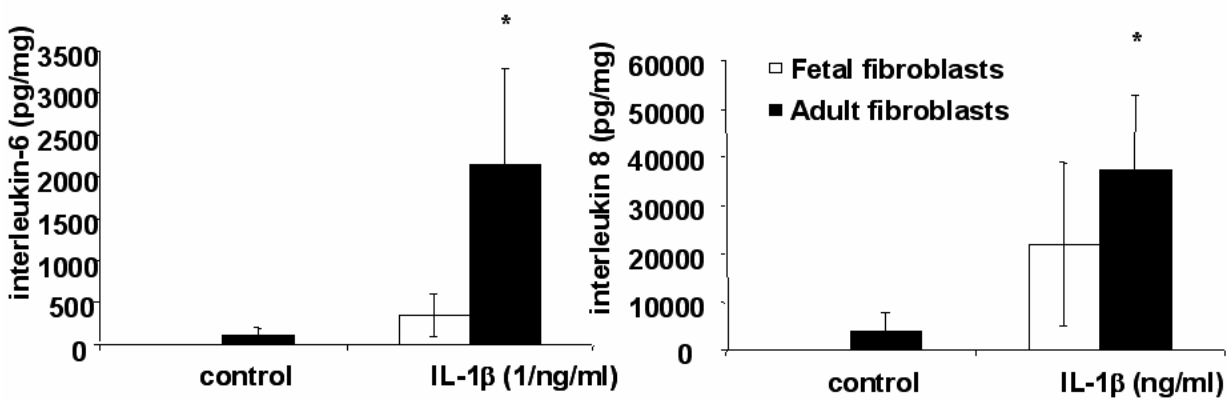


Figure 5. IL-1β up-regulates dermal fibroblast production of IL-6 and IL-8.

Confluent monolayers of fetal and adult fibroblasts were treated with interleukin-1β (1ng/ml) in the absence of fetal bovine serum for 24 hours. Media was collected and analyzed for interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion using commercial ELISA kits. For these measurements, data were collected from 5 fetal and 5 adult fibroblast cultures and expressed as averages with error bars as standard error of the mean. * represents $p<0.05$

IL-1β up-regulates COX-2 expression in human fetal and adult dermal fibroblasts. Confluent monolayers of adult and fetal fibroblasts were treated with IL-1β for 8 and 24 hours. Quantitative RT-PCR (Taqman) was used to quantify the change in mRNA levels following IL-1β administration compared to untreated levels. As shown in Figure 6, in the absence of IL-1β stimulation, the COX-2 mRNA levels in fetal and adult fibroblast were similar. Following stimulation by IL-1β, COX-2 mRNA levels increased dramatically in both cell types. Additional experiments indicated that COX-2 mRNA up-regulation following IL-1β is dose dependent, with increasing up-regulation following an increase in IL-1β dose from 0.1 to 1.0ng/ml. This up-regulation was detected in response to TNFα, another well known inducer of COX-2 expression, but not by IFNγ. The effect of all three inflammatory mediators was conserved in fetal fibroblasts.

Further analysis addressed the intracellular protein levels for COX-2. As illustrated in Figure 7, stimulation of adult fibroblasts by IL-1β resulted in up-regulation of COX-2 protein levels as detected by Western blotting. Unstimulated COX-2 protein levels were virtually un- detectable, as would be expected given that this is an inducible enzyme. Up-regulation of COX-2 protein levels was conserved in fetal fibroblasts. Dose response experiments indicated that as in the case of message, COX-2 protein up-regulation occurs in both adult and fetal fibroblasts. In addition, time course experiments indicated that exposure of fetal and adult fibroblasts to IL-1β at 1ng/ml for 8, 16 and 24 hours results in up-regulation of COX-2 as early as 8 hours in both fibroblast phenotypes.

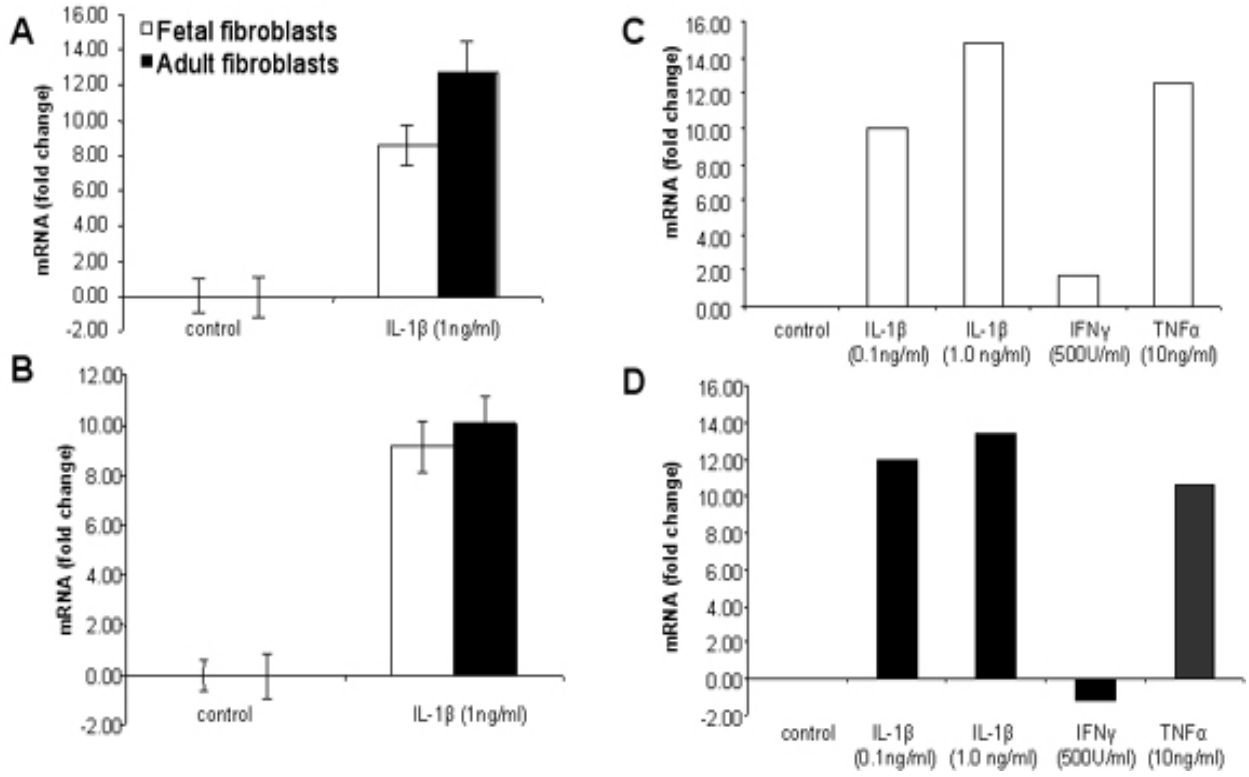


Figure 6. IL-1β up-regulates COX-2 expression in human fetal and adult dermal fibroblasts.

Confluent monolayers of adult and fetal fibroblasts were treated with IL-1β for 8 or 24 hours. The cell layer was isolated and processed for RNA isolation. Quantitative PCR (Taqman) using specific human primers for COX-2 was used to measure the level of enzyme expression. For these measurements, data were collected from 5 fetal and 5 adult fibroblast cultures and expressed as averages with error bars as standard error of the mean. IL-1β up-regulates COX-2 expression in both adult and fetal fibroblasts at 8 (panel **A)** and 24 (panel **B)** hours post treatment. This is a dose dependent response which is mimicked by TNFα, but not IFNγ in both cell types (panels **C, D)** (data from only 1 fibroblast cultures for each phenotype).

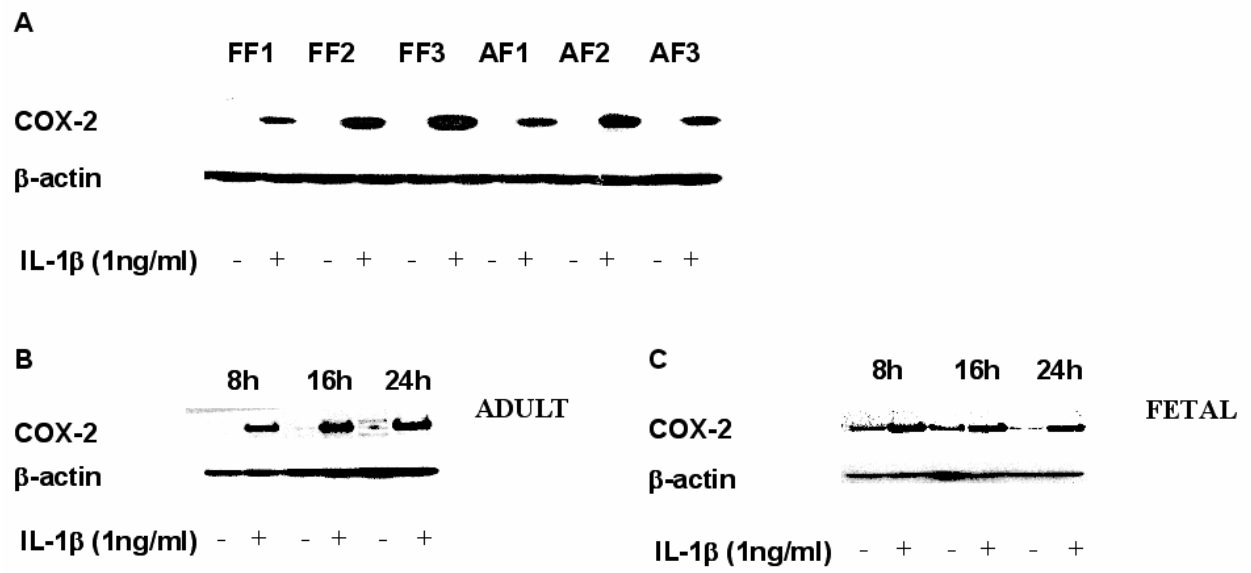


Figure 7. IL-1β up-regulates COX-2 protein levels in human fetal and adult dermal fibroblasts.

Confluent monolayers of fetal and adult fibroblasts were treated with IL-1β for 8, 16 or 24 hours. The cell layer was isolated and processed for protein isolation. Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human COX-2. COX-2 protein levels are increased by IL-1β to a similar degree in both fetal (FF) and adult (AF) fibroblasts above basal levels Panel **A).** COX-2 induction was detectable at 8, 16 and 24 hours post IL-1β treatment in both adult (panel **B)** and fetal (panel **C)** fibroblasts.

IL-1β up-regulates mPGES1 expression in and PGE2 secretion by fetal and adult fibroblasts. Confluent monolayers of fetal and adult fibroblasts were treated with IL-1β at 0.1ng/ml and 1.0ng/ml. Whole cell lysates were analyzed by Western blot for human mPGES1 (Figure 8). Data indicate that while basal levels of mPGES1 protein are relatively low in both fetal and adult fibroblasts, they are rapidly induced as early as 8 hours following stimulation with IL-1β. Both fetal and adult fibroblasts exhibit a similar dose response profile with respect to IL-1β up-regulation of mPGES1 protein levels. Media from all experiments were collected and analyzed for PGE2 secretion by fibroblasts using a high sensitivity commercial ELISA kit. Data are expressed as averages with error bars as standard error of the mean. Administration of IL-1β caused an increase in endogenous PGE2 secretion by fetal and adult fibroblasts ($p<0.05$ in the case of adult fibroblasts, $p=0.15$ for fetal fibroblasts).

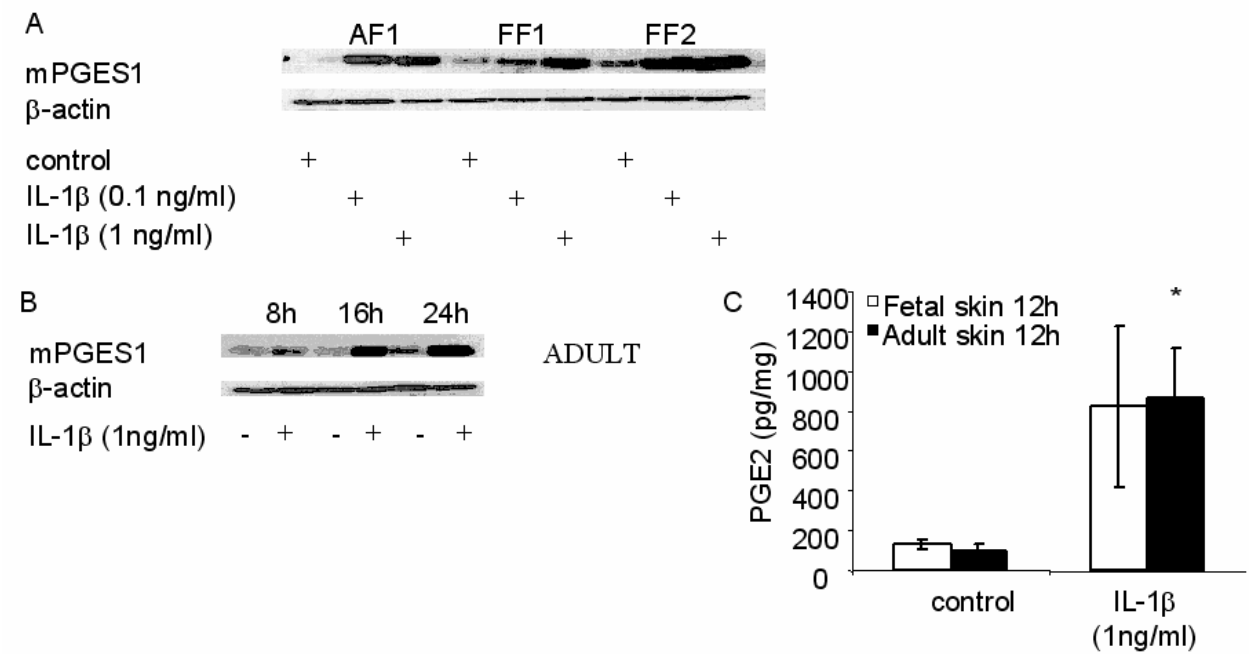


Figure 8. IL-1β up-regulates mPGES1 expression in and PGE2 secretion by fetal and adult fibroblasts.

Confluent monolayers of fetal (FF) and adult fibroblasts (AF) were treated with IL-1β for 8, 16 or 24 hours. Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human mPGES1. mPGES1 expression was up-regulated by IL-1β in a dose dependent manner in both cell types (panel **A),** as early as 8 hours following stimulation (panel **B).** Media was collected and analyzed for PGE2 secretion using a commercial ELISA kit (panel **C).** For these measurements, data were collected from 5 fetal and 5 adult fibroblast cultures and expressed as averages with error bars as standard error of the mean. * represents $p<0.05$ IL-1β causes an increase in PGE2 secretion by fetal and adult fibroblasts ($p<0.05$ in the case of adult fibroblasts, $p=0.15$ for fetal fibroblasts). Basal and stimulated levels were similar between fetal and adult fibroblasts.

IL-1β does not alter expression of 15-PGDH and EP receptors. PGE2 breakdown is an important component of the PGE2 pathway, and has previously been described to contribute to tissue PGE2 levels. Data indicate that treatment of fetal and adult dermal fibroblasts with IL-1β, TNFα or IFNγ (data not shown), does not alter the protein levels of 15-PGDH (Figure 9). Further experiments were aimed at characterizing the effects of IL-1β on EP receptor expression profile of fetal and adult dermal fibroblasts. Compared to basal, untreated levels, EP mRNA levels were unchanged following IL-1β treatment at 8 and 24 hours post stimulation (data not shown). Additional analysis indicated that protein levels for the EP receptors were similarly unchanged following treatment with varying doses of IL-1β (Figure 10). Specifically, EP2 protein expression remains constant following treatment with IL-1β at a dose of 1ng/ml.

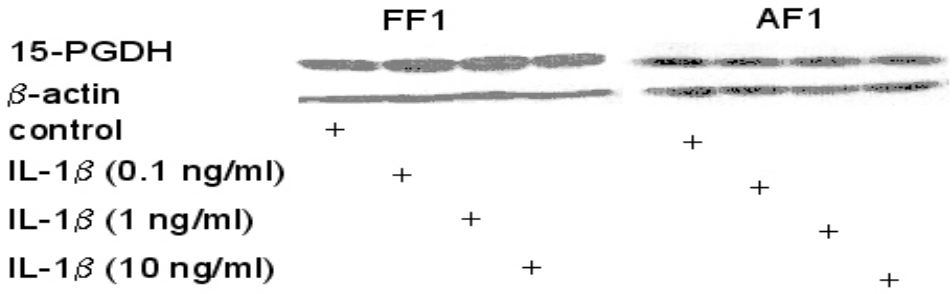


Figure 9. IL-1β does not regulate 15-PGDH protein levels in human fetal and adult dermal fibroblasts.

Confluent monolayers of fetal and adult fibroblasts were treated with IL-1β for 24 hours. The cell layer was isolated and processed for protein isolation. Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human 15-PGDH. 15-PGDH protein levels were not altered by IL-1β in either fetal (FF) or adult (AF) fibroblasts above basal levels.

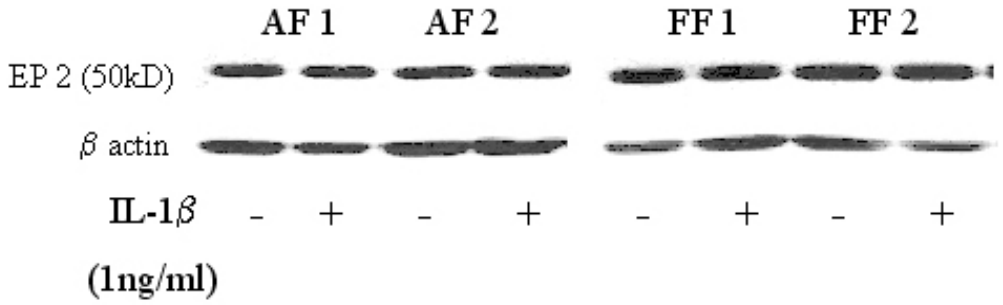


Figure 10. IL-1β does not alter EP protein levels in fetal and adult fibroblasts.

Confluent monolayers of adult and fetal fibroblasts were treated with IL-1β, for 8 or 24 hours. The cell layer was isolated and processed for RNA or protein isolation. Quantitative PCR Taqman using specific human primers for EP 1-4 was used to measure the level of enzyme expression. For these measurements, data were collected from 5 fetal and 5 adult fibroblast cultures and expressed as averages with error bars as standard error of the mean. Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human EP2 receptor. IL-1β fails to alter the expression of EP2 receptor at the protein level in either adult (AF) or fetal (FF) fibroblasts.

PGE2 up-regulates adult dermal fibroblast production of IL-6 but not IL-8. PGE2 has been shown to play an integral role in the IL-1β regulation of secondary inflammatory mediators such as IL-6 and IL-8. Confluent fetal and adult dermal fibroblasts were treated with PGE2 at a dose of 1μM for 24 hours. Secreted IL-6 and IL-8 levels were measured quantitatively using commercial ELISAs as detailed above. Treatment of adult dermal fibroblast cultures with PGE2 resulted in an increase in IL-6 levels (p- value <0.05), but did not produce a

significant increase in IL-8 levels (Figure 11). As detailed above, fetal fibroblasts secreted lower basal levels of both IL-6 and IL-8 compared to their adult counterparts. In response to PGE2, fetal fibroblasts failed to up-regulate their production of either IL-6 or IL-8.

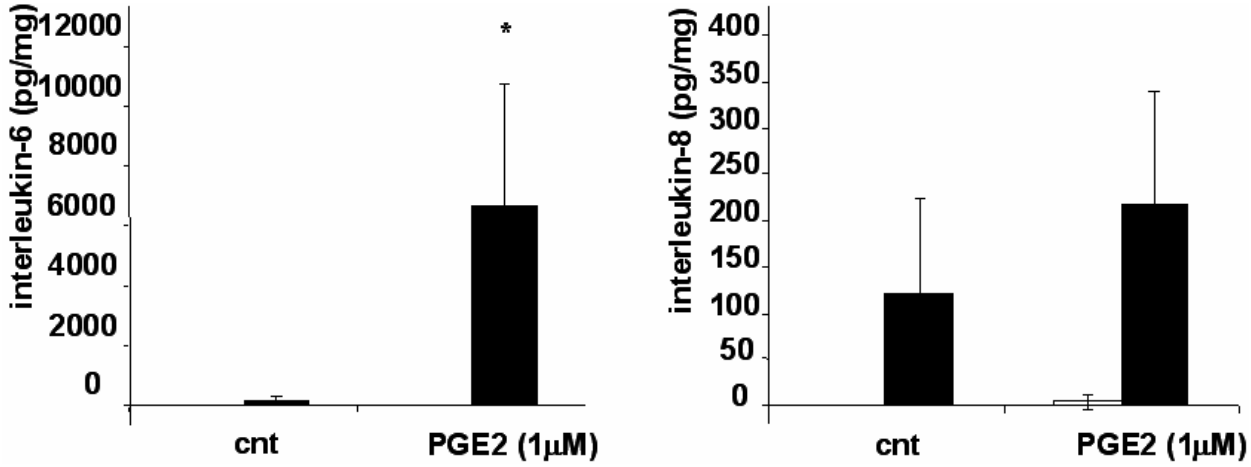


Figure 11. PGE2 up-regulates adult dermal fibroblast production of IL-6 but not IL-8.

Confluent monolayers of fetal and adult fibroblasts were treated with PGE2 (1mM) in the absence of fetal bovine serum for 24 hours. Media was collected and analyzed for interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion using commercial ELISA kits. For these measurements, data were collected from 5 fetal and 5 adult fibroblast cultures and expressed as averages with error bars as standard error of the mean. * represents $p<0.05$ PGE2 causes an increase in IL-6 but not IL-8 secretion by adult fibroblasts, but has no effect on fetal fibroblast IL-6 and IL-8 production.

3.1.4 Discussion

Inflammation is a crucial component of dermal wound healing. As such, it is essential that it be integrated into the larger wound healing response temporally, qualitatively and quantitatively. Inflammatory mediator production, reception and degradation must be closely coordinated throughout the repair process in order to achieve sufficient yet not excessive activation of inflammatory processes required for appropriate restoration of tissue structure and function. One of the most important early inflammatory mediators is IL-1β, which can subsequently activate secondary inflammatory processes in dedicated inflammatory cells as well as fibroblasts.[57,61] Fibroblast participation in the inflammatory phase of wound healing has been previously documented, yet remains poorly understood.[13,14] While dedicated inflammatory cells such as macrophages and neutrophils carry the primary burden of responding to injury in adult tissue, these cells are reduced in both number and maturity in the case of fetal tissue repair. In addition, dedicated inflammatory cells have a limited lifespan within the wound bed, while fibroblasts persist into the latter phases of wound healing. Fibroblasts have been shown to secrete a variety of secondary

inflammatory mediators, including IL-6, IL-8 and PGE2, though the precise regulation of these inflammatory cascades remains unclear.[1,2,64]

While dermal injury has been shown to trigger up-regulation of COX-2 and its main product in the skin, PGE2, neither the mechanism of this up-regulation, nor its contribution to the final outcome of wound healing has yet been clarified.[35,46,47] In vivo studies confirm that COX-2 up -regulation occurs relatively quickly following injury, and that this up-regulation is greater in adult than fetal tissue. This phenomenon correlates with an increase in IL-1β expression, which is similarly larger during adult tissue repair, suggesting that the level of COX-2 expression following dermal injury is partially dictated by upstream triggers such as IL-1β. As such, previously documented alterations of COX-2 following fetal dermal injury may be related to a deficiency in primary stimuli for enzyme expression and not to and intrinsic COX-2 related fetal phenotype. Previous studies in our laboratory have shown that fetal wound healing is also associated with differential regulation of EP4 expression when compared to its adult counterpart. Additional work in our laboratory suggests that this differential regulation may extend to all 4 PGE2 receptors (Li-Korotky, manuscript in preparation). Outstanding questions remain as to what are the triggers and regulatory mechanisms for EP expression.

In order to further address the link between IL-1β and PGE2 signaling during wound healing, the current study investigated how COX-2/PGE2 signaling is regulated by IL-1β in fetal and adult dermal fibroblasts. Fibroblasts were chosen for subsequent experimentation for the following reasons: 1) dedicated inflammatory cells are reduced in number and activity during fetal wound healing, 2) fibroblasts express PGE2 synthetic enzymes and secrete PGE2 and 3) fetal fibroblasts have been shown to have altered secretory capacity with respect to other secondary inflammatory mediators, IL-6, IL-8.[13,14] Recent evidence in multiple tissue types have illustrated that fibroblasts play a very important role in the production of these mediators, though this role does appear to be limited to production of secondary inflammatory mediators such as IL-6, IL-8 and PGE2. In the airway, impaired PGE2 production by fibroblasts has been linked to improper wound healing responses, and the development of excessive fibrosis and scarring.[64,115,116,117] A specific role for fibroblast-produced PGE2 in the dermal wound healing beyond inflammatory signaling remains unclear. While COX-2 up-regulation and PGE2 production has been linked to the development of fibrosis, several issues remain outstanding. First, what is the cellular source of PGE2? Second, what are the mechanisms regulating the PGE2 pathway? Third, how does regulation of the PGE2 pathway fit into the overall wound healing phenotype?

Data presented above confirm that fetal fibroblasts maintain a diminished inflammatory phenotype with respect to secondary inflammatory mediators such as IL-6 and IL-8. Production of the both mediators is diminished under basal conditions as previously reported.[13,14] Interestingly, administration of IL-1β causes significant increases in secreted IL-6 and IL-8 levels in adult fibroblasts, but fails to stimulate fetal fibroblast production of these two mediators. Given the diminished inflammatory capacity of fetal fibroblasts with respect to IL-6 and IL-8, we anticipated that fetal fibroblast production and secretion of PGE2 might also be diminished. To the contrary, our data suggest that fetal dermal fibroblasts maintain a competent synthetic pathway for PGE2. Specifically, COX-2 expression can be induced appropriately by exogenous IL-1β at the level of both message and protein, in a manner indistinguishable from adult fibroblasts. The effects of IL-1β on COX-2 expression are specific, and can be

mimicked by TNFα, another well know activator of COX-2, but not by IFNγ. In addition to COX-2, IL-1β up-regulates the expression of the inducible isoform of PGE2 synthase, mPGES1 and increases the secreted levels of fibroblast PGE2. While increased PGE2 production by fetal fibroblasts does not reach the level of statistical significance, this appears to be primarily a result of large variability in the secreted levels. It should be noted that the cells used in these experiments are primary cultures, derived from specimens of varying gestational ages. It is therefore, not surprising, to observe some degree of variability in the phenotype of these cells. However, we contend that varying gestational ages should be used in these studies, given that the scarless fetal wound healing phenotype spans an extended gestational range. The goal is to determine the fetal fibroblast phenotype as it relates to secretion of inflammatory mediators.

In contrast to the synthetic arm of the PGE2 pathway, the degradative and receptive components of this pathway appear to be constitutively expressed and are not regulated by inflammatory mediators such as IL-1β. Previous studies have indicated that degradative enzymes such as 15 HPGDH can be regulated at both tissue and cell levels by exogenous stimuli.[74,73,76] These changes in expression have been associated with specific tissue phenotypes and are thought to cause increased tissue levels of PGE2. Data presented above indicate that while fetal and adult dermal fibroblasts express detectable levels of this degradative enzyme, the protein levels of this enzyme are not altered by administration of exogenous IL-1β. Previous studies have indicated that PGE2 receptor expression levels are altered subsequent to tissue injury, and these changes may play a role in the inflammatory process. These data are confirmed by in vitro studies by other groups which have demonstrated that EP receptor expression as well as localization can be regulated at the cell level by exogenous cues.[77,78,85] To date, no study has determined whether these changes occur in fibroblasts generally, and in dermal fetal and adult fibroblasts specifically. Data presented here suggest that EP receptors are constitutively expressed in dermal fetal and adult fibroblasts. Exogenous IL-1β does not appear to alter the expression profile of these receptors.

Taken together, the results may be interpreted as follows. Dermal injury triggers up- regulation of IL-1β and COX-2, and to a lesser degree in fetal wounds. In dermal fibroblasts, the expression of PGE2 synthetic enzymes COX-2 and mPGES1 in response to exogenous IL-1β is similar in fetal and adult fibroblasts with respect to qualitative, quantitative and temporal components. In contrast, IL-1β-stimulated production of secondary inflammatory signals IL-6 and IL-8 production by fetal fibroblasts in response to IL-1β is reduced compared to their adult counterparts. These data indicate that: 1) fetal fibroblasts retain a functional PGE2 synthetic pathway, which is regulated by exogenous IL-1β, and as such the diminished activation of COX-2 at the tissue level may not be indicative of a specific fetal fibroblast phenotype and 2) fetal fibroblasts do not maintain a generalized impaired secretory phenotype. Previous studies have demonstrated a link between secondary inflammatory mediators activated by IL-1β, particularly that IL-1β induction of IL-6 is partially dependent on intermediate activation of PGE2. In conclusion, IL-1β activation of PGE2 occurs appropriately, but PGE2 does not turn up-regulate subsequent production of IL-6 by fetal fibroblasts. Surprisingly, none of the PGE2 receptors appear to be regulated by IL-1β at either the message or protein level, in contrast with suggestions derived from in vivo data. There are two possible explanations for this discrepancy. First, changes in EP expression levels found in animal wound healing studies may be attributable to a cell type other than dermal fibroblasts. Second, the primary regulator for EP

expression may not be IL-1β, but rather another early wound healing mediator, a growth factor perhaps or a product of blood coagulation or the complement cascade. A comprehensive analysis of putative triggers for EP receptor regulation during wound healing is currently being conducted. Preliminary analysis indicates that EP receptor expression can be altered by stimulation with various concentrations of fetal bovine serum, following serum starvation. Surprisingly, EP expression appears to be differentially altered, with EP2 levels decreasing and EP4 levels increasing with higher serum concentrations (data not shown).

It was somewhat surprising that while IL-1β is a potent activator of fibroblast PGE2 synthetic enzymes, it fails to significantly alter the expression profile of either the degradative enzyme 15-PGDH, or the EP receptors. This phenomenon is interesting in that it implies that not all arms of the PGE2 signaling pathway (synthetic, degradative, receptive) are regulated by the same primary trigger, and that it is possible that at the tissue level, IL-1β regulation of COX-2 and mPGES1 may be integrated with regulation of the EP receptors by other primary triggers such as growth factors. From a research perspective, such a possibility would dramatically alter the approach to studying specific signaling pathways and force us to consider more complex networks of inflammatory cascades which are neatly integrated into the larger wound healing response. From a clinical perspective it suggests the possibility that portions of the PGE2 pathway may be amenable to exogenous manipulation with minimal side effects on other components of the pathway.

3.2 PROSTAGLANDIN E2 DIFFERENTIALLY MODULATES HUMAN FETAL AND ADULT DERMAL FIBROBLAST MIGRATION AND CONTRACTION: IMPLICATIONS FOR WOUND HEALING

3.2.1 Introduction

In adult dermis, repair is initiated by hemostasis and inflammation and results in the formation of a scar, which partially recapitulates the initial tissue structure and function. Failure of the repair process to recover tissue structural integrity is primarily due to the inability of wound fibroblasts to synthesize and reorganize extracellular matrix (ECM) components in a manner identical to uninjured dermis. In contrast, fetal wounds have been shown to heal in a regenerative manner, without scar formation, up to the third trimester of human gestation. This result is thought to be the product of both intrinsic fetal fibroblast properties and extrinsic soluble mediators such as growth factors, cytokines and chemokines.[25,24] Previous studies have demonstrated that fetal wound healing contains a minimal inflammatory component. Macrophages and neutrophils, which contribute to the inflammatory response in adult-type wound healing, are diminished in both number and activity in the fetal wound bed. Soluble inflammatory mediators, including interleukin (IL)-6, IL-8 and IL-10, have also been shown to be altered during fetal wound healing leading to an overall diminished inflammatory response. It has been suggested that such alterations in the inflammatory phase of wound healing are partially responsible for the decreased or absent scarring associated with fetal dermal wound healing.[13,14,15] More specifically, inflammatory mediators have been shown to have differential effects on fetal fibroblast activities, including migration and ECM synthesis.[24,25]

Cyclooxygenase-2 (COX-2) has long been established as an important component of connective tissue inflammation subsequent to injury. Its products, derivatives of arachidonic acid, regulate inflammatory processes during tissue repair.[64,116,117,53] In the dermis, COX-2 up-regulation follows the initial injury, participates in the subsequent inflammatory phase and appears to contribute to the degree of eventual fibrosis [35,119], primarily through one of its products, prostaglandin E2 (PGE2). [83,120,121] PGE2 in turn modulates the activity of a wide variety of cells via four E-prostanoid (EP) receptors, EP1-EP4, coupled to calcium and cAMP intracellular signaling, which belong to the larger family of G coupled protein receptors.[44,45,77,78] The precise receptor expression profile appears to be important in determining the overall effect of PGE2, which can be either pro- or anti- inflammatory, and the EP profile is differentially regulated during fetal and adult wound healing. While EP1 activation modulates fibroblast proliferation, EP2 and EP4 activation is primarily responsible for migration or chemotaxis. The effects of PGE2 on fibroblast proliferation and migration are thought to be important to its overall role in dermal repair, though the precise contributions of specific cellular effects to the overall wound healing process are unclear. Effects on fibroblast migration are of particular interest to wound repair in that they may dictate the pace of fibroblast migration into the wound bed as well as subsequent wound activity including contraction of wound extracellular matrix (ECM).

While the importance of PGE2 in adult dermal wound healing is well established, evidence is scant as to its role in fetal dermal repair. Wilgus et al reported that COX-2 expression and PGE2 production in the wound bed

increase as dermal wound healing transitions from scarless to a scarring phenotype. At a cellular level, PGE2 was shown to regulate the proliferation of fibroblasts from both wound healing phenotypes. Interestingly, the effects of PGE2 appear to be qualitatively, but not quantitatively conserved between the two fibroblast phenotypes, a finding that may have important implications for the overall wound healing process. The current study was designed to extend these findings by utilizing early passage human dermal fetal and adult fibroblasts to qualitatively and quantitatively compare the effects of exogenous PGE2 on the migratory and contractile properties of both cell types. This analysis combines a traditional two-dimensional motility assay with a modified floating fibroblast populated collagen lattice (FPCL) contraction assay designed to highlight fibroblast interactions with a bioactive collagen matrix as are encountered during migratory and remodeling phases of wound healing**.** To date, no studies have been conducted directly comparing human fetal and adult fibroblast contraction in response to PGE2. PGE2 has been suggested as an autocrine/paracrine mediator of cytokine-induced inhibition of human fetal lung fibroblast contraction by IL-1β and TNFα, predominantly using a cAMP-based signaling cascade.[122] Exogenous PGE2 decreases collagen gel contraction by human fetal lung fibroblasts in a dose-dependent manner without collagen gel degradation.[123,124,125] Adult dermal fibroblast contraction is also diminished by PGE2 by a process that also alters cell morphology.[126,127,128,129]

This study is focused on the effects of PGE2 on human fetal and adult dermal fibroblast migration and contraction. First, it seeks to establish whether both cell types express any or all four PGE2 receptors. This is particularly important, given previous work regarding differential expression of PGE2 receptors during wound healing. In addition, the expression of all four receptors has yet to be characterized in fetal and adult dermal fibroblasts. Second, it extends previous findings, by comparing and contrasting the effects of PGE2 on fetal and adult dermal fibroblast migration with analysis focused on both qualitative and quantitative differences in its effects on fetal and adult dermal fibroblast migration. Third, it uses a combination of horizontal locomotion and modified FPCL contraction in order to relate in vitro cell migration with processes relevant to in vivo wound healing. As such, it should provide insight into the precise role PGE2 plays during dermal repair and fibrosis.

3.2.2 Materials and methods

Chemicals: Prostaglandin E2 (PGE2), 11-deoxy-prostaglandin E1, butaprost (Cayman Chemical, Ann Arbor MI), 17-phenyl-trinor-prostaglandin E2 (Biomol, Plymouth Meeting, PA), N6, 2'-O-dibutyryladenosine 3':5'-cyclic, Rp-Cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt, isoproterenol (Sigma, St Louis MO) and forskolin (Calbiochem, San Diego CA).

Cell culture: Dermal fibroblasts were obtained from human tissues produced during standard medical procedures, with institutional review and approval for work with human-derived materials. For this series of experiments, three human fetal dermal fibroblast cultures of 15, 16 and 18 weeks gestation and six human adult dermal fibroblast cultures were obtained from frozen stocks in our laboratory. A fourth human fetal fibroblast culture (15 week gestation) was obtained from the Coriell Institute for Medical Research (Camden, NJ) and used to validate specific experiments. Frozen cells were thawed and cultured as detailed above.

mRNA analysis: As detailed above (section 3.1.2).

Protein analysis: As detailed above (section 3.1.2).

Cell imaging: Fibroblasts were grown on glass coverslips under normal growth conditions. Media was removed, cells were fixed using paraformaldehyde and permeabilized using Triton-X 100. For EP receptor localization, cells were incubated with anti- EP1, EP2, EP3 or EP4 antibody at 1:250 dilution for 1 hour at room temperature followed by an Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Eugene OR), and TOPRO-3 1:1000 (Molecular Probes, Eugene OR) for nuclear localization. The actin cytoskeleton was visualized using rhodamine phalloidin 1:100 (Molecular Probes, Eugene OR) to highlight the actin fibers. Digital images were captured using an inverted microscope equipped with phase contrast objective (Nikon Inc., Melville, NY), prepared and labeled using Adobe Photoshop® 7.0 software.

Migration assay: Cells were plated in 24 well plates (Costar; Corning Incorporated, Corning, NY) at a density of approximately $3x10^6$ cells/plate. This concentration allowed the fibroblasts to reach confluency overnight. All wounding assays were performed using confluent cell monolayers. A 200-µl plastic pipette tip was used to scratch the cell monolayer following a pre-drawn grid on the underside of each plate creating a standard area devoid of cells. The wells were then filled with the desired experimental medium. In each experiment, 4 wells were assigned to each experimental condition. Each well was photographed using an inverted microscope equipped with phase contrast objective (Nikon Inc., Melville, NY). The image was saved and used as a template for images of the same well as subsequent time points. The images were stored and analyzed using imaging software (Metamorph; Universal Imaging Corporation, Downington, PA). Changes in the denuded area over time were converted into average speed of fibroblast migration for the various conditions tested. Statistical significance was derived using average speeds, standard error of the mean, and Student's t-test.

Collagen gel contraction: The collagen gel contraction assay used in these studies was a modification of the standard FPCL method and an alternative method using Teflon rings. Collagen gels with a volume of 200µl were cast in ½" Teflon rings (Seastrom Manufacturing Company, Inc., Twin Falls, ID) in 60×15 mm Petri dishes (company). The collagen source, Vitrogen (Angiotech Biomaterials Corp., Palo Alto, CA), was prepared using the manufacturer's protocol of a 8:1:1 ratio of Vitrogen, 10X PBS (Sigma, St. Louis, MO), and 0.1 M NaOH (Sigma, St. Louis, MO) while adjusting the final pH with a small amount of 0.1 M HCl (Sigma, St. Louis, MO), to a final concentration of 2.4 mg/ml. The Petri dishes were placed in a 37°C water bath for one hour to allow for collagen gelation. Fibroblasts were placed on top of the collagen gels inside the rings in a volume of 200µl containing 2.88×10^4 cells. The dishes were incubated for 30 minutes to ensure fibroblast adhesion to the collagen gels and floated in 7 ml of medium containing various amounts of fetal bovine serum and pharmacological agents. The Teflon rings were then removed and the collagen gels loosened from the surface with a pipette tip. Collagen gels were imaged at 2, 4, and 24 hours with a CCD camera connected to a dissecting microscope. Changes in collagen gel area were analyzed using the above described imaging software.

cAMP measurement: Intra-cellular cAMP levels were assayed using a commercially available enzyme linked immuno-assay (ELISA) (R&D Systems, Minneapolis, MN). For these experiments, 2 fetal and 2 adult fibroblast cultures were used. Cells were plated and allowed to reach confluency under standard growth conditions in 6 well

plates (Costar; Corning Incorporated, Corning, NY). Once at confluence, cell media was replaced with DMEM (control) or DMEM supplemented with either PGE2 (1 μM), butaprost (1 μM) or forskolin (25 μM) for 30 minutes. Cells were lysed and cAMP levels assayed according to the manufacturer's protocol. cAMP levels were standardized to total protein levels measured using a bicinchoninic acid assay (described above). Triplicate measurements were conducted for each experimental condition.

3.2.3 Results

EP receptor profile

The receptor expression profile of fetal and adult dermal fibroblasts was measured at the mRNA and protein levels. Quantitative PCR indicated no significant difference in the receptor expression profile between fetal and adult dermal fibroblasts (Figure 12). Data were pooled and expressed as relative cycle time, i.e., the number of amplification cycles necessary for each mRNA to be detected, which produces a value inversely related to the relative abundance of the mRNA. In both fetal and adult fibroblasts, mRNA levels for EP3 were highest, followed by EP4 and EP2, with EP1 mRNA levels lowest of the four.

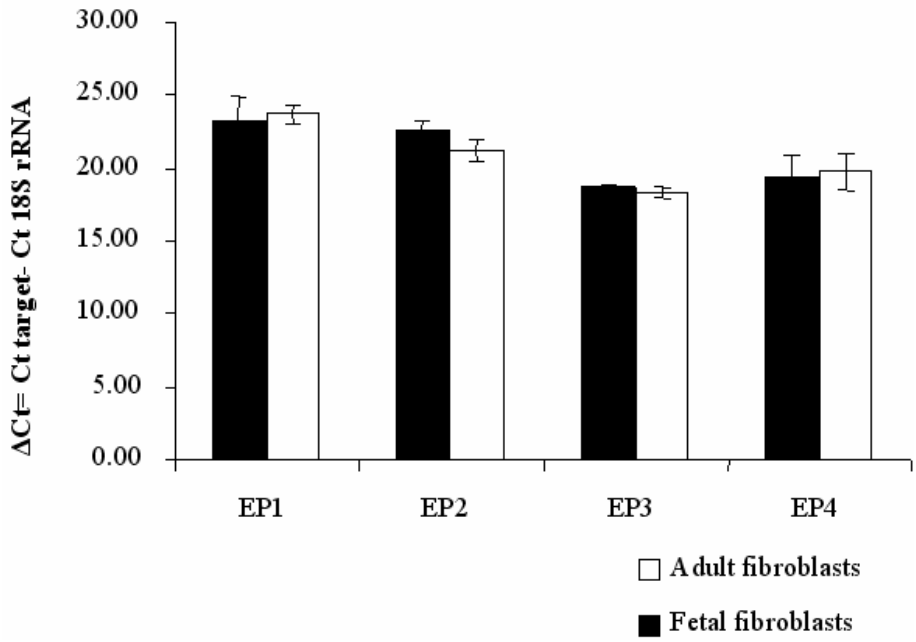


Figure 12. Fetal and adult fibroblasts express mRNA for all four PGE2 receptors.

Fibroblasts were grown under standard conditions, harvested and mRNA levels for each of the prostaglandin E2 receptors measured. The values indicate the EP1-EP4 mRNA levels following standardization to

internal 18S mRNA levels. Data were pooled and expressed as relative cycle time, which produces a value inversely related to the relative abundance of the mRNA, with the error bars representing standard deviations.

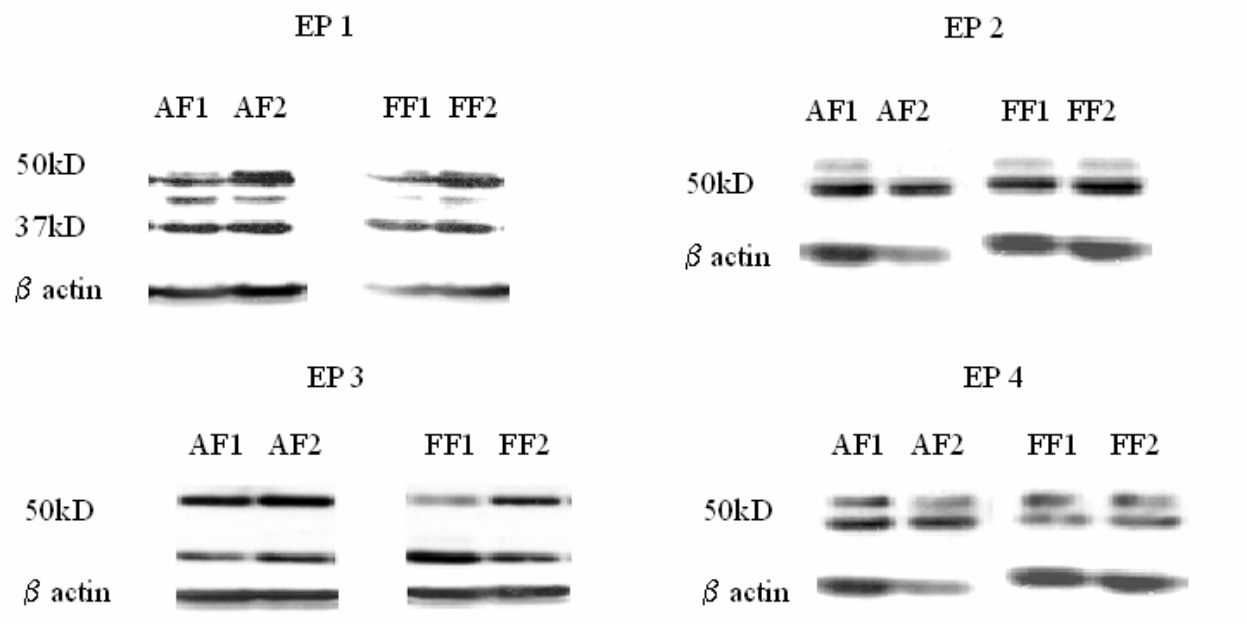


Figure 13. Fetal and adult fibroblasts express protein for all four PGE2 receptors**.**

Whole cell lysates from both adult (Af) and fetal (Ff) dermal fibroblasts were probed using commercial antibodies for each of the four prostaglandin E2 receptors (EP1-EP4). EP2 and EP4 were determined to migrate in the 50kD molecular weight range. Blotting for EP3 revealed two primary protein bands at the 55kD and 45kD molecular weights, which may represent multiple splicing variants. Similarly, blotting for EP1 revealed two primary bands in the 48kD and 37kD molecular range. In order to standardize for protein loading, β-actin was identified using a monoclonal primary antibody (Sigma, StLouis MO) at 1:30,000 dilution and secondary goat anti-mouse (Jackson ImmunoResearch, West Grove PA) at 1:10,000.

Further analysis using standard Western blotting revealed similar levels of EP protein expression in both fibroblast types. Figure 13 is a composite of representative blots of whole cell lysates probed using polyclonal antibodies against EP1, EP2, EP3 or EP4. Protein bands were found to run at the expected molecular weights, as previously reported.[77,105] Analysis of multiple immunoblots revealed no significant difference in the levels of EP receptor expression between the two fibroblast types (data not shown).

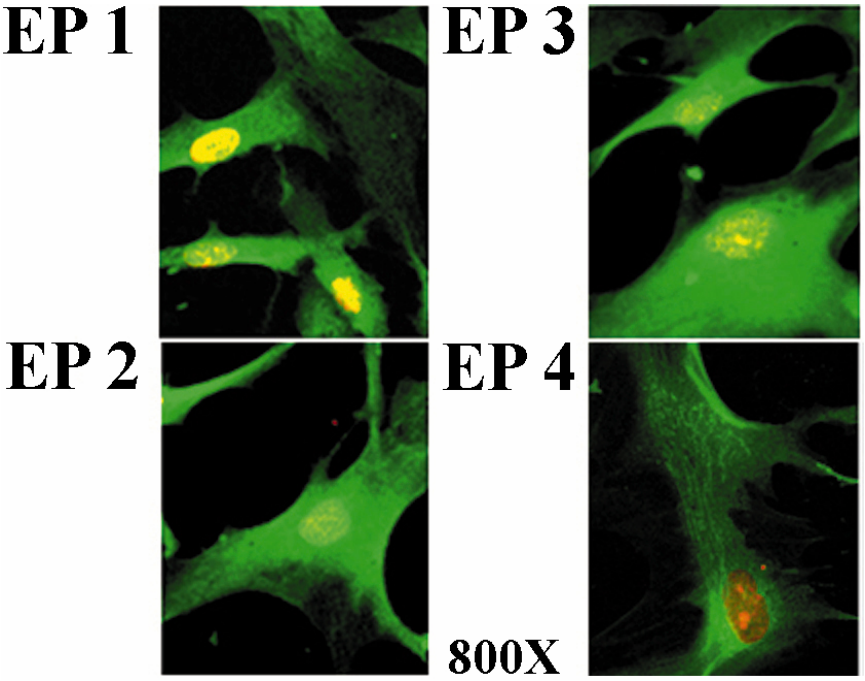


Figure 14. PGE2 receptors have a pan cellular distribution.

Adult fibroblasts were fixed using paraformaldehyde and permeabilized using Triton-X 100. For EP receptor localization, cells were incubated with anti- EP1, EP2, EP3 or EP4 antibody at 1:250 dilution for 1 hour at room temperature followed by an Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Eugene OR), and TOPRO-3 1:1000 (Molecular Probes, Eugene OR) for nuclear localization.

With respect to cellular distribution of the four EP receptors, immunofluorescence revealed localization of all four receptor subtypes throughout the cell in both adult (Figure 14) and fetal (data not shown) fibroblasts, including the cell membrane, cytosol, nuclear membrane and nucleus, consistent with previous reports.[77,78] While all four receptors were localized at multiple sites throughout the cell, EP4 staining revealed a distinct peri-nuclear, localized pattern, which may indicate endoplasmic reticulum-Golgi staining.

Migration

Previous studies have demonstrated that under certain experimental conditions fetal fibroblasts display a higher speed of migration than their adult counterparts. In this study a standard two-dimensional migration assay was used to confirm and extend previous findings. As shown in Figure 15, fetal fibroblasts migrate faster than adult fibroblasts (under basal conditions, without fetal bovine serum). In addition, fibroblast migration is not a constant process, but rather it exhibits a temporal pattern, with speed of migration increasing over time. This trend is conserved in both fetal and adult fibroblasts with speed of migration increasing from the early (0-2 hours) to the latter (4-8 hours) phases of this experimental window. For this series of experiments migration analysis was limited

to the first 8 hours in order to capture the early response of migrating fibroblasts, as well as the early cellular responses to PGE2 signaling.

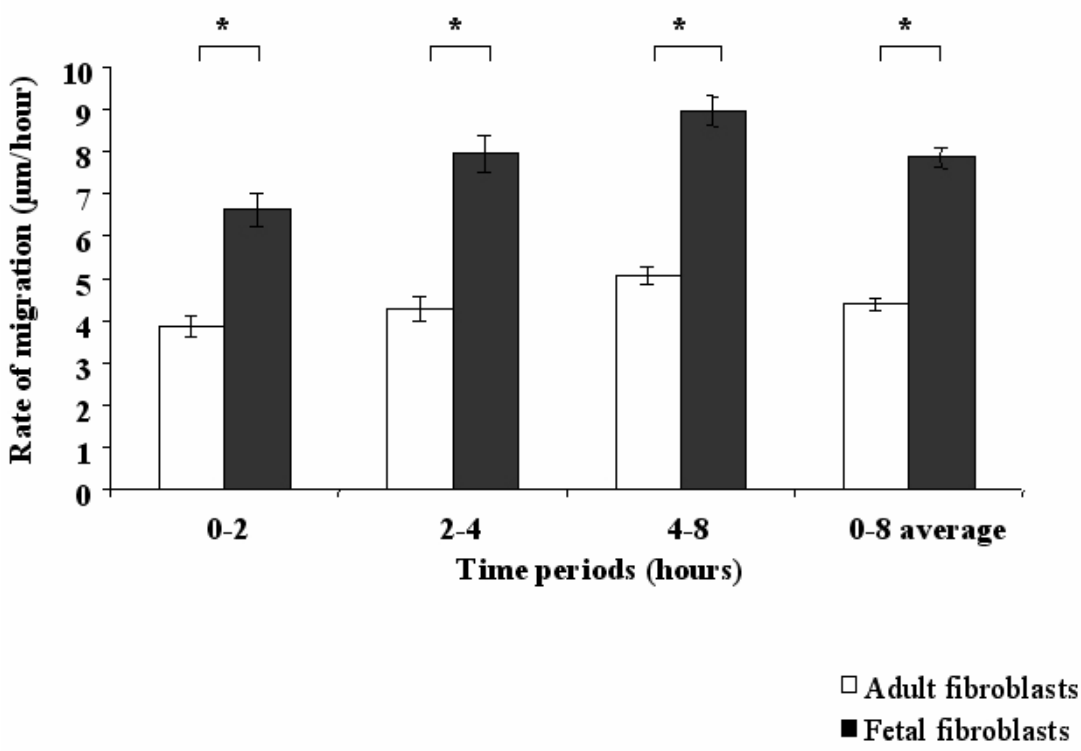


Figure 15. Fetal fibroblasts migrate faster than adult fibroblasts.

All migration assays were performed using confluent cell monolayers in regular fibroblast media (DMEM) with no additional supplementation. Data from multiple fetal or adult fibroblasts were pooled. In each experiment, 4 wells were assigned to each experimental condition. Data are presented as means with error bars representing standard error of the mean. * indicates p-value < 0.01 by Student's t-test.

Using this baseline migratory profile as a starting point, we examined the effects of PGE2 on fetal and adult fibroblast two-dimensional migration. As shown in Figure 16, PGE2 inhibits both fetal and adult fibroblast migration in a dose-dependent manner. The effects on fetal fibroblast migration are consistent with previous reports. While the effects are qualitatively similar, they differ quantitatively. First, fetal fibroblasts migrate faster than adult fibroblasts at each PGE2 concentration tested. Second, while adult fibroblast migration is decreased by more than 80% at the maximal 10µM PGE2 concentration, fetal fibroblast migration is decreased by less than 60%. Fetal fibroblasts exhibit an absolutely greater speed of migration and relatively less inhibition by PGE2.

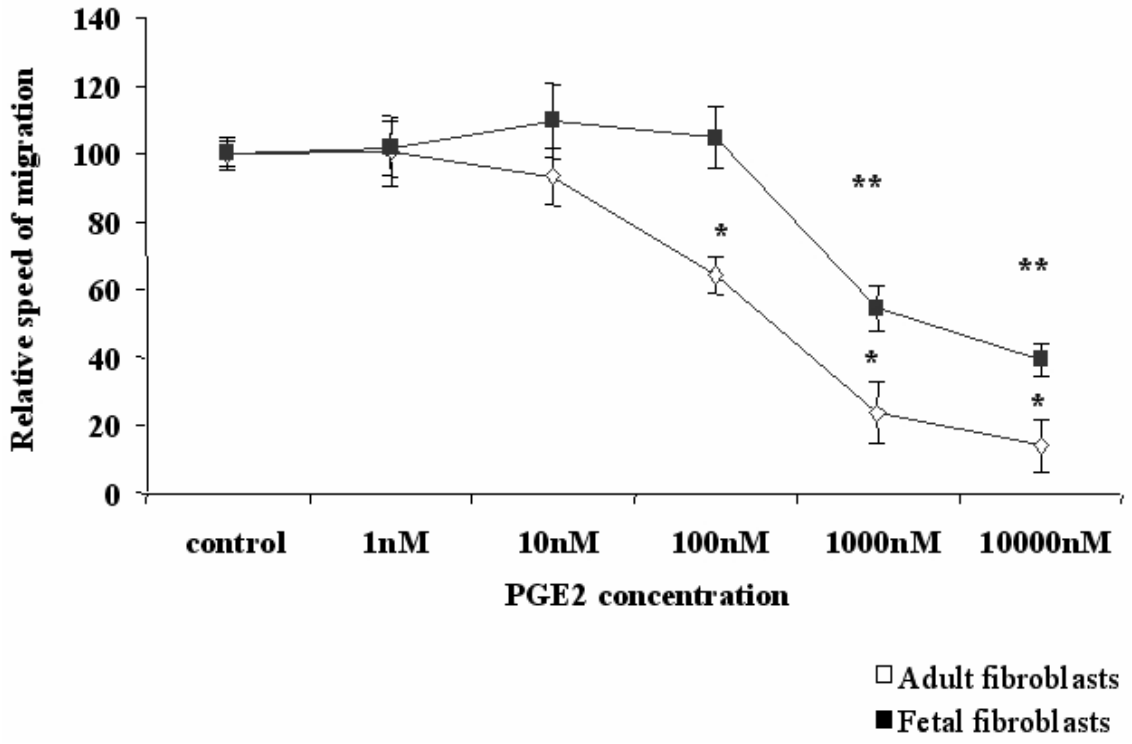


Figure 16. PGE2 inhibits fetal and adult fibroblast migration.

All scratch assays were performed using confluent cell monolayers in regular fibroblast medium (DMEM) with no additional supplementation. Data from multiple fetal and adult fibroblasts were pooled. In each experiment, 4 wells were assigned to each experimental condition. Data are presented as means with error bars representing standard error of the mean. Data points differing from the control value are indicated by asterisks; * indicates adult fibroblast p-value < 0.01 by Student's t-test, ** indicates fetal fibroblast p-value < 0.01 by Student's t-test.

In order to further elucidate the signaling pathway responsible for the effects of PGE2 on fibroblast migration, a variety of pharmacological agents were used in subsequent experiments to inhibit fetal and adult fibroblast migration. Of three EP receptor agonists, 11-deoxy PGE1 (1 μM), an EP2/EP4 agonist, and butaprost (1 μM), an EP2 agonist, inhibited both fetal and adult migration, while 17-phenyl trinor (100 nM) an EP1 agonist had no significant effect on migration for either cell type. Additional analysis suggests that this effect can be mimicked by administration of isoproterenol, a known activator of the beta- adrenergic receptor coupled to downstream activation of Gs proteins and increased levels of intra-cellular cAMP. In all cases, inhibition of adult fibroblast migration is more pronounced than that of fetal fibroblasts (Figure 17).

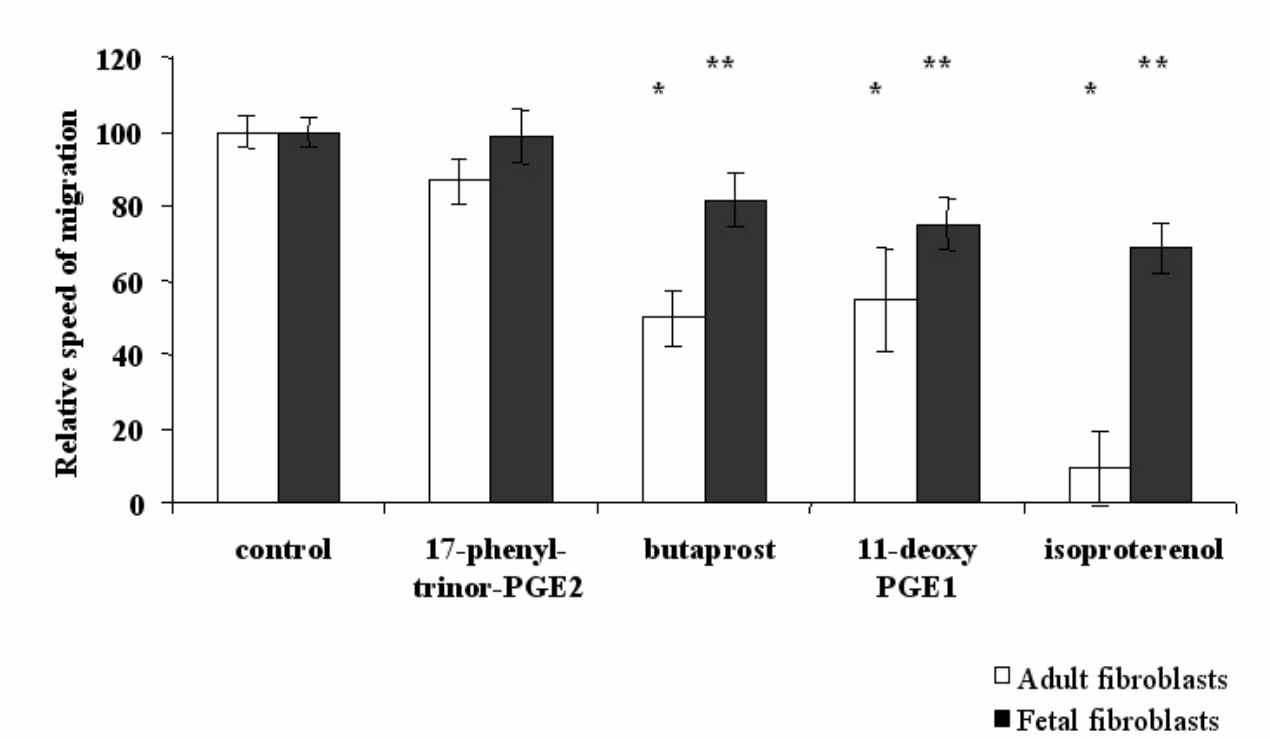


Figure 17. PGE2 signaling via EP2/EP4 receptors inhibits fibroblast migration.

All wounding assays were performed using confluent cell monolayers in regular fibroblast medium (DMEM) under control conditions, or following supplementation with 11-deoxy-prostaglandin E1 (EP2/EP4 agonist) (1μM), butaprost (EP2 agonist) (1μM), isoproterenol (10μM), or 17-phenyl-trinor prostaglandin E2 (EP-1 agonist) (100nM).In each experiment, 4 wells were assigned to each experimental condition. Data are presented as means with error bars representing standard error of the mean. Data points differing from the control value are indicated by asterisks; * indicates adult fibroblast p-value < 0.01 by Student's t-test, ** indicates fetal fibroblast p-value < 0.01 by Student's t-test.

In order to correlate the inhibitory effects of PGE2 to cAMP signaling, intra-cellular cAMP levels were measured under basal or stimulated (PGE2-1 μM, butaprost-1 μM) conditions (Figure 18). Under basal conditions, cAMP levels were indistinguishable between the two cell types (adult fibroblasts-10pmol/mg protein, fetal fibroblasts-10pmol/mg protein). Following stimulation with PGE2 or butaprost, cAMP levels were found to be elevated in adult, but not fetal fibroblasts. The role of intra-cellular cAMP was further demonstrated using forskolin, a direct activator of adenylate cyclase, which was found to inhibit both fetal and adult fibroblast migration. At the chosen pharmacological dose, 25 μM, forskolin inhibited fetal and adult fibroblast migration to a similar degree, which correlated with a dramatic up- regulation in intra-cellular cAMP levels (adult fibroblasts- >1000pmol/mg

protein, fetal fibroblasts- 587.8pmol/mg protein). Direct administration of, dibutyryl cAMP, a membrane-soluble cAMP analog, resulted in a similar inhibition of fibroblast migration, further implicating activation of intra-cellular cAMP cascades in this migration inhibitory effect (Figure 19).

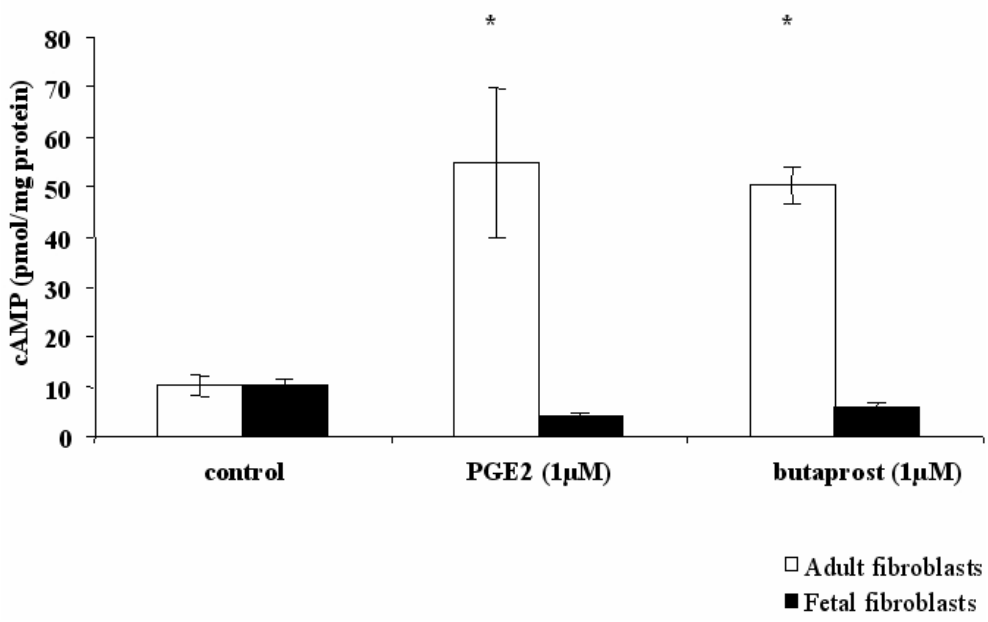


Figure 18. PGE2 up-regulates intra-cellular cAMP levels.

Fetal and adult fibroblast cultures were treated with PGE2 (1µM) or butaprost (1µM) for 30 minutes, cells were lysed and intra-cellular cAMP levels measured. Each experiment was performed in triplicate. Data are presented as means with error bars representing standard error of the mean. * indicates a p-value <0.01 by Student's t-test when compared to the control values for both fetal and adult fibroblast migration.

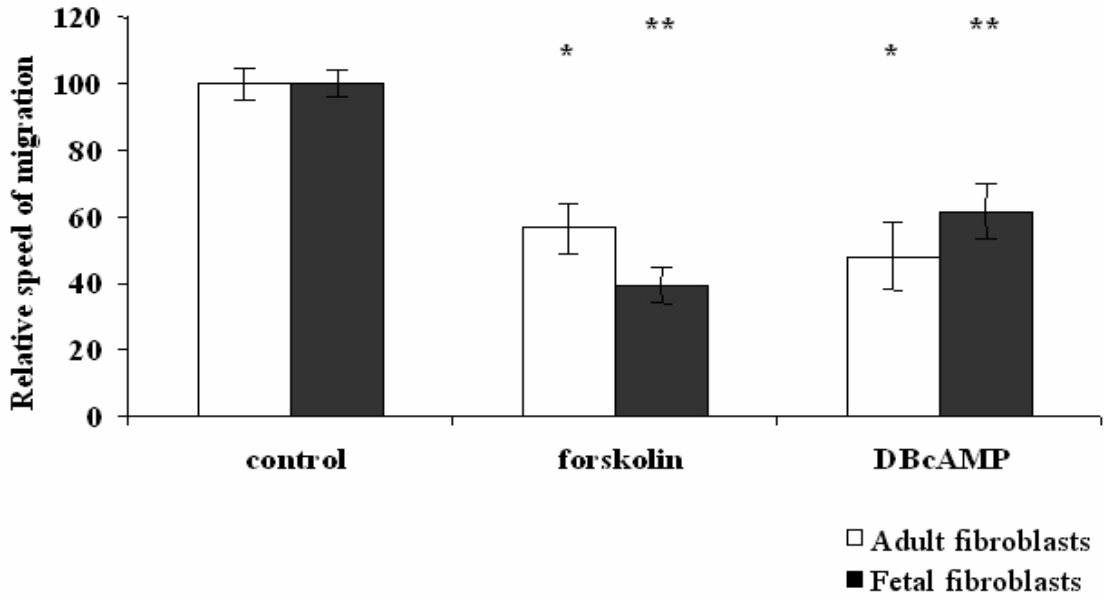


Figure 19. Activation of cAMP signaling inhibits fibroblast migration.

All wounding assays were performed using confluent cell monolayers in regular fibroblast medium (DMEM) under control conditions, or following supplementation with an adenylate cyclase activator, forskolin (25μM) or a soluble cAMP analog, DB-cAMP (500μM). In each experiment, 4 wells were assigned to each experimental condition. Data are presented as means with error bars representing standard error of the mean. Data points differing from the control value are indicated by asterisks; * indicates adult fibroblast p-value < 0.01 by Student's t-test, ** indicates fetal fibroblast p-value < 0.01 by Student's t-test.

Previous studies have indicated a role for protein kinase A (PKA) in modulating the effects of cAMP on fibroblast migration [30] likely by phosphorylating cytoskeleton modifying proteins. The current study confirms that in adult dermal fibroblasts, PKA is partially responsible for the migration inhibitory effects of PGE2. Figure 20 illustrates that rp-cAMP (50 μM), a selective antagonist of PKA, can reverse approximately 50% of the inhibition induced by PGE2 (100 nM). These data are not meant to completely elucidate the precise signaling cascade downstream of cAMP activation, but rather to confirm the implication of PKA in the inhibition of fibroblast migration by PGE2.

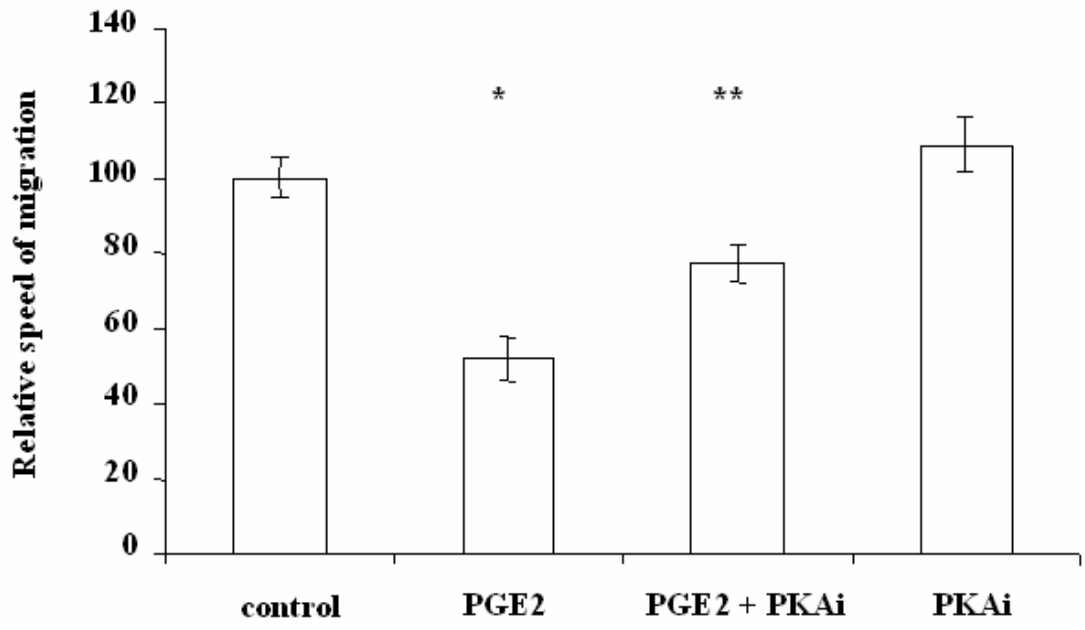


Figure 20. PGE2 inhibition of migration is reversed by blocking PKA activity.

All wounding assays were performed using confluent cell monolayers in regular fibroblast medium (DMEM) with no additional supplementation. Experiments were performed using two adult fibroblast cultures. In each experiment, 4 wells were assigned to each experimental condition. * indicates a p-value <0.01 by Student's t-test when compared to the control values for adult fibroblast migration. ** indicates a p-value <0.01 by Student's t-test when compared to the PGE2 inhibited speed of migration value.

Cellular morphology

Cell migration involves continuous remodeling of the actin fiber cytoskeleton. Disruption of actin structures has been previously shown to inhibit cellular motility [130,131]. Figure 21 illustrates that administration of PGE2 (1μM) or DB-cAMP (500μM) induces a disruption of adult fibroblast actin filamentous structures. Removal of the PGE2 stimulus results in restoration of the resting fibroblast morphology (data not shown). In general, higher concentrations of PGE2 induce this morphological alteration in a higher proportion of cells, a finding consistent with previous reports [132]. In contrast to adult fibroblasts, fetal fibroblast cytoskeletal morphology was not altered by exposure to either PGE2 or DB-cAMP.

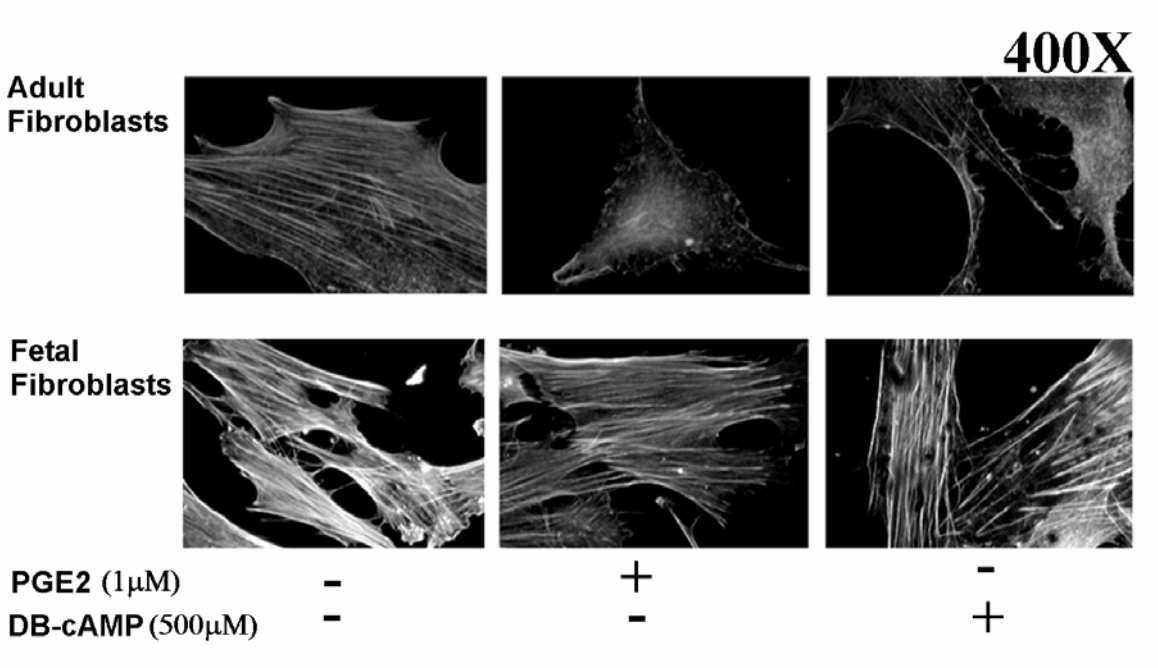


Figure 21. PGE2 and cAMP destabilize the actin cytoskeleton of adult but not fetal fibroblasts**.**

Experiments were performed using confluent cell monolayers in regular fibroblast medium (DMEM) with no additional supplementation. Fibroblasts were fixed using paraformaldehyde and permeabilized using Triton-X 100. The actin cytoskeleton was visualized using rhodamine phalloidin 1:100 (Molecular Probes, Eugene OR) to highlight the actin fibers. Images were captured at 100X magnification and are representative of all the cell cultures used in this study.

Collagen gel contraction

The involvement of actin cytoskeleton remodeling with the effects of PGE2 on fibroblast migration make it likely that the inhibitory effects of PGE2 would be replicated in vivo. To further address this, contraction of fibroblast populated collagen lattices (FPCL) was used to ascertain the effects of PGE2 on fibroblast migration on a bioactive substrate. Under basal conditions (in the absence of fetal bovine serum), fetal fibroblasts exhibit elevated rates of FPCL contraction compared to their adult counterparts (Figure 22).

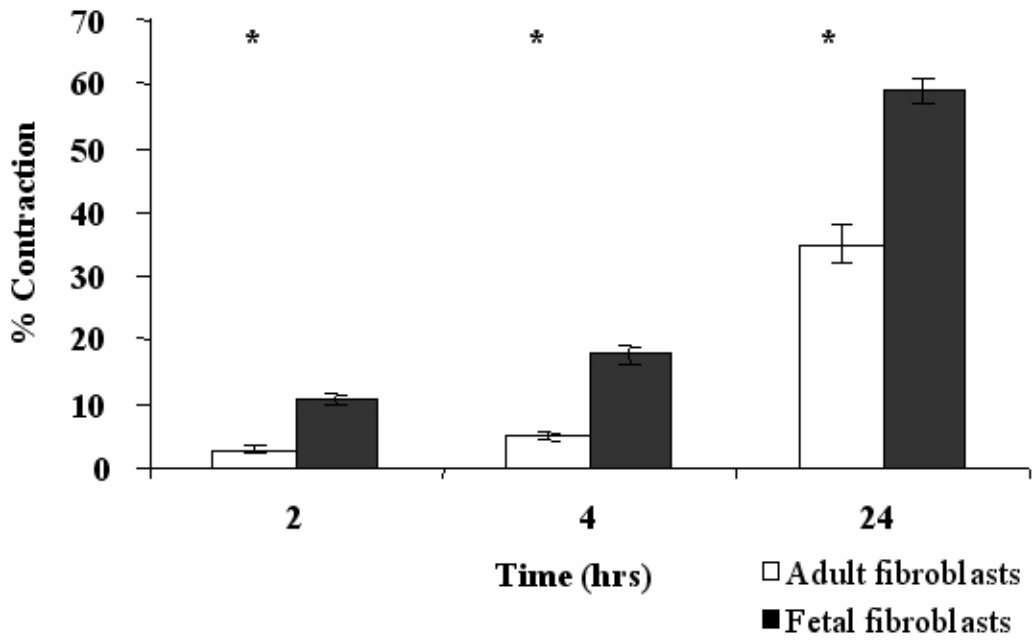


Figure 22. Fetal fibroblasts show greater contractile capability than adult fibroblasts under basal conditions.

All collagen gel contraction assays were performed with the same number of cells and collagen concentration with DMEM but no serum. n=27 and n=20 for the adult fibroblast and fetal fibroblast contraction, respectively. Data are presented as means with error bars representing standard error of the mean. * indicates p-value < 0.01 by Student's t-test when comparing the adult to fetal fibroblast contraction at each time point.

Administration of exogenous PGE2 (1μM) (Figure 23) or forskolin (25μM) (Figure 24) decreases both fetal and adult fibroblast contraction of FPCLs. The effects of PGE2 on FPCL contraction by fetal fibroblasts PGE2 are diminished compared with adult fibroblasts, paralleling the results from the two dimensional migration studies.

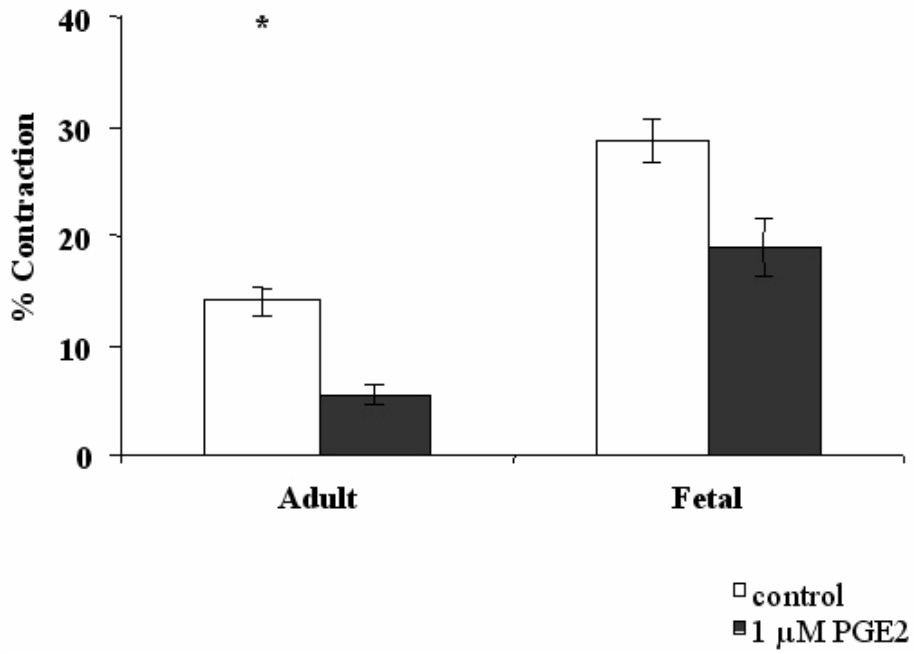


Figure 23. PGE2 inhibits adult fibroblast collagen gel contraction after 2 hours.

All collagen gel contraction assays were performed with the same number of cells and collagen concentration with 2% serum in DMEM with and without 1 μM PGE2. n=24 and 9 and 5 and n=19 and 8 for the adult fibroblast and fetal fibroblast contraction, respectively, for 2% serum and 2% serum with 1 μM PGE2. Data are presented as means with error bars representing standard error of the mean. * indicates p-value < 0.01 by Student's t-test when comparing fibroblast contraction between control and PGE2 cases.

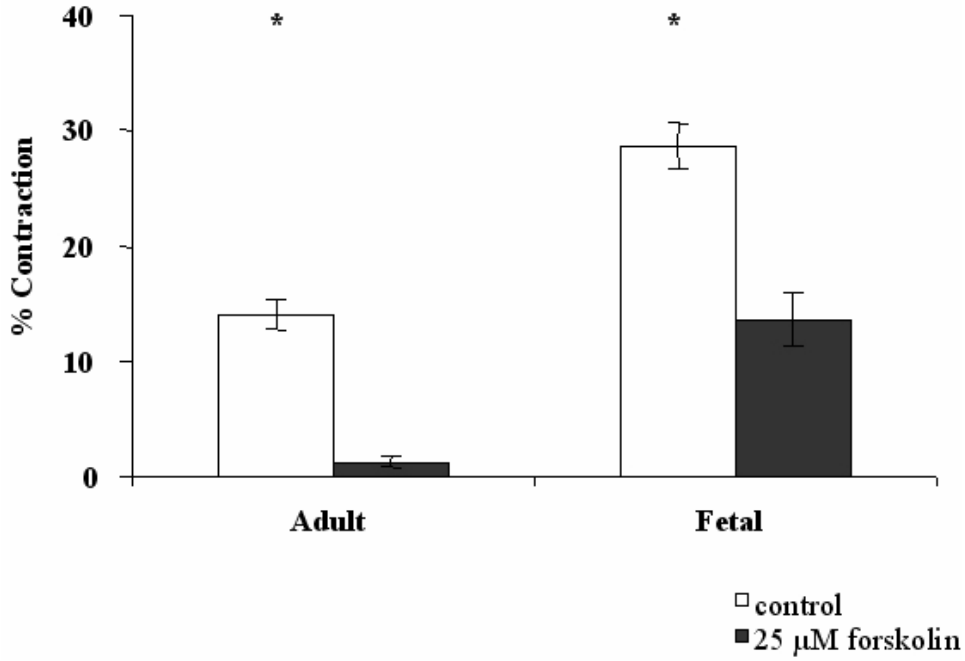


Figure 24. Forskolin inhibits adult fibroblast collagen gel contraction to a larger degree than fetal fibroblast collagen gel contraction after 2 hours.

All collagen gel contraction assays were performed with the same number of cells and collagen concentration with 2% serum in DMEM with and without 25 μM forskolin. n=24 and 5 and 5 and n=19 and 6 for the adult fibroblast and fetal fibroblast contraction, respectively, for 2% serum and 2% serum with 25 μM forskolin. Data are presented as means with error bars representing standard error of the mean. * indicates p-value < 0.01 by Student's t-test when comparing fibroblast contraction between control and PGE2 cases.

3.2.4 Discussion

Dermal injury initiates a cascade of events beginning with hemostasis and inflammation and concluding with ECM deposition and re-modeling. While inflammation eventually subsides as wound healing progresses, it has lasting effects on the final wound healing outcome. Inflammatory mediators, released by macrophages and neutrophils, serve as chemotactic cues for invading fibroblasts and keratinocytes and later regulate cell proliferation and ECM deposition in the wound bed. The importance of inflammation in determining the final outcome of wound healing is highlighted by the different outcomes of fetal and adult dermal repair. While inflammation is a significant component of adult wound healing, it is essentially absent during fetal dermal repair. The diminished inflammatory component of fetal repair is thought to allow fibroblasts to deposit and remodel the wound ECM with organization that is closer to the uninjured tissue.

Injury activates multiple inflammatory cascades including induction of COX-2. PGE2 is thought to be the most important COX-2 product during dermal wound healing. Wilgus et al have indicated that increased COX-2 up-regulation and PGE2 synthesis correlate with the transition of dermal repair from a scarless to a scarring phenotype. In addition, their data indicate that fibroblast sensitivity to PGE2 mitogenic effects increases with gestational age. This study extends these findings in two ways. First, it utilizes early passage human-derived fetal and adult dermal fibroblasts. Given that the gestational switch from scarless healing to scarring is species-dependent, this is an important consideration. Second, it addresses the role of PGE2 in modulating fetal and adult dermal fibroblast migration and ECM contraction. To date, the effects of PGE2 on migration by these two fibroblast phenotypes remain unclear. Our analysis focused on characterizing qualitative and quantitative differences in the response of both fibroblast phenotypes to PGE2.

Data presented here provide insight into the role of PGE2 in regulating fetal and adult dermal fibroblast activity. Human fetal and adult dermal fibroblasts express comparable mRNA and protein levels for all four PGE2 EP receptors with similar cellular distribution patterns. This is particularly important given previous work which indicated that EP4 is differentially regulated during fetal and adult dermal wound healing. Conservation of the EP receptor profile at the level of the fibroblast may be explained in two ways. First, changes in the EP expression profile at the tissue level may be indicative of changes in cells other than fibroblasts. Second, while the basal levels of EP expression are similar, they may change in response to specific cues such as growth factors or inflammatory

mediators. Previous studies have demonstrated that fetal fibroblasts retain a faster speed of migration and differential modulation of migration by extrinsic cues.[133] These data confirm this finding in human-derived dermal cells. Using this as a baseline, the effects of PGE2 on fetal and adult fibroblast migration were determined. Data indicate that PGE2 inhibits both fetal and adult dermal fibroblast migration in a dose-dependent manner. However, this inhibition is significantly lower in fetal fibroblasts. There are consistently higher rates of fetal fibroblast migration at each PGE2 concentration and the maximal relative inhibition of fetal fibroblast migration is less compared to adult fibroblasts. These findings are important, in that they establish a quantitative difference between the response of fetal and adult dermal fibroblast to PGE2. These results are consistent with data reported by Wilgus et al showing that PGE2 effects on dermal fibroblast proliferation were qualitatively conserved, yet quantitatively different.[15,35]

The mechanism for inhibition of migration is conserved in both fetal and adult fibroblasts. PGE2 effects on migration are attributed to the EP2/EP4, but not EP1 receptors. This is in contrast to the mitogenic effects of PGE2, which are thought to be transduced via the EP1 receptor. This study did not focus on PGE2 effects transduced via the EP3 receptor because of the complexity of signal transduction for this receptor; multiple EP3 splice variants have been identified, each tied to a different signaling pathway and subsequent cell activity. Additionally, the pharmacological armamentarium available for a study of EP3 signaling is not as receptor-specific as that available for the other PGE2 receptors.

Previous studies utilizing extensive pharmacological experimentation have demonstrated that activation of EP2 and EP4 receptors results in increases in intra-cellular cAMP via coupling with Gs proteins Here we established a differential role for cAMP in PGE2-induced inhibition of adult and fetal fibroblast migration using several pharmacological agents. Isoproterenol, known to activate a non- PGE2 related cAMP coupled receptor, the beta-adrenergic receptor, was found to mimic the effects of PGE2. Administration of either PGE2 or an EP2 specific agonist (butaprost) resulted in a significant up- regulation in adult fibroblast intra-cellular cAMP levels, but failed to achieve the same result in fetal fibroblasts. This may indicate reduced coupling of PGE2 receptors to intra-cellular components of the cAMP pathway in fetal fibroblasts. In contrast to receptor dependent cAMP up-regulation, direct activation of adenylate cyclase by forskolin inhibited both fetal and adult fibroblast migration and correlated with a significant elevation in intra-cellular cAMP levels in both cell types. Finally, administration of an exogenous cAMP analog duplicated the PGE2-induced inhibition of fibroblast migration. Together, these data confirm that: 1) PGE2 inhibits fibroblast migration in a cAMP-dependent manner in these two fibroblast phenotypes and 2) fetal fibroblasts possess patency of this intra-cellular signaling pathway. However, it is possible that in fetal fibroblasts, PGE2 coupling to cAMP activation is quantitatively diminished.

Inhibition of fibroblast migration was found to correspond to an obvious morphological alteration in the actin cytoskeleton. Immunofluorescence revealed that PGE2, likely through a cAMP mediated pathway, destabilizes the actin cytoskeleton and depolymerizes existing actin stress fibers. This is consistent with previous studies linking cAMP release to PKA activation and cytoskeletal rearrangement.[130,131,132,134,135,136] It is likely that this process is responsible for the impairment of fibroblast motility. Data indicate that pharmacological inhibition of PKA can partially restore adult fibroblast motility. Interestingly, actin fiber depolymerization does not appear to

occur in fetal dermal fibroblasts in the tested range. For the purpose of this study, analysis was limited to those concentrations of various pharmacological agents that were either previously reported or were close to the dissociation constants (K*d*) of the various receptors.

These data indicate that fetal fibroblast migratory properties may rely on different cytoskeletal dynamics and render these cells less responsive to the effects of exogenous cues. They also allow us to begin to extrapolate these in vitro results to in vivo cellular responses. Two dimensional in vitro migration does not completely recapitulate the many dynamic processes of wound healing, such as interactions with other cell types and extracellular matrix components. However, both in vitro and in vivo cell motility are dependent upon appropriate cytoskeletal dynamics. These data indicate that fetal fibroblasts may be partially refractory to anti-migratory signals (PGE2) and that this is partially dependent on intrinsically different actin cytoskeleton stability. In order to begin to address the in vivo implications of the in vitro data, a modified free floating collagen lattice contraction assay was used to determine whether PGE2 impairment of fibroblast motility is conserved in the presence of a bioactive substrate. While most migration studies to date focus on horizontal cell locomotion over an inert surface, there exist in vitro methods that reproduce some of the complex in vivo processes underlying wound repair. More specifically, it is possible to mimic the cellular forces exhibited by locomoting fibroblasts using a well-established protocol of floating fibroblast populated collagen lattice (FPCL).[137]

The FPCL assay has previously been used as an in vitro tool to study fibroblast-driven collagen fiber organization and contraction. Collagen fibril reorganization is driven primarily by mechanical and noncovalent interactions of cells and collagen molecules, respectively, and shows little dependence on collagen degradation or production, cross-linking of fibrils, or enzymatic modification.[138,139] More specifically, contraction and remodeling of collagen gels results from tangential shearing forces generated within the actin cytoskeleton via a family of β1 integrin receptors that bind directly to sites on the collagen molecules causing the reorganization of the collagen fibrils.[140,141,142] In FPCLs, contraction and remodeling have been hypothesized to be mainly due to forces exerted via cellular locomotion since fibroblasts in this model do not exhibit α-smooth muscle actin or spindle bipolar morphology characteristic of myofibroblasts in anchored collagen gel models. [126,143] Few studies have been conducted comparing human fetal and adult dermal fibroblast contraction.[28,31,144] It is generally accepted that fetal and adult fibroblasts have different contractile properties, which are exacerbated by specific extrinsic cues including growth factors and inflammatory mediators. In this study, a modified version of the FPCL was used to quantify fibroblast contraction of collagen gels over a 24 hours period, with additional 2 and 4 hour observation intervals. Data indicate significant contraction of collagen gels over the entire 24 hour period, with fetal fibroblasts maintaining a higher rate of contraction compared to their adult counterparts. This is important in that it correlates well with the two dimensional migration data and suggests that a similar mechanism underlies both processes. Addition of exogenous fetal bovine serum increased the rates of fetal and adult fibroblast contraction, though fetal fibroblasts achieve a maximal contractile rate at lower FBS concentrations.

Given the dramatic effects of PGE2 on adult fibroblast migration and cytoskeletal re-arrangement, it was expected that PGE2 would inhibit adult fibroblast contraction. Results showed that PGE2 (1μM) reduces adult fibroblast contraction by more than 50%, an effect mimicked by forskolin, once again implicating activation of

cAMP signaling as being responsible for these effects. In contrast, both mediators had a quantitatively diminished effect on fetal fibroblast contraction of collagen lattices. These data clearly indicate an intrinsic ability of fetal fibroblasts to retain certain aspects of the cytoskeleton responsible for force generation and less sensitivity to soluble mediators.

CONCLUSIONS. In the fetal dermis, injury is accompanied by a diminished inflammatory response, exemplified by decreased COX-2 activation and PGE2 production. The precise cellular effects resulting from this altered inflammatory response remain unclear. This study addresses the role of PGE2 in regulating fibroblast migration and collagen contraction, two crucial components of the fibrotic phase of dermal repair. The results indicate that fetal fibroblasts are partially refractory to exogenous PGE2, though they retain functional signaling pathways for this molecule. These results suggest that lower endogenous production of PGE2 during fetal dermal repair may combine with diminished fibroblast responsiveness to this mediator to reduce the degree of inflammation-triggered fibroblast activation in fetal wound healing.

3.3 PROSTAGLANDIN E2 REGULATES NORMAL AND KELOID FIBROBLAST MIGRATION AND COLLAGEN SYNTHESIS

3.3.1 Introduction

Keloid formation is an important dermatological concern that remains incompletely understood. Keloids are excessive scars that extend beyond the boundaries of the initial dermal wound, and occur most frequently on the chest, shoulders, arms, head and neck areas of patients. Their presence in the dermis represents a significant clinical concern, with associated patient morbidity. Current treatment modalities for keloids are only partially effective. Recurrence rates following surgical excision range between 45and 100%. Addition of single fraction radiotherapy following excision reduces but does not eliminate the risk of recurrence. Non-surgical approaches, including corticosteroids, immuno-modulatory agents (interferon, imiquimod) or cytotoxic agents (5-fluorouracil), have shown variable success rates, with significant accompanying side effects.[33,34]

Understanding the cellular and molecular processes underlying keloid formation is crucial to the development of more successful therapeutic interventions. Existing research indicates that this dermal pathology is primarily due to aberrant fibroblast activity in the wound bed. Keloid fibroblasts have been shown to have altered rates of proliferation, migration and collagen synthesis, properties crucial to wound healing.[39,50,**145**] The keloid fibroblast phenotype is further exacerbated by altered responses to extrinsic cues. Keloid fibroblast DNA synthesis is refractory to inhibition by phorbol esters, through a mechanism partially dependent on endogenous prostaglandin production.[7] Keloid fibroblast migration in response to epidermal growth factor (EGF) is reduced due to limited activation of intracellular motility pathways.[40] Collagen synthesis by keloid fibroblasts is regulated by extrinsic factors, including transforming growth factor-beta1 (TGF-β1), interleukin (IL)-13 and multiple keratinocyte-derived factors, in a manner different from normal dermal fibroblasts.[146,147,148]

Inflammation is an early and important component of wound healing. In the dermis, macrophages and neutrophils invade the wound bed, debride necrotic tissue and stimulate subsequent keratinocyte and fibroblast invasion of, and activity in, the wound bed. Wound fibroblasts also participate in the inflammatory phase of wound healing, by secreting a variety of inflammatory mediators, ranging from IL-6 and IL-8 to prostaglandins.[13,14,116] Keloid formation is accompanied by an abnormal inflammatory component involving both the secretory and responsive properties of keloid fibroblasts with respect to inflammatory cues [149,**150**]**.** Local aberrations in inflammation are accompanied by systemic abnormalities in the immune/inflammatory states of keloid patients.[7,36] These findings raise the possibility that an altered inflammatory state may exacerbate the abnormal keloid fibroblast phenotype and result in an exuberant, up-regulated wound healing response.

Prostaglandin E2 (PGE2) is an important inflammatory mediator, synthesized from arachidonic acid by cyclo-oxygenases (COX) and synthases (PGES). Multiple studies have demonstrated that COX-2 expression and PGE2 secretion increase subsequent to tissue damage, though the precise role of PGE2 in connective tissue repair has not yet been fully elucidated. In the lower airway (lung) mucosa, PGE2 appears to have a protective, anti-fibrotic effect.[53,64,115,116,117] In contrast, during dermal repair, PGE2 appears to have pro-fibrotic effects. Studies

conflict as to the importance in dermal wound repair of endogenous PGE2 and other cyclooxygenase products.[46,47,82,83] The precise contribution of PGE2 to the final outcome of dermal wound healing remains unclear, particularly because in vitro experiments have demonstrated that PGE2 has anti-fibrotic effects, reversing the transition of fibroblasts to the myofibroblast phenotype induced by TGF-β1. It is possible that the pro-fibrotic events associated with increased PGE2 in the dermis result indirectly from PGE2-driven inflammation, and not from direct PGE2 effects on fibroblast activity in the wound bed.

PGE2 synthesis is highly regulated by exogenous cues, including IL-1β, at both the COX-2 and mPGES levels.[64,66,118] This regulation results in a highly integrated inflammatory response to injury that links production and secretion of multiple secondary inflammatory mediators such as PGE2, IL-6 and IL-8 throughout the wound healing process.[13,35] Fibroblasts are an important component of this process. They can secrete PGE2, IL-6 and IL-8 in response to primary inflammatory cues. In addition, they can modulate their activity in response to exogenous administration of secondary inflammatory mediators.

The current study attempts to advance understanding of the role of PGE2 in dermal fibrosis, specifically keloid formation. Few studies to date have addressed this issue and they have failed to provide a consensus. The current approach used in vitro methodology to analyze two aspects of PGE2 signaling in keloid fibroblasts. First, it investigated whether endogenous production of PGE2 by keloid fibroblasts is impaired in a manner similar to fibrotic lung mucosal fibroblasts to determine whether PGE2 production during keloid formation might be impaired. Second, it investigated the effects of exogenous PGE2 on keloid fibroblast migration and collagen synthesis, activities crucial to keloid scar formation. It is important to note that despite previous studies which have examined the effects of PGE2 on fibroblast activity in other tissue types, it would be incorrect to simply extrapolate such findings to this abnormal dermal fibroblast phenotype. This is particularly important given the demonstrated paradoxical effects of PGE2 in lower airway and dermal repair. This represents the first comprehensive analysis of the role of PGE2 in regulating keloid fibroblast activity, and the results may broaden understanding of the role of PGE2 in keloid formation and shed light on novel therapeutic approaches that modulate inflammatory processes during keloid formation.

3.3.2 Materials and methods

Chemicals: as described above

Cell culture: Human fibroblasts from normal skin and from keloids were obtained from tissues excised during standard surgical procedure, according to a protocol approved by the Institutional Review Board of the University of Pittsburgh and Children's Hospital of Pittsburgh. Six keloid and 5 normal dermal fibroblast cultures derived from human biopsies were obtained from in house frozen stocks. These cells were obtained from normal (N) dermis of 4 female and 1 male individual (age range 20-66), or keloid (K) scars from the dermis of 5 male and 1 female individual (age range 8-57). Frozen cells were thawed and cultured as described above.

mRNA analysis: as described above (section 3.1.2).

Protein analysis: as described above (section 3.2.2).

Cell imaging: as described above (section 3.2.2).

Migration assay: as described above (section 3.2.2).

3.3.3 Results

Inflammatory mediator secretion

The secretory profile of normal and keloid dermal fibroblasts was established by measuring endogenous production of two key secondary inflammatory mediators (IL-6 and IL-8). Both mediators have previously been shown to play an important role in the inflammatory phase of wound healing. Data (Figure 25) indicate that under basal condition, normal fibroblasts secrete higher concentrations of both IL-6 and IL-8 than their keloid counterparts, though in neither case, do these rise to the level of statistical significance (IL-6 p= 0.09, IL-8 p= 0.15). Stimulation of both normal and keloid fibroblasts with a primary inflammatory mediator, IL-1β resulted in increased secretion of both IL-6 and IL-8 in both fibroblast phenotypes.

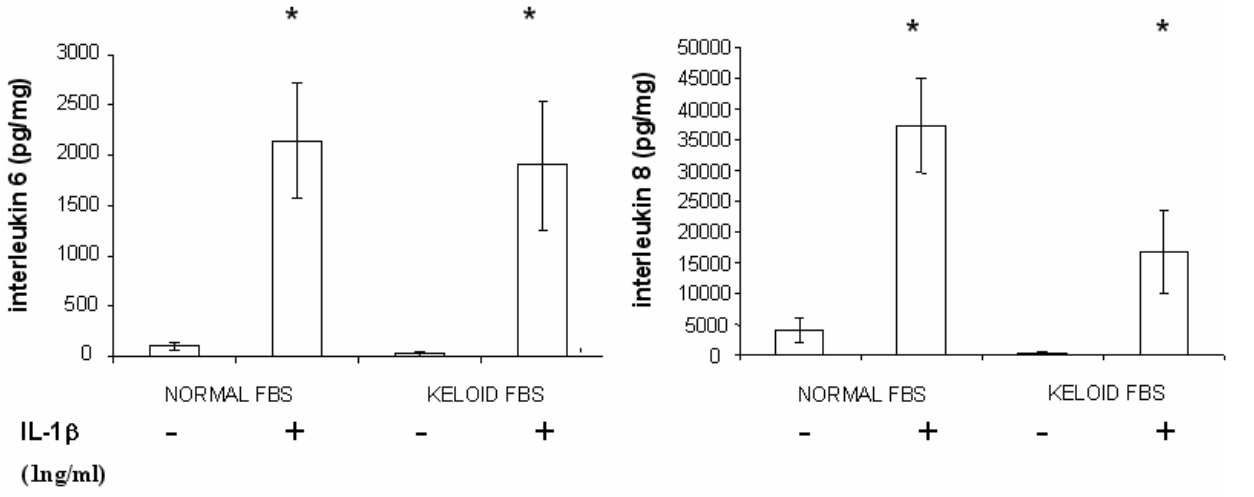


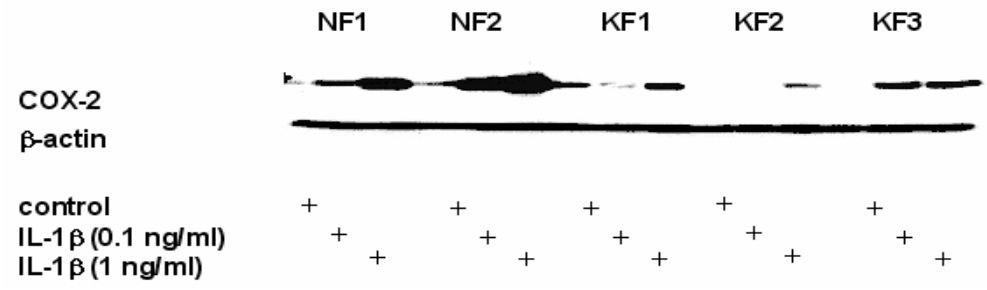
Figure 25. IL-1β increases normal and keloid fibroblast production of IL-6 and IL-8.

Confluent monolayers of normal and keloid fibroblasts were treated with interleukin-1β (1ng/ml) in the absence of fetal bovine serum for 24 hours. Media was collected and analyzed for interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion using commercial ELISA kits. Each experiment was performed using 4 different normal and keloid fibroblast cultures. Data are expressed as averages with error bars representing standard error of the mean. * represents $p < 0.05$

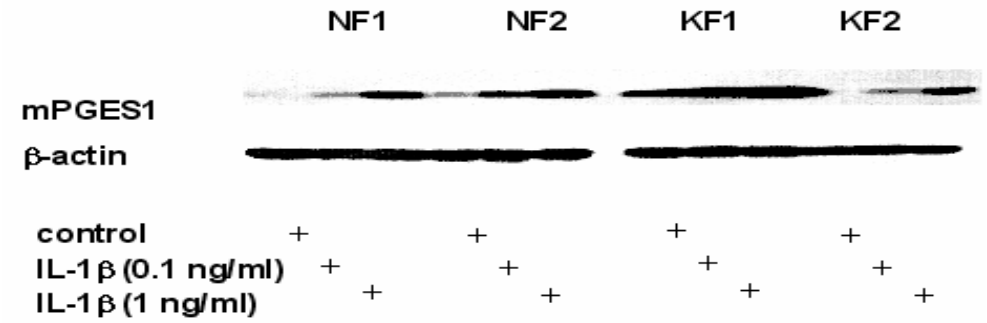
With this result as a starting point, the secretory pathway for PGE2 was investigated (Figure 26). Administration of IL-1β (1ng ml $^{-1}$) up-regulated the intracellular levels of COX-2 in both normal and keloid fibroblasts (Fig 26A), though the keloid response was attenuated. While stimulation with IL-1β resulted in a significant increase in endogenous PGE2 levels in normal fibroblasts ($p < 0.05$), it failed to evoke a similar

significant increase in keloid fibroblasts (Fig 26C). Protein levels of mPGES1 were up- regulated to a similar degree in both keloid and normal fibroblasts (Fig 26B).

A



B



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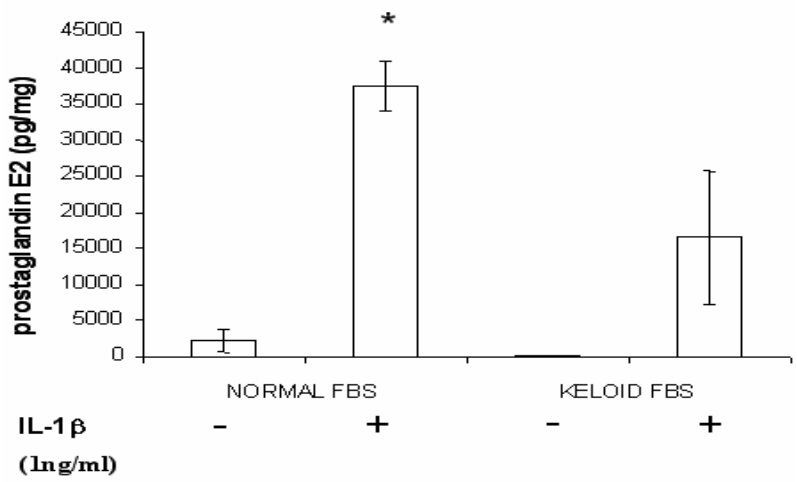


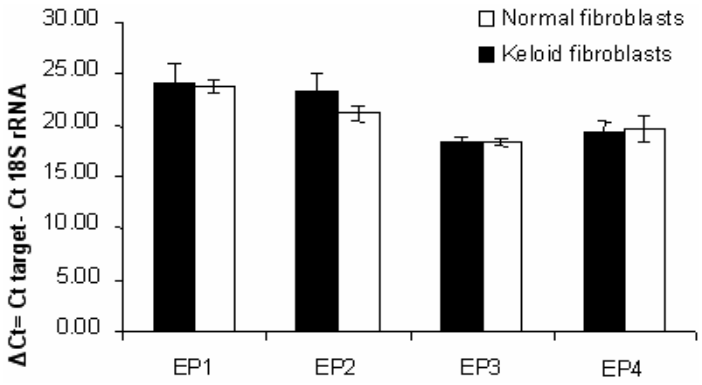
Figure 26. IL-1β up-regulates COX-2 and mPGES1 expression and PGE2 production by fibroblasts.

Confluent monolayers of normal and keloid fibroblasts were treated with interleukin-1β for 24 hours. The cell layer was isolated and processed for protein isolation. Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human COX-2 (panel **A**) and mPGES1 (panel **B**). Panel **C)** Media was collected and analyzed for PGE2 secretion using a commercial ELISA kit. Data are expressed as averages with error bars representing standard error of the mean. * represents $p<0.05$

EP receptor profile

The PGE2 receptor expression profile of normal and keloid dermal fibroblasts was measured at the mRNA and protein levels. With quantitative PCR, there was no significant difference in the expression profile of normal and keloid dermal fibroblasts (Figure 27A). In both fibroblast types, mRNA levels for EP3 were highest, followed by EP4 and EP2, with EP1 mRNA levels lowest of the four (data not shown). Further analysis using standard Western blot analysis revealed similar levels of EP protein expression in both fibroblast types (Figure 27B). Protein bands were detected at the expected molecular weights, as previously reported. [19,22] Analysis of multiple immunoblots revealed no significant difference in the level of EP receptor expression and cellular localization between the two fibroblast types (data not shown).

A



B

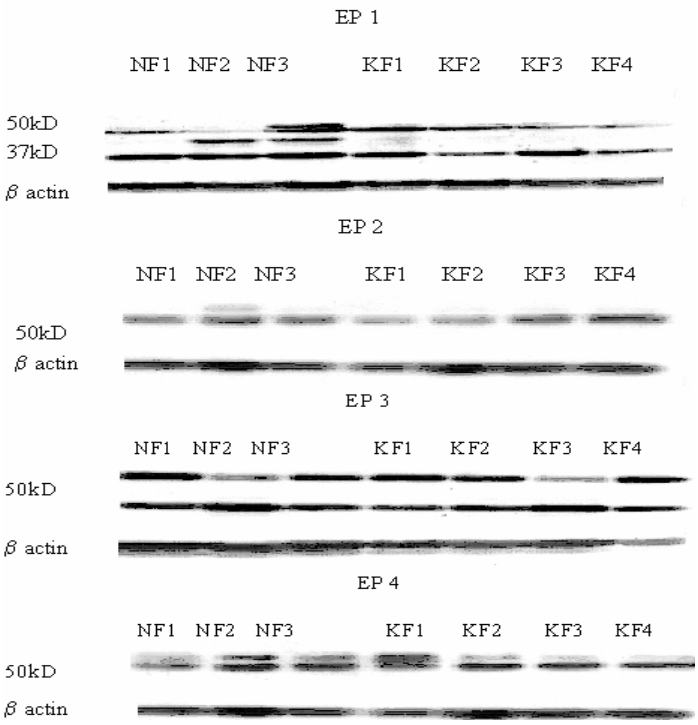


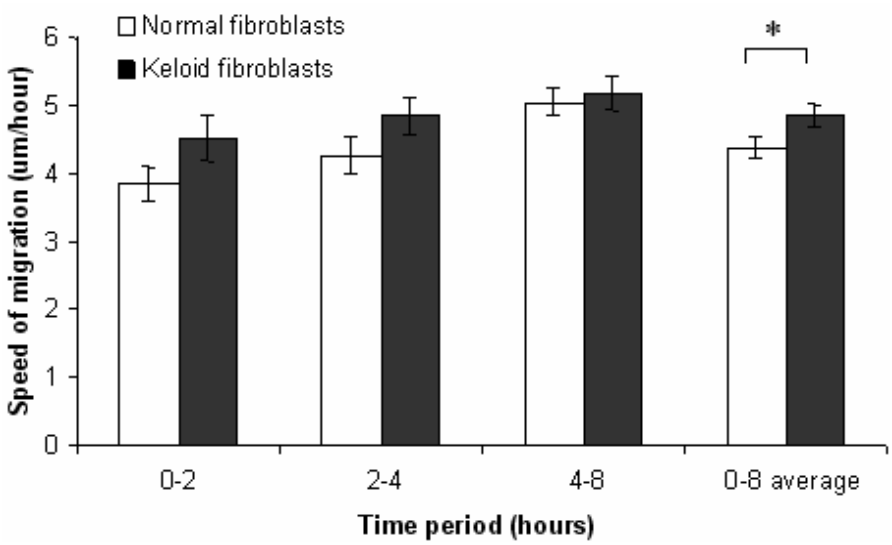
Figure 27. Keloid fibroblasts express EP receptor mRNA and protein levels similar to their normal counterparts.

Confluent monolayers of normal and keloid fibroblasts were processed for RNA and protein isolation. Panel **A)** mRNA was reverse transcribed and the resulting cDNA was amplified using human EP specific primers. The levels of keloid EP receptor mRNA are expressed relative to normal EP receptor mRNA after normalization to a uniformly expressed gene, 18S. Panel **B)** Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against each of four PGE2 receptors (EP1-EP4).

Migration

A well established, two-dimensional migration assay was used to measure and compare the speeds of migration of keloid and normal dermal fibroblasts. As shown in Figure 28, keloid fibroblasts migrate slightly, but significantly faster than normal fibroblasts (under basal conditions in the absence of fetal bovine serum). The temporal migration pattern (with speed of migration increasing over time) is conserved in both keloid and normal fibroblasts (Figure 28A). For this series of experiments migration analysis was limited to the first 8 hours in order to capture the early response of migrating fibroblasts, as well as the early cellular responses to PGE2 signaling. The migration speed of both normal and keloid fibroblasts was similarly modulated by the addition of varying amounts of fetal bovine serum, a common fibroblast media additive (Figure 28B). Fetal bovine serum exhibited a biphasic effect on both normal and keloid fibroblast migration.

A



B

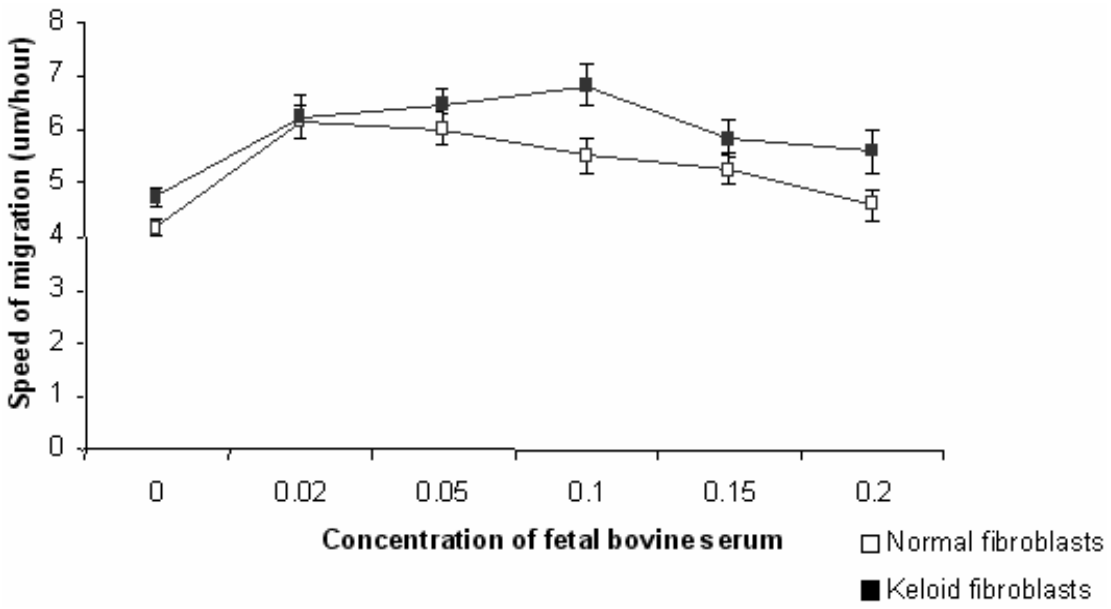


Figure 28. Keloid fibroblasts exhibit a similar migratory profile to their normal counterparts.

Scratch assays were performed on normal and keloid fibroblast monolayers and the speed of migration measured. Panel **A)** Speed of migration for each of the time periods studied is represented as averages with error bars representing standard error of the mean. * represents p-value <0.05 Panel **B)** Speed of normal and keloid fibroblast migration in the presence of increasing concentrations of fetal bovine serum (0% to 20% concentration in DMEM) was measured and compared over the entire 8 hour experimental period. Data are expressed as averages with error bars representing standard error of the mean.

Using this baseline migratory profile as a starting point, we examined the effects of PGE2 on keloid and adult fibroblast two dimensional migration. As shown in Figure 29, PGE2 inhibits both keloid and adult fibroblast migration in a dose dependent manner. In order to further elucidate the signaling pathway responsible for the effects of PGE2 on fibroblast migration, pharmacological agonists were used in subsequent experiments. Of three EP receptor agonists tested, 11-deoxy PGE1 (an EP2/EP4 agonist) and butaprost (an EP2 agonist) inhibited both normal and keloid migration, while 17-phenyl trinor (an EP1 agonist) had no significant effect on fibroblast migration. Subsequent analysis revealed that PGE2 inhibition of migration is mimicked by stimulation of the cAMP signaling pathway. Isoproterenol (a beta adrenergic agonist), forskolin (an adenylate cyclase activator) and dibutyril cyclic AMP (a membrane permeable cAMP analog) were shown to inhibit both normal and keloid fibroblast migration (Figure 30).

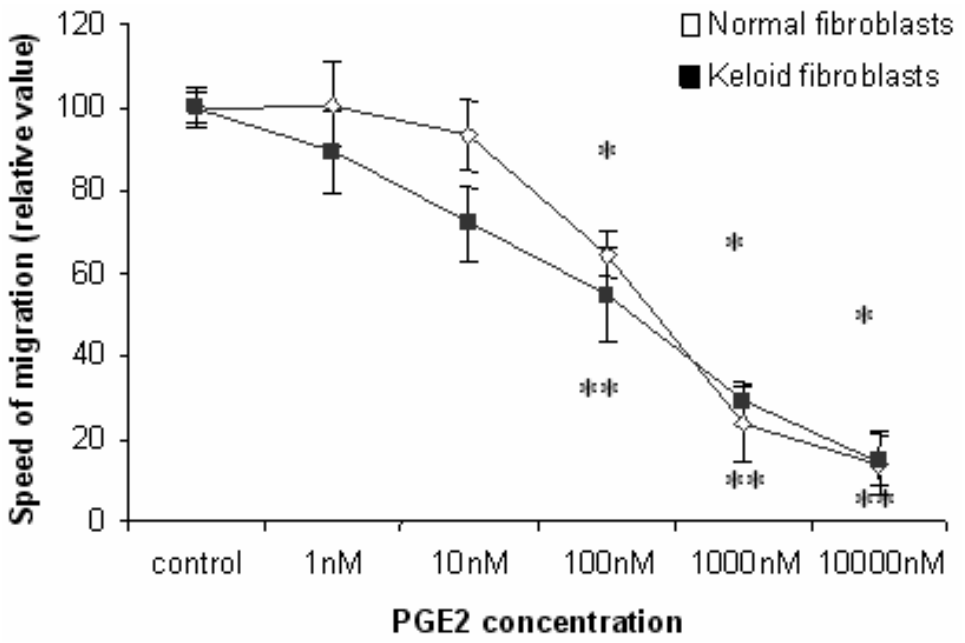


Figure 29. PGE2 inhibits keloid fibroblast migration in a dose dependent manner.

Scratch assays were performed using confluent normal and keloid fibroblast monolayers in regular fibroblast medium in the absence of fetal bovine serum. Data from multiple normal and keloid fibroblasts were pooled. In each experiment, 4 wells were assigned to each experimental condition. Data are presented as means with error bars representing standard error of the mean. Data points differing from the control value are indicated by asterisks: * indicates normal fibroblast p-value < 0.05 ** indicates keloid fibroblast p-value < 0.05

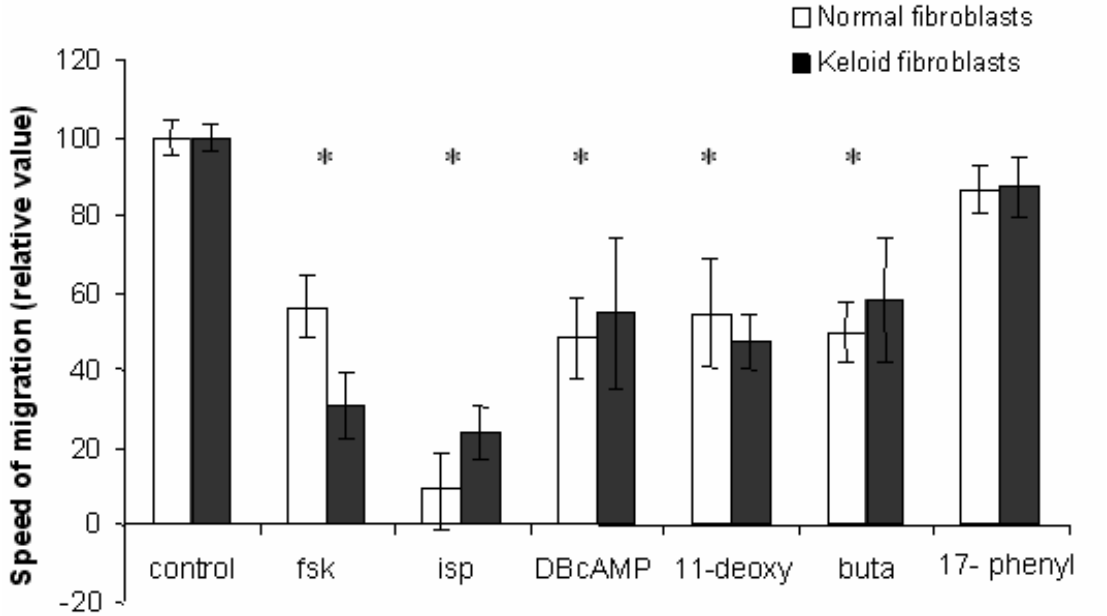


Figure 30. Activation of intracellular cAMP via EP2/EP4 but not EP1 inhibits keloid fibroblast migration.

Scratch assays were performed using confluent normal and keloid fibroblast monolayers in regular fibroblast medium in the absence of fetal bovine serum. Data from multiple normal and keloid fibroblasts were pooled. In each experiment, 4 wells were assigned to each experimental condition. Concentrations for pharmacological agents used were: forskolin (25μM), isoproterenol (10μM), DB-cAMP (500μM), 11-deoxy-prostaglandin E1(EP2/EP4 agonist) (1μM), butaprost (EP2 agonist) (1μM) and 17-phenyl-trinor prostaglandin E2 (EP-1 agonist) (100nM). Data are presented as means with error bars representing standard error of the mean. Data points differing from the control value are indicated by asterisks: * indicates normal fibroblast p-value < 0.05 ** indicates keloid fibroblast p-value < 0.05

Cellular morphology

Cell migration involves continual remodeling of the actin cytoskeleton. Disruption of actin structures has been previously shown to decrease cellular motility. Figure 31 illustrates that administration of PGE2 can induce a disruption of fibroblast actin filamentous structures in both keloid and normal dermal fibroblasts.

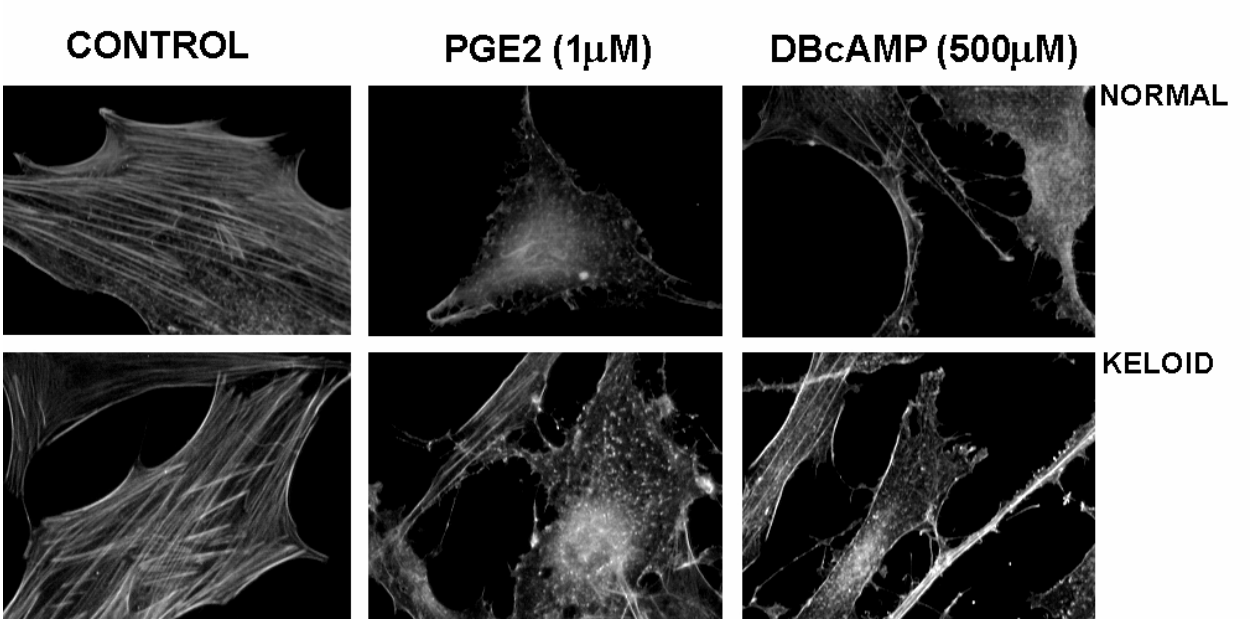


Figure 31. PGE2 and a soluble cAMP analog disrupt the actin cytoskeleton of keloid fibroblasts.

Normal and keloid fibroblasts were treated with PGE2 (1μM) or DBcAMP (500μM), fixed using paraformaldehyde and permeabilized using Triton-X 100. The actin cytoskeleton was visualized using rhodamine phalloidin 1:100 to highlight the actin fibers. Images were captured at 400X magnification and are representative of all the cell cultures used in this study.

Collagen synthesis

Keloid formation is an abnormal wound healing phenotype, in which the primary characteristic is excessive/ abnormal production of extracellular matrix (ECM). Previous studies have linked abnormal collagen production to the keloid fibroblast phenotype, including altered sensitivity to TGF-β1. [41,145,148] In this study, administration of TGF-β1 resulted in an increase in collagen I and III production by both normal and keloid

fibroblasts. This increase was partially reversed upon administration of either PGE2 or forskolin in conjunction with TGF-β1 (Figure 32).

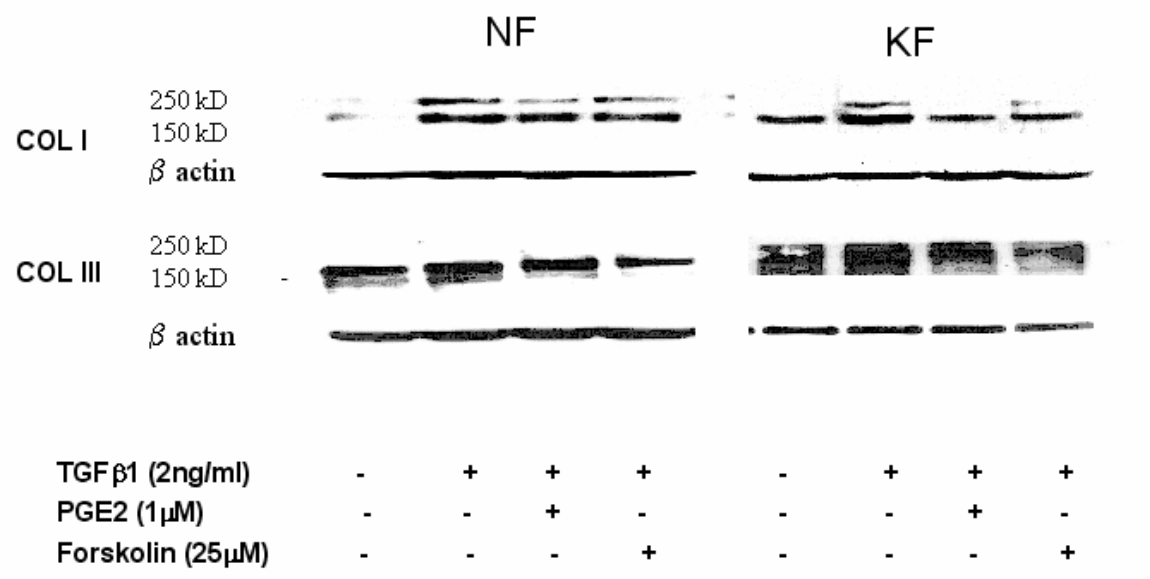


Figure 32. PGE2 reverses the up-regulation of collagen I and III production by TGF-β1 in keloid fibroblasts

Confluent monolayers of normal and keloid fibroblasts were treated with TGF-β1 in the absence or presence of PGE2 (1μM) or forskolin (25μM). Whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies against human collagen type I and III.

3.3.4 Discussion

Fibroblast participation in wound healing is regulated in part by the surrounding environment, of which inflammatory mediators are a crucial component. This study was designed to examine the role of PGE2 signaling in keloid formation with respect to two issues: 1) endogenous keloid fibroblast production of PGE2 in response to inflammatory stimuli and 2) keloid fibroblast response to exogenous PGE2. Addressing these issues will help to define the role of PGE2 signaling in the process of keloid formation. In addition, it will determine whether control of PGE2 signaling might represent a viable therapeutic approach to modulating keloid formation.

Fibroblasts are generally regarded primarily as a target of inflammatory mediators. However, multiple studies have shown that fibroblasts have the capacity to secrete secondary inflammatory mediators, including interleukins and prostaglandins. While the precise contribution of fibroblast-secreted inflammatory mediators in directing the wound healing response remains unclear, impaired fibroblast PGE2 production has been linked to abnormal wound healing outcomes. Specifically, studies of the lower airway (lung) mucosa have indicated that impaired COX-2 activation in and PGE2 secretion by mucosal fibroblasts in response to lipopolysaccharide (LPS) and/or IL-1β are associated with the development of chronic fibrosis in the lung.[53,64,115,116,117] These data strongly suggest that in the lung, PGE2 plays a protective role by down-regulating fibrosis following mucosal injury or

trauma. In contrast, in the dermis, increased PGE2 levels have been associated with scar formation and fibrosis, though there are no data regarding the role of the PGE2 pathway in keloid formation. Recent evidence suggests that induction of COX-2 expression in keloid fibroblasts by exogenous triggers may be impaired.

This study was designed to address the synthetic profile of keloid fibroblasts as it relates to PGE2. The results indicate that IL-1β stimulation produces weaker up-regulation of COX-2 in keloid fibroblasts compared to their normal counterparts. In contrast, a downstream enzyme, mPGES1, also inducible, is activated to a similar degree in both fibroblast phenotypes. Impaired stimulation of PGE2 secretion by IL-1β appears to correlate with the COX-2 but not the mPGES1 levels, indicating that COX-2 activation may be the rate limiting step in this synthetic cascade. Diminished COX-2 activation and PGE2 secretion does not appear to be indicative of a more generalized impaired secretory phenotype, as IL-6 and IL-8 production are up-regulated by IL-1β in both normal and keloid fibroblasts. This discrepancy is significant, given that PGE2 and IL-6 have been previously linked. Specifically, PGE2 is thought to be the intermediary signal triggering IL-6 production subsequent to IL-1β stimulation.[56,57,90] As such, it is important to note that diminished up-regulation of PGE2 secretion by keloid fibroblasts does not translate into impaired production of IL-6. This discrepancy is not unexpected. Activation of inflammatory pathways is often redundant, and there is no existing evidence that fibroblast IL-6 synthesis is wholly dependent on preliminary activation of the PGE2 pathway. In conclusion, keloid fibroblasts appeared to have an impaired ability to up-regulate COX-2 expression in response to a primary inflammatory mediator, IL-1β, which does not necessarily reflect a larger impaired secretory phenotype. The precise mechanisms by which IL-1β regulates COX-2 expression in keloid fibroblasts will be examined in further detail in subsequent studies.

While fibroblasts participate in the inflammatory phase of wound healing, their primary function is to invade the wound bed, synthesize the replacement ECM and restore tissue integrity. Their activity in the wound bed is influenced by inflammatory mediators available via both autocrine and paracrine production. PGE2 has been shown to modulate both fibroblast migration and synthesis of ECM components.[94,105] To date however, no study has demonstrated whether PGE2 effects on fibroblast migration and ECM synthesis are conserved in keloid fibroblasts. This study addressed three specific questions: 1) do keloid fibroblasts express all four PGE2 receptors? 2) does exogenous PGE2 alter in vitro keloid fibroblast migration and collagen production? and 3) are the effects of PGE2 on keloid fibroblasts comparable to its effects on normal dermal fibroblasts? Results indicate that keloid fibroblasts retain a functional signaling pathway for PGE2, expressing levels of EP receptor mRNA and protein comparable to their normal counterparts, with an indistinguishable cellular localization pattern. This is important, in that appropriate receptor expression determines the precise effects of PGE2 on cell activity.

Fibroblast invasion of the wound bed is crucial to proper dermal repair, and is highly regulated by soluble mediators, including PGE2. Previous studies have indicated that keloid fibroblast migration is differentially regulated by extrinsic cues such as epidermal growth factor (EGF). This difference is the result of altered activation of intracellular pathways regulating motility. However, to date, no study has addressed the effects of PGE2 on keloid fibroblast migration. The current study indicates that under basal conditions, keloid fibroblast migration is marginally faster than that of their normal counterparts. While this difference is statistically significant, it is unclear whether it translates into a physiologically significant difference in keloid fibroblast transit into or out of the wound

bed. Addition of serum, an in vitro culture additive and a relevant component of dermal repair, was found to biphasically increase both normal and keloid fibroblast migration.

In contrast to serum, PGE2 has been shown to inhibit fibroblast migration. Data suggest that PGE2 can inhibit the migration of both normal and keloid fibroblasts in a dose-dependent manner. In both keloid and adult fibroblasts, decreased levels of migration appear to be achieved through activation of the EP2/EP4 but not EP1 receptors, coupled to an intracellular cAMP second messenger pathway. These findings suggest that regulation of cell migration via the cAMP pathway are conserved in keloid fibroblasts, and stand in contrast with previous work which indicated that phospholipase C (PLC)γ and MAP kinase/ m- calpain regulation of motility is impaired in keloid fibroblasts. This discrepancy suggests the involvement of redundant pathways regulating fibroblast motility in response to various exogenous cues.

PGE2 inhibition of fibroblast migration via up-regulation of intracellular cAMP, was found to correspond to a morphological alteration in the actin cytoskeleton. Immunofluorescence revealed that PGE2 destabilizes the actin cytoskeleton and depolymerizes the existing actin stress fibers. This is consistent with previous studies linking cAMP release to protein kinase A (PKA) activation and cytoskeletal re-arrangement.[134,135] It is likely that this process is responsible for the impairment in fibroblast motility in both normal and keloid fibroblasts, indicating that keloid fibroblasts do not exhibit altered cytoskeletal dynamics. These results also suggest that changes in cell motility should be considered with respect to three general issues: extracellular stimulus, intracellular pathways and effector systems (cytoskeletal elements, focal adhesions, integrins, etc.). PGE2 modulation of keloid fibroblast migration may be accomplished via activation of a signaling cascade similar to normal dermal fibroblasts. Involvement of the actin cytoskeleton in this process makes it very likely that in vitro migration results can be extrapolated to in vivo processes.

Improper collagen deposition and organization are characteristic features of keloid formation. Keloid fibroblast collagen synthesis can be enhanced by exogenous modulators including TGF-β1, an important mediator of wound healing, with demonstrated pro-fibrotic effects. In mucosal fibroblasts, TGF-β1 stimulation of fibroblast collagen production can be reversed by administration of prostaglandin E2.[105,145] However, data are lacking regarding the ability of PGE2 to reverse the pro-fibrotic effects of TGF-β1 in a fibrotic dermal fibroblast phenotype such as the keloid. Here we demonstrate that up-regulation of collagen type I and III production by TGF-β1 can be partially reversed by administration of PGE2 in both normal and keloid fibroblasts. Once again the mechanism of action appears to be activation of intracellular cAMP pathways as demonstrated by reconstitution of the PGE2 effects by forskolin. These results are important in that they indicate that the most pathophysiologically relevant keloid fibroblast trait, collagen production, can be regulated by exogenous PGE2. It is important to note that wound healing is a dynamic process involving not only ECM component synthesis, but also degradation and re- modeling. As such, it will be important for future studies to consider the effects of PGE2 on the production and secretion of matrix metalloproteinases by keloid fibroblasts.

CONCLUSION. This is the first study to begin to comprehensively address the effects of PGE2, a key inflammatory mediator, on the keloid fibroblast phenotype. These data indicate that the previously demonstrated effects of PGE2 on fibroblast migration and collagen synthesis are conserved in human dermal fibroblasts, and

importantly in an abnormal subset, keloid fibroblasts. Endogenous keloid fibroblast production of PGE2 is reduced, likely due to diminished up-regulation of COX-2. Given that PGE2 has demonstrated putative anti- fibrotic effects, reduced PGE2 production during keloid formation may account for excessive fibroblast invasion and ECM synthesis. This study adds an important dimension to existing work which has suggested that increased PGE2 levels in dermal wounds contribute to scar formation. [37] Previous studies do not precisely describe the mechanisms by which PGE2 can lead to the development of dermal fibrosis. Given that PGE2 can act as both an inflammatory mediator and a fibroblast modulator, there are several potential mechanisms by which in vivo PGE2 effects can be explained. These data demonstrate, using a fibrotic dermal fibroblast phenotype, that PGE2 activity appears to be anti-fibrotic with respect to multiple fibroblast activities. It is therefore possible that in vivo PGE2 induction of a fibrotic response may be indirect and related to its inflammatory role.

The effects of PGE2 on keloid fibroblast migration and collagen synthesis demonstrated in this study have interesting implications for possible therapeutic interventions. To date, no single therapy has proven successful at managing keloid formation/ recurrence. Exogenous PGE2 may be used to modulate the wound healing process, specifically, by retarding fibroblast invasion of and collagen synthesis in the dermal wound bed. Our lab is currently pursuing animal models of hypertrophic scar formation and the potential modulation by exogenous PGE2 of in vivo wound healing processes.

3.4 PART I SUMMARY

For the first time, endogenous fetal dermal fibroblast production of PGE2 has been shown not to be differentially regulated by primary inflammatory mediators such as IL-1β. This is in contrast to fetal fibroblast production of select cytokines (IL-6) and chemokines (IL-8) which has been shown by us and other to be diminished compared to that of adult fibroblasts. These findings suggest that tissue level differences in COX-2 activation and PGE2 production observed during fetal, in contrast to adult wound healing are likely the result of reduced primary trigger (IL-1β) levels. Lower IL-1β levels could explain why fetal dermal wound healing does not exhibit PGE2 associated fibrosis.

An additional explanation for the minimal role of PGE2 in fetal dermal repair would be an intrinsically altered fetal fibroblast response to this soluble mediator. The current analysis indicates that this is indeed the case. Fetal fibroblasts have a diminished response to exogenous PGE2 with respect to two key wound healing activities: migration and contraction. What is puzzling is that PGE2 effects on these two activities are inhibitory and would thus be anti- fibrotic should they extend to the in vivo setting. This contradiction can be explained by the dual role PGE2 plays during dermal repair: inflammatory mediator and fibroblast modulator. While its direct fibroblast activity may be anti- fibrotic, its pro- inflammatory effects may trigger increased tissue fibrosis via secondary effects. Our data suggest that this may be the case, as PGE2 can induce adult dermal fibroblast production of a pro-inflammatory cytokine associated with increased scar formation (IL-6). In contrast, fetal fibroblast production of IL-6 is diminished under basal levels and fails to up-regulate in response to exogenous PGE2.

These data suggest that PGE2 may act as both an anti- fibrotic and a pro- inflammatory mediator. During dermal wound healing, the pro- inflammatory role predominates and results in increased overall fibrosis. In order to further address the fibrotic effects of PGE2, analysis was extended to a specific fibroblast phenotype associated with excessive scar formation: the keloid fibroblast. Data presented above indicate that keloid fibroblasts respond to exogenous PGE2 with decreased migration and collagen synthesis. Indeed, PGE2's effects could be termed antagonistic to the fibroblast-myofibroblast transition thought to be crucial to scar formation. As such, the anti-fibrotic effects of PGE2 can be generalized to dermal fibroblasts regardless of cell source (fetal, normal/adult and fibrotic/keloid). Surprisingly, our data suggest that the only abnormality in keloid PGE2 signaling exists with respect to endogenous PGE2 production due to diminished COX-2 up-regulation. The precise implications of this finding are unclear and do not lend themselves to easy clarification due to experimental difficulties which will be detailed in subsequent sections (Discussion).

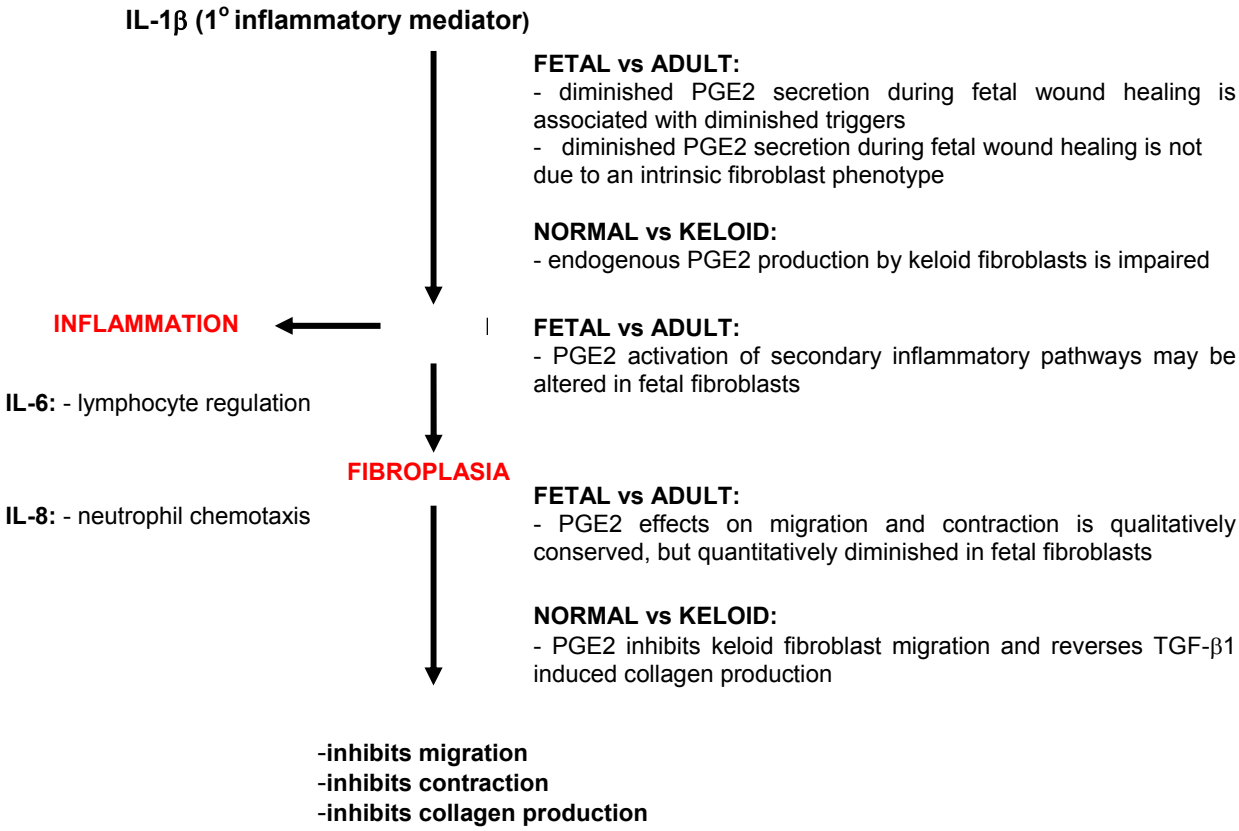


Figure 33: IL-1β and PGE2 signaling in fetal, normal/adult and keloid fibroblasts.

4.0 PART II: INTERACTIONS BETWEEN FIBROBLASTS AND EXTRA-CELLULAR MATRIX COMPONENTS CAN ALTER THE INTRINSIC FIBROBLAST PHENOTYPE

It has been demonstrated that fetal, normal/adult and keloid fibroblasts maintain different phenotypic properties with respect to a variety of cellular activities. Our laboratory and others have shown that fetal fibroblasts have higher rates of migration and contraction compared to their adult counterparts. These phenotypic differences can be maintained, diminished or enhanced by exogenous factors**.** Data presented above demonstrate that the addition of exogenous PGE2 inhibits both fetal and adult fibroblast migratory and contractile rates, likely through alterations in the actin cytoskeletal dynamics. The elevated rates of fetal fibroblast migration and contraction compared to adult fibroblasts are maintained in the presence of PGE2. This phenotypic difference with respect to a specific signaling pathway is important, in that it may be relevant to the larger wound healing response. However, it is important to keep in mind that PGE2 modulation of fibroblast activity in the dermal wound bed occurs in the context of a complex multi- factorial process representing additional extracellular constraints.

Extracellular constraints are thought to significantly impact cell activity. In the context of wound healing, the extracellular matrix has been shown to dramatically alter fibroblast activities such as motility, contractility and protein synthesis. We suggest that it is insufficient to analyze fibroblast phenotypic differences as they relate to a particular signaling pathway using limited in vitro methodology. We further suggest that it is important to determine the putative effects of increasingly complex environmental conditions such as the addition of extracellular matrix (ECM) components on specific phenotypic differences. Data detailed above indicates that the phenotypic differences described with respect to fibroblast motility are conserved upon the addition of a bioactive matrix (i.e. fibroblast populated collagen lattice contraction). Studies described below present a more comprehensive system for challenging fetal and adult fibroblasts with increasingly complex matrices in order to determine the robustness of their intrinsic phenotype.

4.1 FETAL AND ADULT FIBROBLAST MIGRATION AND CONTRACTION: EXTRA-CELLULAR MATRIX ALTERS INTRINSIC PHENOTYPIC DIFFERENCES

4.1.1 Introduction

Fibroblast migration is a critical component of wound healing. Invasion by fibroblasts leads to extracellular matrix synthesis, contraction, and remodeling, all key events that dictate the final outcome of tissue repair. In postnatal wound healing, these events lead to scar formation due to the inability of fibroblasts to completely recapitulate the natural structure and function of the tissue.[3,5] In contrast, fetal wound healing is characterized by appropriate organization of the newly deposited matrix that leads to regenerative, scarless repair.[1,151,152] The initial invasion of the wound bed by fibroblasts, through migration, is a result of cytoskeletal traction forces generated by actin stress fibers bound to the matrix via integrin receptors.[153,154,155] In postnatal wound healing, closure of the wound has been theorized to be migratory fibroblasts exerting traction forces at the edges of the newly deposited matrix.[126,156] As tension builds within the wound bed, fibroblasts differentiate into the myofibroblast phenotype, characterized by an increase in the number of α-smooth muscle (SM) actin filaments, resulting in larger contractile forces responsible for wound bed closure and remodeling.[157,158,159,160] Once the wound has closed and tension is no longer present, myofibroblasts revert back to the fibroblast phenotype and continue to remodel the wound long term. Interestingly, myofibroblasts are either not detected or only transiently expressed in fetal wounds.[161,162,163] This observation implies that the traction forces involved in early migration and late remodeling may be important factors in dictating the final pathological outcome of scar formation and may be sufficient for matrix organization leading to scarless or regenerative healing. To date, the contribution of events associated with migration and contraction at the individual fibroblast level to the larger population dynamic remains unclear.

Cellular migration and events associated with focal adhesion formation have mostly been studied on two-dimensional substrates consisting of either inert, biological, or conjugated surfaces. Three-dimensional collagenous matrices have been used to more closely approximate the in vivo wound healing environment, such as fibroblast populated collagen lattices. In these systems, traction forces and subsequent cell migration lead to translocation and remodeling of the collagen fibrils, in a manner similar to early invasion of a wound bed as well as final remodeling once the wound has contracted and closed. This technique has been used to elucidate differences between populations of human adult and fetal fibroblasts, with inconsistent results. Additional techniques have been developed to investigate individual fibroblast characteristics in three-dimensions. Collagen constructs populated by embedded fibroblasts are thought to mimic the native environment since cell adhesion structures and interactions evolve toward in vivo-like conditions.[28,31,144]

In general, approaches to the study of fetal and adult fibroblast dynamics during wound healing suffer from several generalized draw-backs: 1) a reliance on bulk (large population) measurements, 2) extrinsic constraints, and 3) excessive reduction. Bulk measurements allow for hypothesis directed studies which provide easily interpretable

data, but often fail to correlate individual cell activity with larger population dynamics or to address multiple processes occurring within the same time frame. Under standard in vitro conditions, exogenous constraints include cell crowding, inter-connectedness, excessive paracrine and autocrine signaling, circumstances not generally reflected of in vivo conditions. Excessive reduction is often encountered in studies which address the effects of growth factors or other soluble mediators on specific cell activities. Although the reductionist approach can delineate specific signaling pathways, it creates a loss of context. While intracellular signaling pathways may be activated in response to specific triggers, the relative importance of this effect in vivo remain unclear due to elimination of more subtle qualities of signaling such as timing and geographic gradients.

The goal of this study is to create an analytical paradigm which contains a progressively complex test of intrinsic fibroblast properties: 1) analysis and comparison of fetal and adult fibroblast migratory and contraction dynamics using standard bulk measurements, 2) analysis of individual fetal and adult fibroblast morphological dynamics on inert and bioactive substrates and 3) analysis of population and individual cell morphological dynamics under 3 dimensional constraints using a novel 3D collagen gel construct. These approaches are meant to complement each other and provide insight into both subtle phenotypic differences between fetal and adult dermal fibroblasts and the interactions of these cells with the surrounding ECM. The two questions addressed in this study are: 1) how much of the intrinsic fibroblast phenotype is preserved under an increasingly complex environment and 2) how does individual cell activity correlate with population wide dynamics?

4.1.2 Materials and methods

Cell culture: As detailed above (section 3.1.2). Migration and fibroblast populated lattice contraction experiments were performed using all fibroblast populations. Real-time and stacked collagen gel experiments were performed using 2 fibroblast populations from each cell phenotype.

Directional migration assay: As detailed above (section 3.2.2).

Collagen gel contraction: As detailed above (section 3.2.2).

Real time measurements of cell dynamics: Time lapse imaging was conducted using the imaging equipment described above. Cells were seeded onto either a glass surface (LabTek Flaskette Chamber Slide, Nalge Nunc Inc, Rochester, NY) or a thin collagen gel of 100 μl (see above for preparation) adherent to an underlying glass surface (LabTek Chambered coveglass, Nalge Nunc Inc, Rochester, NY) and allowed to settle, attach and spread for 60 min at 37^{O}C 5% CO2 in complete medium (DMEM supplemented with 10%FBS, AB/AM) with 5mM HEPES buffer. Cells were then placed on a heated stage (custom built); a representative region of the slide was chosen and cell activity was imaged at 200X (phase contrast) for an 8 hour period. Images were collected every 3 minutes. Cell activity was assessed using Metamorph imaging software as follows. Images were collated into movies of 1 hour in duration, and each movie was analyzed independently. The following parameters were measured:

cell attachment= the formation of visible extensions of the cytoplasm to the underlying surface *cell spreading*= increase in cell area by the equivalent of 1 cell nucleus or greater

cell shrinking= decrease in cell area by the equivalent of 1 cell nucleus or greater

cell detachment= loss of connections to the underlying surface

membrane ruffling= alterations in cell shape without cell locomotion

cell locomotion= translocation of the cell body by 1 cell nucleus or more in any direction.

These parameters were measured for the cells present in the chosen field within each defined time period. Multiple events for one individual cell were treated as individual events. The number of events was standardized to the total number of cells in the chosen field at the end of each experimental period. The top five distances traversed by the cells in the chosen field were measured and compared across experimental conditions for a total of 20 measurements per fibroblast phenotype.

3D stacked collagen constructs: Collagen gels were polymerized as described above. The entire construct was synthesized as follows (Figure 34A). A volume of 200 µl was polymerized in an11 mm cloning tower inside of the well of a 12 well plate. A total of 28,800 fibroblasts of either fetal or adult origin were suspended in 50 µl of complete media and allowed to attach to the collagen gel for 30 min. A second collagen gel, identical to the first, was poured on top of the cell layer and allowed to polymerize. The entire construct was left inside the cloning tower in complete media for 3 days to assure fusion of the two collagen layers. The stacked collagen constructs were then removed from the cloning towers and allowed to free float for 1, 3 or 7 days post- removal. Gels were then fixed in 10% buffered formalin, paraffin embedded and sectioned (10 µm sections). Sections were processed and stained with haematoxylin so as to highlight the cell nuclei, then imaged at various magnifications using bright field microscopy coupled to Metamorph (version 4.6r10 Universal Imaging Corp, Downington PA) imaging software for subsequent analysis. The width of each gel was captured in 2-3 sequential 100X images. The number of cell nuclei in zones adjacent to (zone A) and away from the gel interface (zone B) were recorded and compared. For all statistical analysis, the number of individual gels determined the number of independent observations.

3D collagen gel image analysis: Sections were analyzed for: 1) fibroblast distribution within the volume of the gel and 2) changes in visible collagen fiber organization and orientation. Fibroblast distribution was assessed as illustrated in Figure 34B. The interface between the collagen gels was identified and cell nuclei were counted in each of the identified gel regions (A1, A2, B1, B2) defined as a 200 µm X 200 µm area captured at a magnification of 100X. The invasion coefficient, C, was defined as the ratio of nuclei in area B to its corresponding area A. The directionality coefficient was defined as the ratio of the two invasion coefficients. All measurements described in the subsequent section rely on counts of cell nuclei. Organization of visible collagen fibers was determined as follows. Representative areas were imaged at 200 and 400X using DIC microscopy. Images were processed so as to: convert color in grayscale images, maximize contrast of black to white objects and reduce noise. Metamorph generated images were analyzed for multiple image parameters described in the following section.

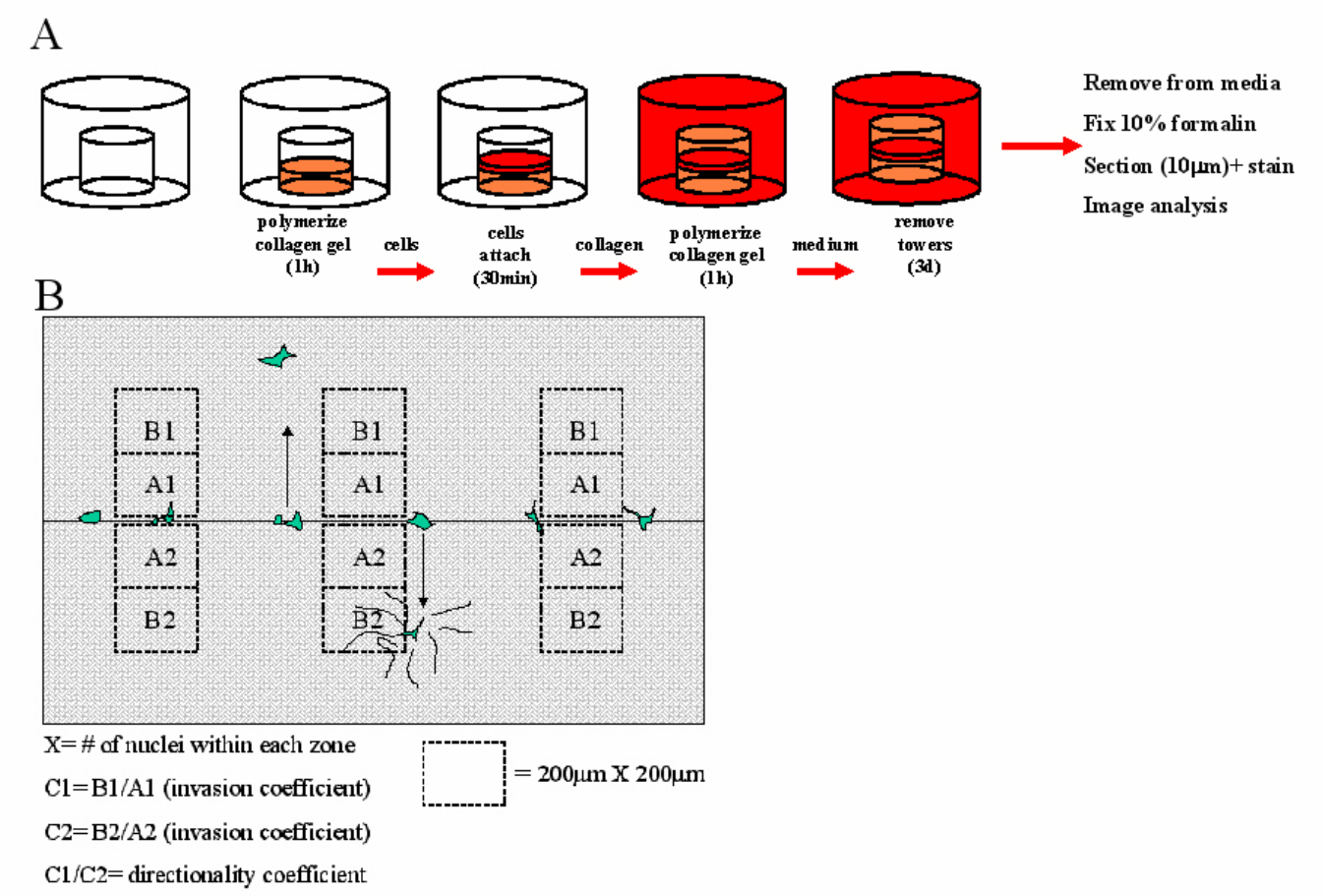


Figure 34. 3D collagen gel invasion model.

Panel **A)** Fibroblasts were seeded at the interface between two collagen gels and allowed to interact with gels undisturbed inside 11mm cloning towers for 3 days. Stacked gel constructs were then removed and allowed to free float for various time periods. Gels were then formalin fixed, paraffin embedded, sectioned and stained with haematoxylin. Panel **B)** Sections of maximal cell invasion were used for subsequent image analysis and nuclei counts. The ratio of cell nuclei in zones away from (zone B) to cell nuclei in zones adjacent to (zone A) the interface was expressed as an invasion coefficient for each of the two gels (C1 and C2). The ratio of these two coefficients was used to define a directionality coefficient (C1/C2).

4.1.3 Results

Directional migration. Two-dimensional migration assays have been used to address the migratory profile of various cell types under both basal and stimulated conditions.[40,164] In this study, we have used this assay to establish the migratory profile of human fetal and adult dermal fibroblasts with respect to two parameters: 1) exogenous stimulatory signals (fetal bovine serum -FBS) and 2) temporal cell dynamics. Data indicate that fetal fibroblasts

migrate approximately 1 fold faster than their adult counterparts (Figure 35A). This statistically significant speed differential is maintained in the presence of increasing concentrations of FBS stimulation, indicating that while motility is enhanced, intrinsic phenotypic differences between the two fibroblast phenotypes are maintained. Within the time frame chosen for this study, fibroblast speed of migration was shown to generally increase with time in the absence of FBS (Figure 35B). The 1 fold higher speed of fetal fibroblast migration was maintained throughout the tested time points.

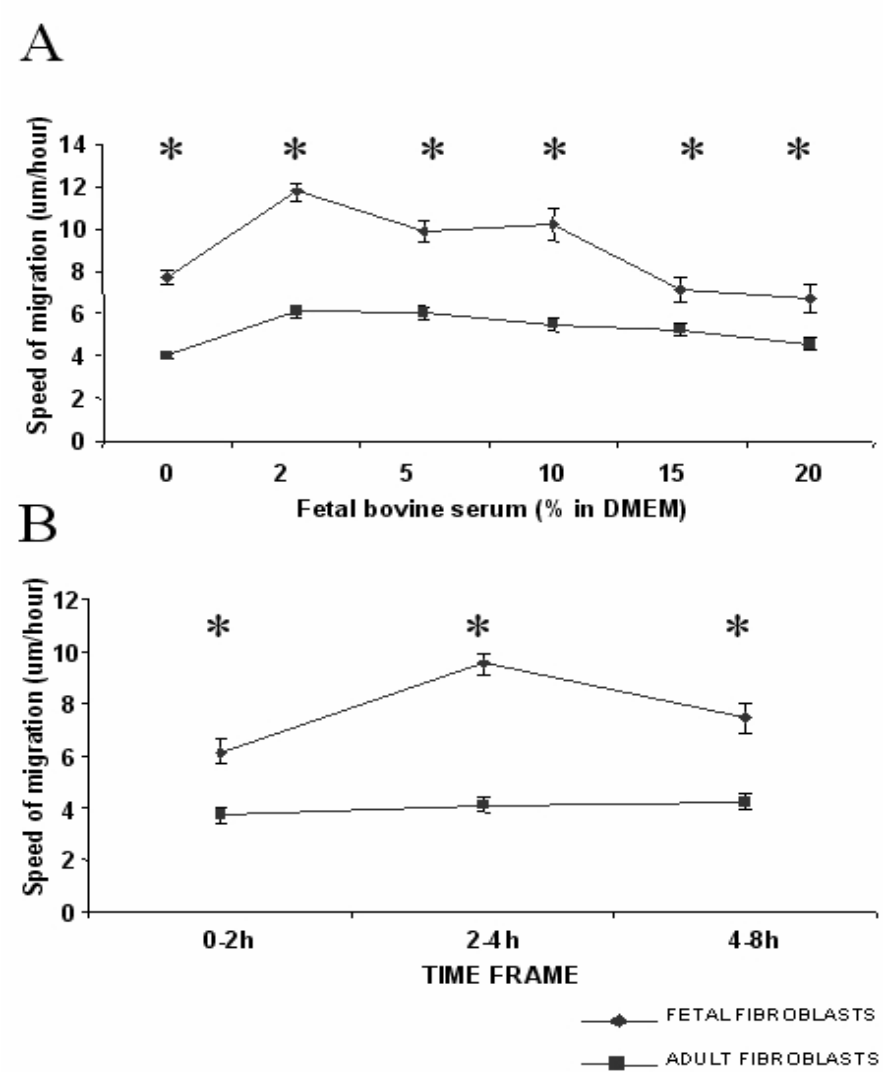


Figure 35. Fetal fibroblasts have a different migratory profile from their adult counterparts.

Confluent monolayers of fetal and adult fibroblasts were subjected to a directional migration assay in which cells invade a central denuded area over time. All migration assays were performed in regular fibroblast media (DMEM) supplemented with various concentrations of fetal bovine serum (FBS). Panel **A)** Both cell types exhibit a biphasic migratory response to FBS over a 24 hour time period. Panel **B)** Speed of migration in the absence of FBS changes throughout the tested time period. Data from multiple fetal or adult fibroblast populations were pooled and

presented as means with error bars representing standard error of the mean. * indicates p-value < 0.05 by Student's t-test.

Contraction of fibroblast populated collagen lattices. Further analysis of fetal and adult fibroblast migratory properties was conducted using the fibroblast populated collagen lattice (FPCL), a method which allows fibroblasts to migrate over a bioactive collagen based substrate, whose high compliance allows gross compaction via cell locomotion. Data indicate that as in the case of migration, fetal fibroblast contraction of collagen lattices is higher than that of their adult counterparts (Figure 36 A-C). Similar to migration data, fetal fibroblast contraction in the absence of FBS is approximately 1 fold higher than that of adult fibroblasts throughout the tested time frame (Figure 36D). However, unlike migration over an inert substrate, this phenotypic difference is ablated by the addition of increasing concentrations of FBS.

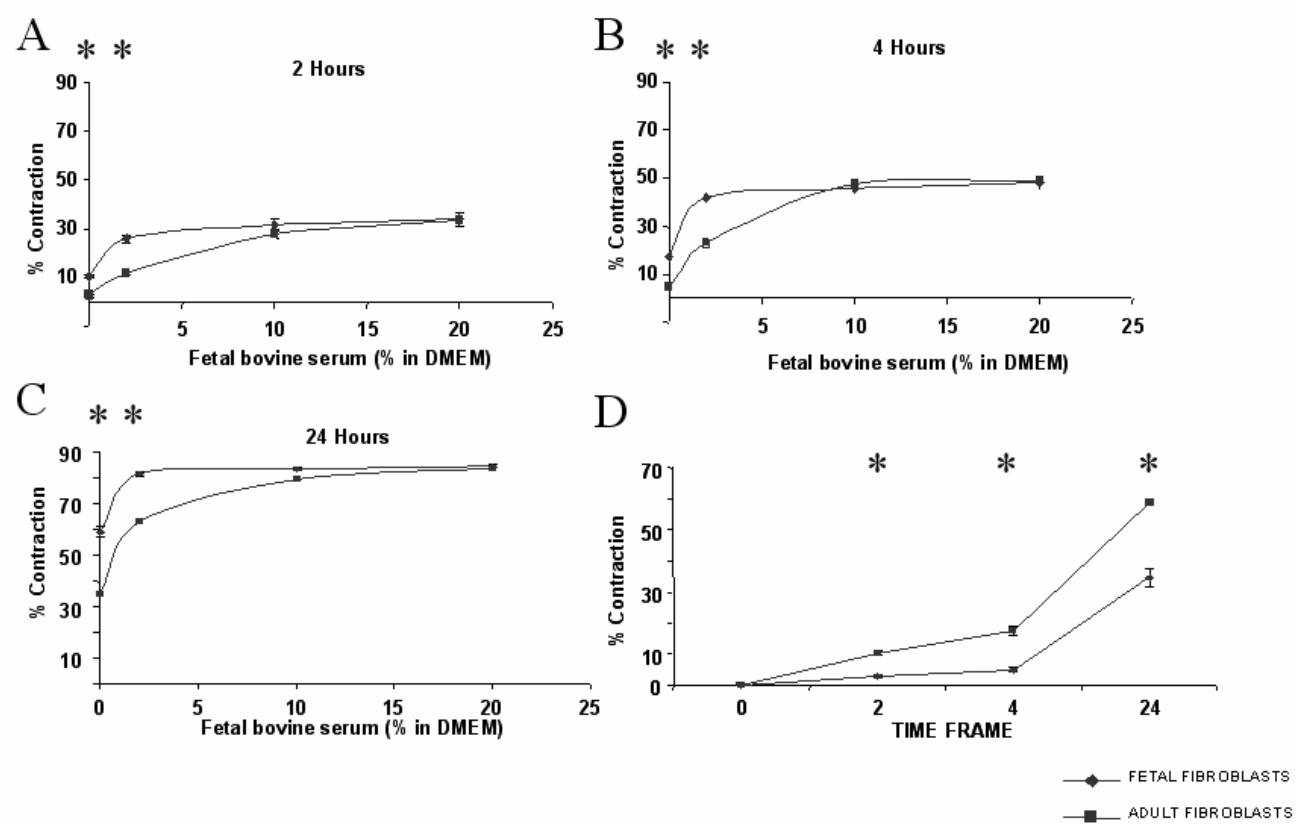


Figure 36. Fetal fibroblasts show greater contractile capability than adult fibroblasts under basal conditions.

Cells were seeded onto thin collagen gel lattices and allowed to attach. Fibroblast populated lattices were allowed to contract under free- floating conditions. All collagen gel contraction assays were performed with the

same number of cells and collagen concentration in regular fibroblast growth medium DMEM supplemented with various concentrations of fetal bovine serum (FBS). Fibroblast contraction is serum dependent for both cell types at 2hrs (panel **A**), 4hrs (panel **B**) and 24 hrs (panel **C**). Panel **D)** Contraction is time dependent, with fetal fibroblasts displaying elevated rates of contraction at all tested time points. Data from multiple fetal or adult fibroblast populations were pooled and presented as means with error bars representing standard error of the mean. * indicates p-value < 0.05 by Student's t-test.

Stochastic cell activity. The migration experiments described above rely upon a specific dynamic dictated by confluent fibroblast population dynamics of non- transformed cells. In order to determine whether the elevated fetal fibroblast migration speed is truly representative of an intrinsic phenotype rather than experimental constraints (i.e. increased contact inhibition), we decided to characterize fibroblast dynamics in a less constrained system. Under the conditions detailed in the previous section, we measured ratios of cell attachment, spreading, shrinking, detachment, locomotion and membrane ruffling over a time period designed to minimize other cell activities such as proliferation and protein synthesis (i.e. collagen). The relatively low cell density employed allowed cells to undergo any or all of the above mentioned morphological dynamics throughout the tested time period. The distribution of these morphological states of fetal and adult fibroblasts on an inert glass surface is illustrated in Figure 37 (A, B). Both fetal and adult fibroblasts transition from predominantly attaching and spreading to locomotion and membrane ruffling as time progresses. In addition, both cell types display a measurable amount of cell shrinking events, but only fetal fibroblasts display detachment events during the tested time period. In addition, fetal fibroblasts appear to have an intrinsically hyperactive state, with elevated rates of attachment and spreading even in the latter time periods. An additional analysis of the top 20 distances traversed by cells from each fibroblast phenotype indicates that fetal fibroblast locomotion is faster (~1.5 fold) than that of adult fibroblasts under non- constrained conditions (16.5μm/8hour vs 10.8μm/8 hour, respectively) (Figure 37C).

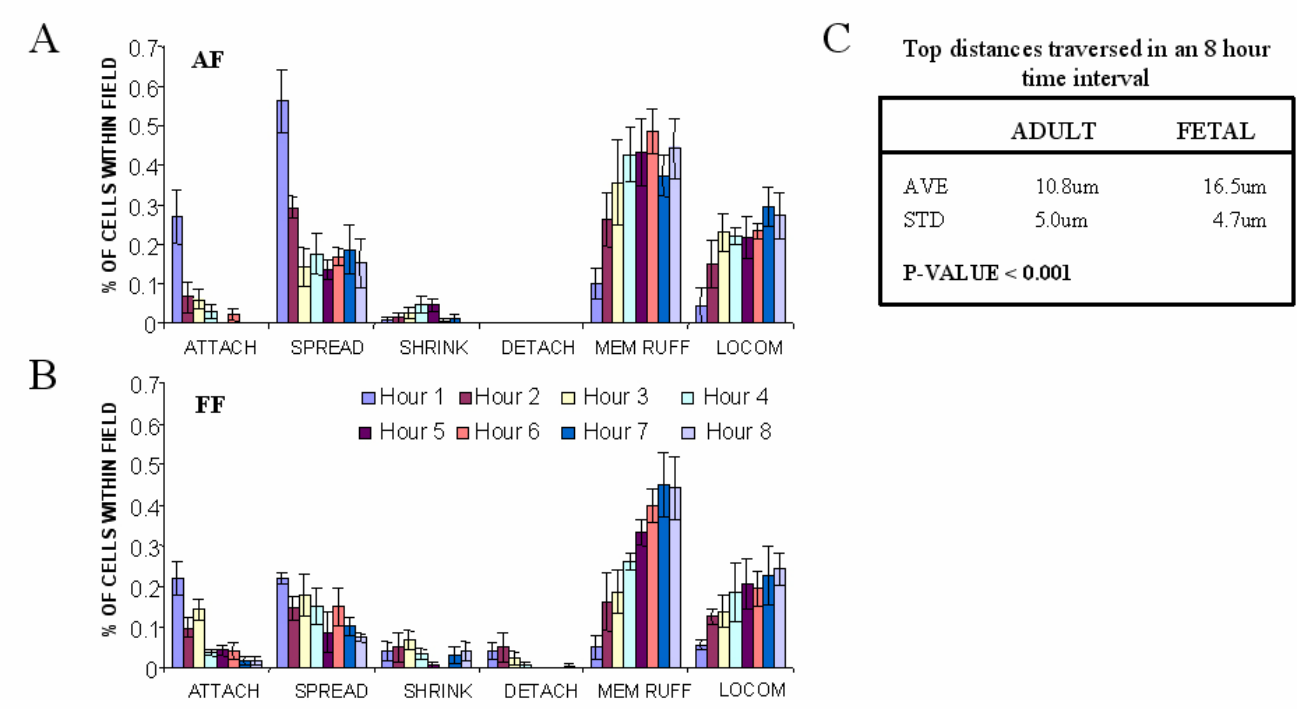


Figure 37. Fetal fibroblasts display a hyperactive morphological dynamic.

Adult and fetal fibroblasts were seeded at low density on a glass surface and allowed to attach in DMEM (with 10% FBS and 5mM HEPES buffer) for 1 hour at 38ºC, 5%CO2. Cells were then placed on a heated stage and imaged at 200X using bright field microscopy every 3 minutes, 1 hour image stacks were created and fibroblast activities measured and compared between the two fibroblast phenotypes. Data for both adult (panel **A)** and fetal (panel **B)** fibroblast activities are presented for each 1 hour period as a % of cells within the captured field. Data are presented as averages of 4 experiments conducted with 2 populations of each fibroblast phenotype (> 100 fibroblasts/phenotype), and error bars representing standard error of the mean. Panel **C)** The top 20 distances traversed over the entire 8 hour time period for each cell type were measured and compared. ATTACH= cell attachment, SPREAD= cell spreading, SHRINK= cell shrinking, DETACH= cell detachment, MEM RUFF= membrane ruffling, LOCOM= cell locomotion.

The addition of a bioactive collagen substrate dramatically altered the cellular dynamics of both fetal and adult fibroblasts (Figure 38). Specifically, fetal fibroblast morphological dynamics on a collagen substrate resembled those of adult fibroblasts on a glass substrate in that: 1) the number of detachment events was substantially diminished and 2) the transition from adhesion and spreading to membrane ruffling and locomotion occurred more rapidly. These dynamics were further augmented in the case of adult fibroblasts, with the transition from attachment and spreading to membrane ruffling and locomotion occurring on a faster time frame, with a substantially lower number of shrinking events. In the case of both cell types, the transition from the inert glass to the bioactive collagen substrate accelerated the initial cellular attachment and resulted in a less dynamic state.

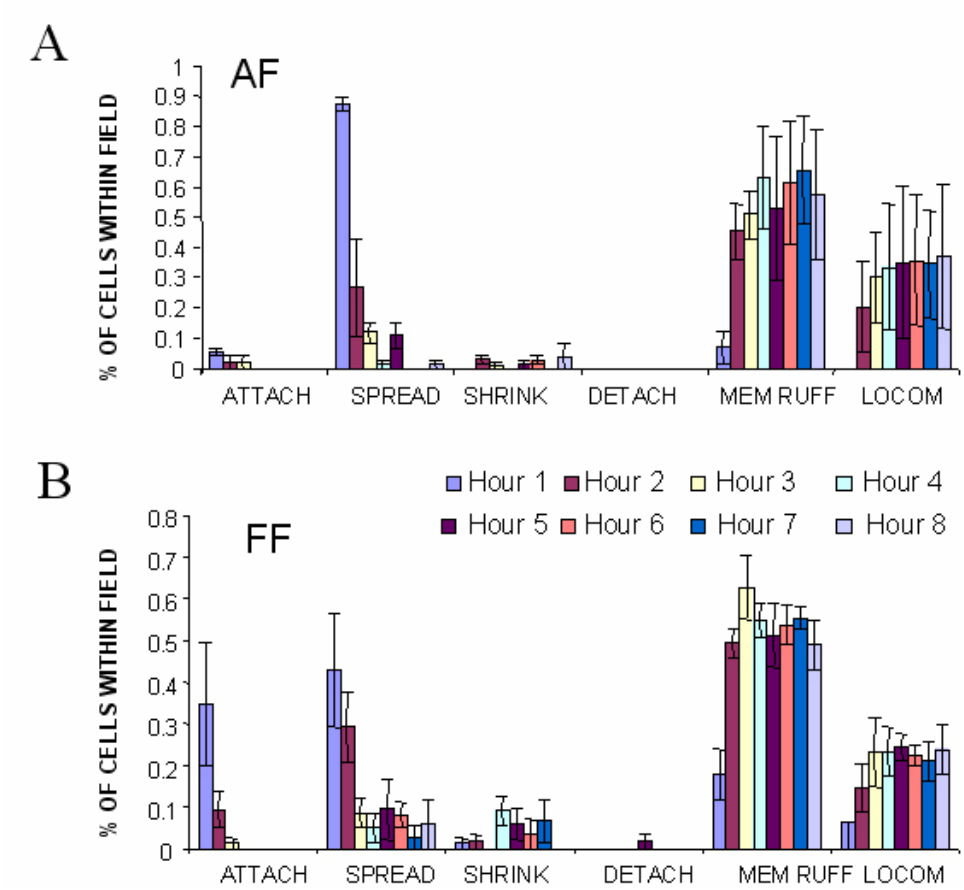


Figure 38. Fibroblasts display a less hyperactive morphological dynamic on a collagen gel compared to a glass surface.

Fetal and adult fibroblasts were seeded at low density on a glass surface or collagen gel and allowed to attach in DMEM (with10% FBS and 5mM HEPES buffer) for 1 hour at 38^{o}C and 5%CO2. Cells were then placed on a heated stage and imaged at 200X using bright field microscopy every 3 minutes, image stacks collected at one hour were created and fibroblast activities measured and compared between the two fibroblast phenotypes. Data for adult (panel **A)** and fetal (panel **B)** fibroblast activities on collagen are presented for each 1hour period as the percentage of cells within the captured field. Data are presented as averages of 4 experiments conducted with 2 populations of each fibroblast phenotype (> 100 fibroblasts/phenotype), and error bars representing standard error of the mean.

3D stacked collagen constructs. The population wide and individual cell dynamic measurements were complemented by additional studies examining fetal and adult fibroblast activity in a 3D matrix. Using the experimental protocol detailed in previous sections, fetal and adult fibroblast invasion of 3D constructs was analyzed and compared. Additional image analysis was used to ascertain the activity of individual cells embedded

inside the constructs. Fetal and adult fibroblasts were found to invade a collagen matrix in a time dependent manner. Specifically, fibroblast nuclei were localized primarily at the collagen gel interface, and progressively away from the interface over the course of the experimental period (Figure 39A, B, C). The fibroblast invasion coefficient (B/A), representing the ratio of cell nuclei away from the interface (B) to cell nuclei close to the interface (A), increased over time, from ~5% at day 1, to ~30% at day 3, to ~50% at day 7 post removal from cloning towers. Interestingly, the invasive process was bidirectional, indicating the absence of any exogenous constraining factors that would provide directionality, as indicated by the fact that the directionality ratio is generally around 1. In the absence of embedded fibroblasts, the gel interface was found to the homogeneous with respect to collagen fiber organization, and the two gels were found to be in close contact with one another (Figure 39E).

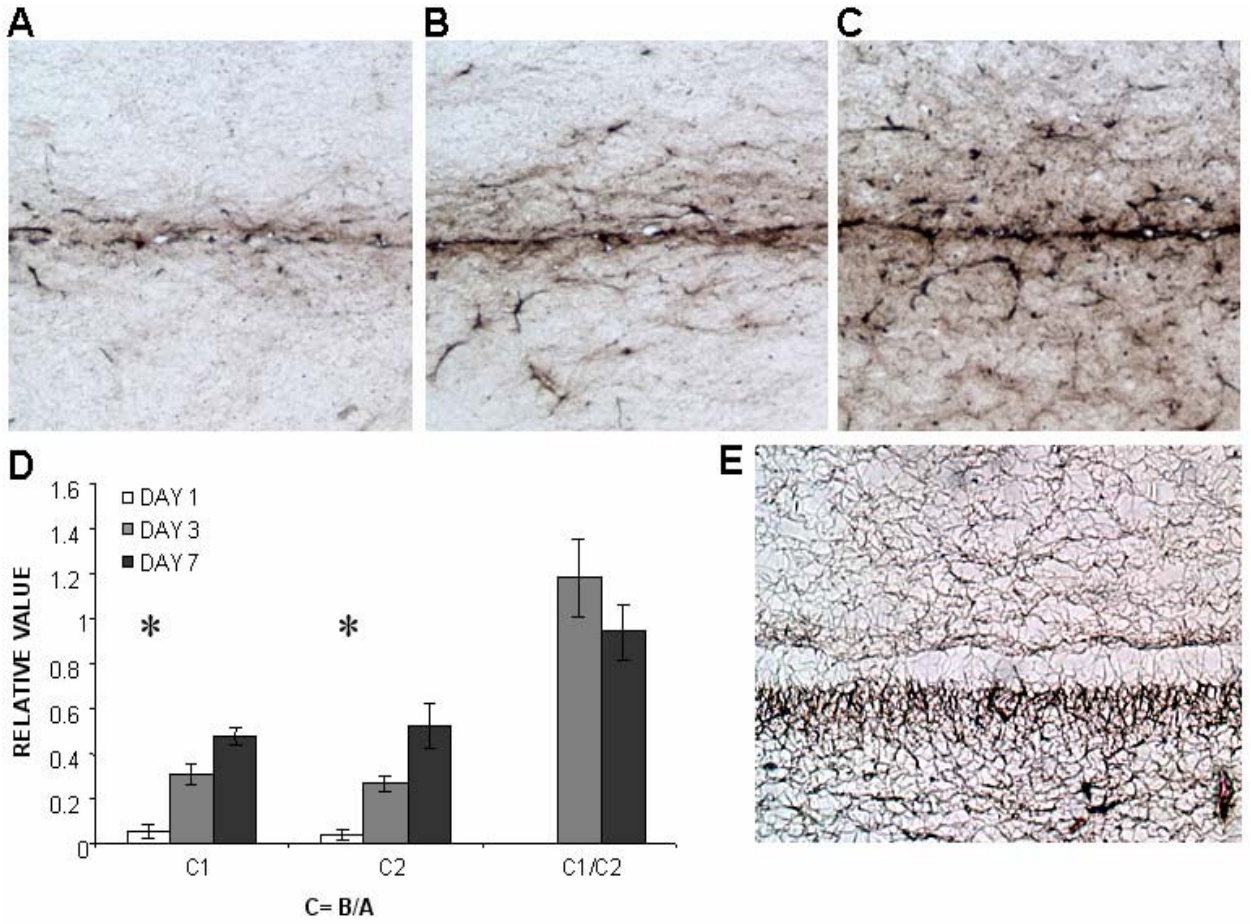


Figure 39. Fibroblasts invasion of 3D collagen gel construct.

Adult fibroblasts were initially (panel **A**; day 1) seeded at the collagen gel interface and allowed to invade the gel over subsequent days (panel **B**; day 3, panel **C**; day 7) (magnification 100X). Panel **D)** Nuclei counts near (zone A) and away from the interface (zone B) indicate that fibroblasts invade the collagen gel in a time dependent manner (invasive coefficients C1 and C2 increase over time), without a directional preference (C1/C2). * indicates p value <0.05 by Student's t-test. Panel **E)** Collagen gel interface at day 7 in the absence of seeded fibroblasts (magnification 400X)

Fetal fibroblast invasion of a 3D collagen gel construct was faster than that of adult fibroblasts at all three time points, with invasion rates of ~25%, 60% and 85% at days 1, 3 and 7 respectively (compared to 5%, 30% and 50% for adult fibroblasts) (Figure 40). Additional analysis, using total nuclei counts indicates that throughout the tested time period, the number of adult fibroblasts present inside the collagen gels increases, particularly in zone B. In contrast, the number of fetal fibroblast nuclei actually decreases in zone A, while increasing in zone B. These data may be indicative of differential roles of fetal and adult fibroblast proliferation in this larger collagen gel invasive process in addition to differences in migratory rates.

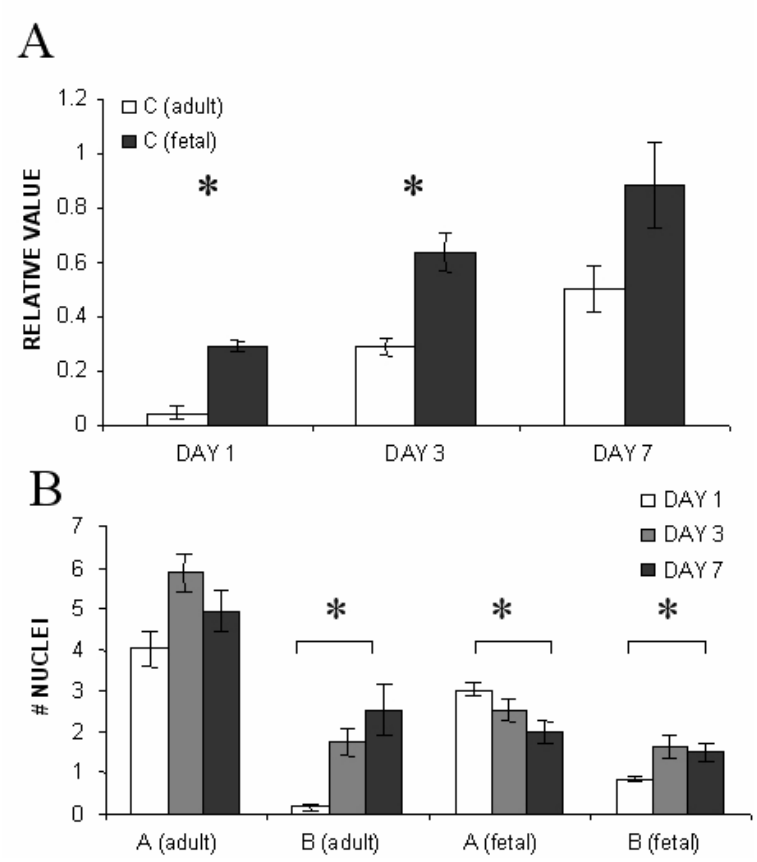


Figure 40. Fetal fibroblasts display greater invasive potential in a 3D stacked gel construct.

Following removal of cloning towers, stacked gels containing fetal or adult fibroblasts were allowed to free float for 1, 3 and 7 days. Panel **A)** Fetal fibroblast gel invasion was faster than that of adult counterparts (higher invasive coefficient C). Panel **B)** The total nuclei counts per section indicate that the number of adult fibroblast nuclei increases both near (zone A) and away from the interface (zone B). In contrast, the number of fetal fibroblast nuclei decreases at the interface and increase away from the interface. Two fetal and two adult fibroblast cultures were used for these experiments with multiple gels per time point. Data are represented as averages with error bars indicating standard error of the mean. * indicates p value <0.05 by Student's t-test

Individual fibroblast activity within collagen construct. The analysis detailed above focused primarily on population wide dynamics within the 3D collagen gel constructs. In this section we present additional data regarding the activity of individual fetal and adult fibroblasts within the bioactive matrix. Specifically, sections of adult and fetal fibroblast populated collagen constructs were imaged using high magnification (600X) DIC microscopy.

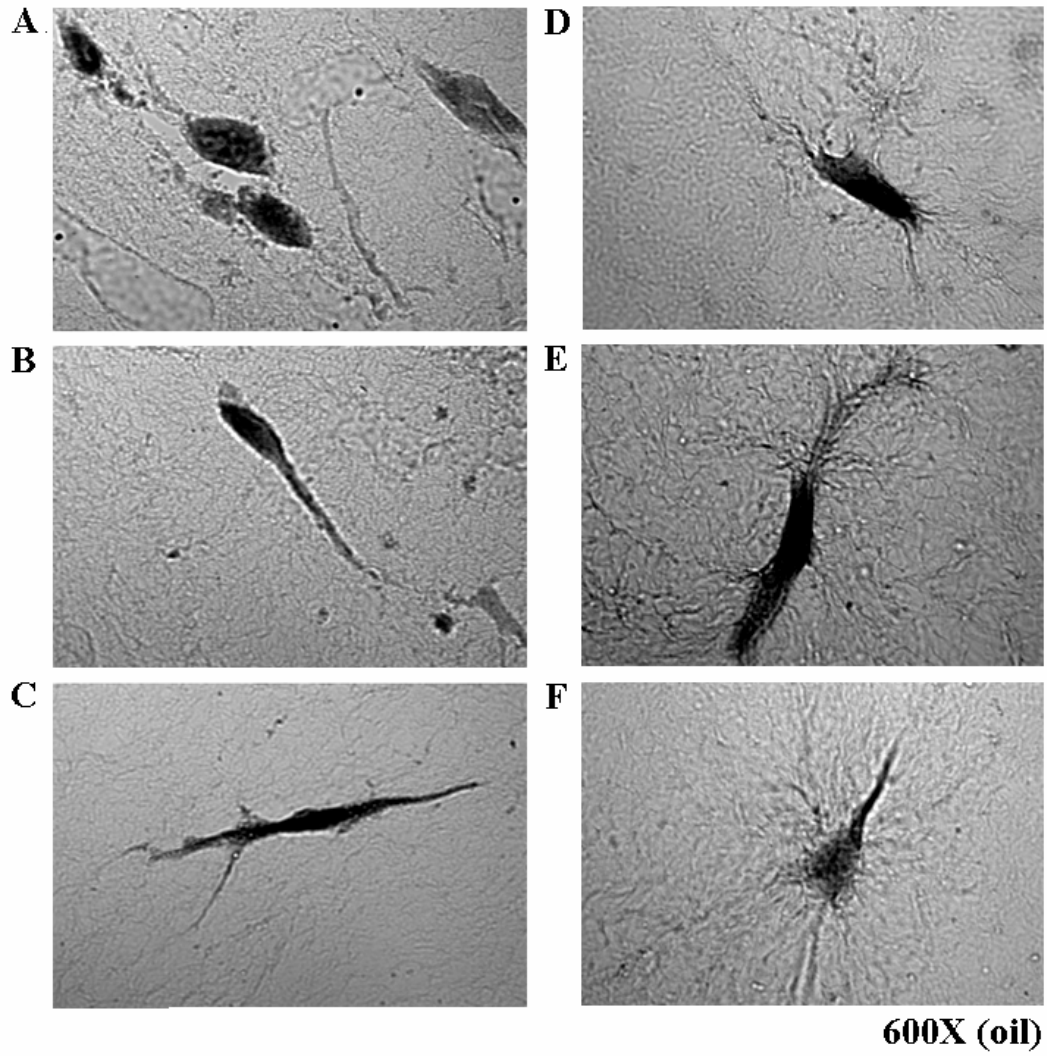


Figure 41. Fibroblast remodeling of collagen gels.

Following adult or fetal fibroblast invasion of constructs for 1, 3 and 7 days post release, high magnification (600X oil) DIC imaging (of 10micron sections stained with haematoxylin) was used to ascertain the organization of visible collagen fibers within the zone of fibroblast invasion. Adult fibroblast invasion of the collagen constructs corresponded with a dramatic alteration in the cellular morphology over time: day 1 (panel **A**), day 3 (panel **B**), day 7 (panel **C**). Fetal fibroblasts embedded within the collagen constructs displayed an increasing number of cell extensions beginning as early as day 1 (panel **D**), and increasing in subsequent time periods day 3 (panel **E**), day 7 (panel **F**).

As illustrated in Figure 41 (A-F) adult and fetal fibroblasts display different morphologic dynamics under 3D constraints. Specifically, adult fibroblasts exhibit few cytoplasmic protusions at one day following release, with an increase in cell area and cell protusions over time. In general, adult fibroblasts were found to display a fusiform morphology with relatively few cytoskeletal protusions. In contrast, fetal fibroblasts exhibited multiple cellular protusions as early as one day release release, with a dramatic increase in cell area and protusions over time. In general, fetal fibroblasts displayed a stellate morphology with a larger number of cellular protusions compared to their adult counterparts. Additional analysis suggests that the collagen fibers surrounding fetal fibroblasts are re-organized to a greater degree than those surroundting embedded adult fibroblasts.

4.1.4 Discussion

Fibroblast dynamics within the healing wound bed are crucial to the eventual resolution of the wound healing response. Whereas in adult tissue fibroblast driven ECM synthesis and remodeling results in imperfect reconstruction of the tissue, fetal fibroblasts appear to posses the ability to completely restore the initial tissue structure and function.[1,2] To date, it remains unclear how much of this cell phenotype is due to intrinsic fibroblast properties and how much is due to the milieu of soluble mediators directing fibroblast activity within the wound bed. Previous studies have demonstrated that: 1) there appear to exist measurable intrinsic phenotypic differences between fetal and adult dermal fibroblasts and 2) these intrinsic phenotypic properties can be differentially enhanced or retarded by exogenous cues.[18,24,25] Intrinsic and exogenously stimulated differences between fetal and adult fibroblasts revolve around: 1) cytoskeletal dynamics (i.e. actin fiber polymerization/ de-polymerization and stability), 2) dynamics of cell attachment to substrates (i.e. integrin receptor expression and distribution) and 3) whole cell morphological dynamics (i.e. attachment, migration, contractility).[28,31] While such studies have provided a wealth of information and expanded our understanding of the fetal fibroblast phenotype, they have yet to elucidate how these measurable differences contribute to the larger wound healing dynamic.

The current study was designed to address outstanding issues regarding phenotypic differences between fetal and adult dermal fibroblasts, by building upon existing work in several ways. First, it combines 2 dimensional dynamic population measurements (migration, contraction) with individual cell tracking. As such, it offers much needed insight into how individual cell dynamics correlate with and contribute to population-wide morphological dynamics. Second, it illustrates how transitions from inert (glass) to bioactive (collagen) substrates alter these morphological dynamics. Third, it utilizes a novel 3 dimensional collagen construct to determine whether phenotypic differences measured in 2 dimensional assays translate to a 3 dimensional environment approaching the in vivo environment.

Data suggest that fetal fibroblasts maintain a fairly robust intrinsic morphological dynamic with respect to migration and contractility. At the population level, this is reflected in elevated rates of directional migration and FPCL contraction, which persist across multiple observational time frames and increasing concentrations of a stimulant (FBS). The basal rates of fetal and adult fibroblast migration and contraction parallel each other, in so far

as fetal fibroblast dynamics are elevated approximately 0.5-1.5 fold over those of their adult counterparts. This observation suggests that fetal fibroblasts may posses intrinsically different rates of cytoskeletal re- arrangement which lead to elevated locomotion and increased traction force development. It also indicates that the change from an inert to a bioactive substrate does not change this intrinsic difference. The effects of FBS on fetal and adult fibroblast migration have been previously addressed, though they remain somewhat unclear. In our laboratory, FBS was found to dramatically stimulate both fetal and adult dermal fibroblast migration in a biphasic manner. With respect to FPCL contraction, FBS stimulatory effects appear to be limited by a saturation effect which occurs at a lower concentration in fetal fibroblasts. At the highest FBS concentrations tested, adult fibroblast contraction mimics that of their fetal counterparts, suggesting that the effects of FBS on cellular locomotion and contraction may not be completely analogous.

Population-wide dynamics such as migration and contraction offer a highly quantitative tool for elucidating cellular phenotypes. However, these types of bulk measurements suffer from several drawbacks which have been addressed in earlier sections. First, cells are largely constrained either by specific physical parameters (direction migration), or high cell density (contraction). As such, specific cell behaviors may reflect non-innate cell activity, but rather the limited choices for cell behavior in the chosen environment. Second, population-wide measurements offer a reduced ability to consider variability within a given cell population. This consideration is particularly important in cases such as this, in which the cell populations used are not derived from a clonal expansion and are somewhat heterogeneous. Third, population measurements provide us with average values for the migratory or contractile properties of the chosen cell population. While average values are generally considered to be an important representation of a cell behavior, they may not always be truly reflective of physiological dynamics in a process such as wound healing. Specifically, it may be possible that during dermal repair, the initial arrival of a small number of fibroblasts is sufficient to trigger subsequent cellular events. If these phenomena are relevant, the average speed of fibroblast migration would be less important than the maximum rate of invasion of the most invasive fibroblasts recruited from the wound margin.

Individual cell tracking has been previously used to address both basal cellular migratory dynamics and the involvement of specific pathways in cytoskeletal dynamics. Here, we utilized a modified live cell tracking technique, coupled to a new analytical paradigm to characterize the behavior of small populations of fetal and adult fibroblasts on both inert and bioactive substrates. Data indicate that, at the individual cell levels, fetal fibroblasts maintain what could be termed a hyperactive state. Specifically, fetal fibroblasts engage in a substantial number of detachment events, which is significant given the physiological dependence of fibroblasts on substrate attachment. These detachment events are accompanied by a generalized temporal delay in the transition from attachment and spreading to membrane ruffling and locomotion. Under relatively non- constrained conditions, the rate of stochastic fetal fibroblast locomotion over an inert surface is significantly elevated over that of adult fibroblasts by approximately 0.5 fold. This is a surprising correlation to the population-wide directional migration data on a similar surface. Overall, analysis of individual cell activity revealed that while fetal fibroblast dynamics may be noticeably different from that of adult fibroblasts, the ability to easily discriminate quantifiable differences between the two

phenotypes is reduced. Nevertheless, we believe that these experiments indicate that individual cell dynamics do somewhat correlate with larger population behaviors.

Fibroblast interactions with a bioactive matrix differ greatly from interactions with an inert surface. Fibroblast binding to inert surfaces such as glass and plastic is generally mediated by interactions with fibronectin. Fibroblasts rely on either endogenous or plasma fibronectin during attachment and contraction of collagen gels but can also bind directly to collagen itself.[165,166,167] Fibroblast attachment, spreading, and subsequent migration on a substrate are mediated by the dynamics of the cytoskeleton and transduced by β1 integrins.[153,154,155] Fibroblast migration in vitro is mediated by a complex interplay of cytoskeletal proteins that interact with the surrounding environment through focal adhesion complexes, akin to fibronexus junctions found in vivo.[168] The tractional forces required for cellular migration are exerted by nascent focal adhesions at the leading edge of lamellipodia that propel the cell body forward; these focal adhesions mature as the cell moves forward and provide anchoring under the cell body.[169] Force and rigidity of the matrix plays a role in increasing the strength of both integrin- cytoskeleton and integrin-matrix linkages as well as directing migration to stiffer areas of a substrate.[170,171,172] Maturation of these linkages and stress fiber formation has been associated with decreases in cell migration.

Data presented above are consistent with these existing studies, in that a dramatic change in both population-wide and individual cell dynamics is noted upon transition from inert surfaces to a collagen substrate. Although basal fetal fibroblast FPCL contraction rates are higher than those of adult fibroblasts, increasing concentrations of FBS negate this difference, in a manner distinct from the effects of FBS on directional migration over inert surfaces. At the individual cell level, the presence of a collagen substrate dramatically alters the overall morphological dynamics, by speeding up the initial phase of cell attachment and spreading, and reducing the number of detachment events. Although these effects transcend the fibroblast phenotype, fetal fibroblast dynamics remain relatively hyperactive compared to those of adult fibroblasts further confirming the robustness of this phenotype.

The appropriateness of extrapolating results obtained using 2D in vitro systems to the in vivo wound healing process remains unclear. In this study we describe a novel 3 dimensional construct which may be used to describe the invasive characteristics of fetal and adult fibroblasts as well as other cell types. This system differs from previously described precursors in several ways: 1) the thickness of the construct provides a true third dimension, 2) the gel interface allows for a consistent starting point for measurements regarding the extent of fibroblast invasion, 3) the construct is amenable to multiple imaging modalities. Analysis using this 3D construct indicates that the cellular dynamics described by both population and individual cell 2D experiments are generally conserved in 3 dimensions. Specifically, fetal fibroblast invasion of the 3D constructs occurs more quickly than that of adult fibroblasts, in the range of 0.5-1.5 fold. Population-wide measurements reveal a surprising difference in the overall cell dynamics of fetal and adult fibroblasts embedded within the collagen constructs. Although fetal fibroblasts display an elevated rate of invasion, their proliferative rate within the construct may be diminished compared to adult fibroblasts, as indicated by the absence of a significant increase in cell number over the week long time frame. Future experiments should attempt to clarify and extend this observation. At the individual cell level, fetal fibroblasts appear to have an increased remodeling potential manifested by the increased zone of collagen fiber bundling surrounding individual cells. In a manner consistent with previous studies, specific, geographically distinct

collagen fiber re- arrangement does not correspond to a generalized re- alignment of collagen fibers throughout the entire construct.[173] As described above, fetal fibroblasts display a highly dendritic morphology, previously assigned to dynamic interactions with the surrounding matrix. While low fibroblast density matrices have been shown to display dendritic cell morphology resulting in local but not global remodeling, high fibroblast density matrices exhibit significant global remodeling and bulk matrix contraction.[174] Cell protrusions are associated with β1 integrin localization and increased local matrix remodeling in low fibroblast density matrices.[175] Fibroblast transitions in cell morphology from a dendritic network to a stellate/bipolar shape have also been observed in collagen gels that were restrained with accompanying increases in focal adhesion and stress fiber formation leading to increases in local remodeling.[174,176]

Conclusions. The fetal fibroblast phenotype consists of a hyperactive morphological state, exemplified by population-wide elevated rates of migration, contraction and invasion and a more dynamic profile of individual cell dynamics. This phenotype is maintained despite exogenous manipulations which include: 1) stimulating factors, 2) transitions from inert to bioactive substrates and 3) transitions from 2 to 3 dimensional environments. Although these findings do not fully explain fetal fibroblast dynamics during fetal wound healing, they do suggest that intrinsic fetal fibroblast properties are crucial to the overall wound healing phenotype.

4.2 IMPACT OF TRANSPLANTED FIBROBLASTS ON RABBIT SKIN WOUNDS

4.2.1 Introduction

Cell- cell and cell-matrix interactions are important in overall body homeostasis. Understanding and characterizing these interactions can serve to elucidate the pathways by which tissue heals, dies, or regenerates. The process of wound healing is complex, involving numerous extracellular components, cell types, and mediators. Inflammation, angiogenesis, and reepithelialization, are important components of this process. However, it is fibroplasia that allows a healing tissue to regain form and function similarly to how it functioned prior to the injury.[177] Fibroblast migration into the wound area is followed by production of matrix components and remodelers. This function is modulated by chemical messengers secreted by macrophages, platelets, and lymphocytes.[178] The collection of these signals directs fibroblasts to fill in the wound with cells and extracellular matrix.

It has been thoroughly established that not all connective tissue is alike in its wound healing response. Fetal skin responds to injury differently than adult tissue; extracellular matrix components such as collagen show differential patterns of expression between the 2 tissue types. [1,179,180,181] This raises the question of whether fetal wound healing is different because of the components of the tissue or the wound environment that surrounds them. This issue has been previously addressed, and it was concluded that the properties associated with fetal wound healing are intrinsic to the tissue.[13,14,15,182,183] Because fibroblasts retain their phenotype in culture and can generate varied wound-healing responses, we hypothesize that transplanting a quantity of cells into a wound may modulate the healing process. We believe that the addition of exogenous fibroblasts into the wound environment will increase wound tensile strength due to earlier deposition and remodeling of the extracellular matrix. We also expect that the transplanted fetal fibroblasts will allow the wound to display a more regenerative healing pattern.

The goal of this study was to determine the effect of injected fibroblasts on full-thickness cutaneous wounds and to distinguish between the properties of fetal vs adult fibroblasts, autogenic (same animal) vs allogenic (same species) vs xenogenic (different species). To accomplish this, we first examined the fate of transplanted fibroblasts, observing survival and migration. Second, we measured the impact of transplanted fibroblasts on the wound healing response using tensiometry. Our findings provide proof of the feasibility of using nonautogenic transplanted fibroblasts for future development of cell therapies for regulating postnatal wound healing, potentially toward a more regenerative healing response.

4.2.2 Materials and methods

Cell culture

The following donor cells were cultured according to existing protocols. Allogenic fibroblast groups included fetal rabbit, neonatal rabbit, and adult rabbit. Xenogenic fibroblast groups included fetal pig on day 75 of gestation and neonatal pig fibroblasts. Autogenic fibroblasts were also prepared from each of the experimental

animals as follows. Full-thickness biopsy specimens (1.5 x 0.5 cm) were obtained from the rabbit hind leg skin after the skin was prepared with 10% povidone-iodine and 70% alcohol swabs. Briefly, fibroblast cultures were established as follows. The tissue was excised aseptically then washed in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY), 100 U/mL of penicillin, 100 U/mL of streptomycin sulfate, and 25 µg/mL of amphotericin C and kept at 4°C overnight. Using aseptic technique, skin sections measuring approximately 1 mm2 were partially digested with collagenase type I (Sigma Chemical Co, St Louis, Mo) and were placed eccentrically in 75-mm2tissue culture flasks and covered with DMEM containing 10% fetal calf serum, 10 mM HEPES buffer, and antibiotics. The cultures were left undisturbed for 1 week to promote tissue explant attachment and incubated in a humidified carbon dioxide incubator maintained at 37°C. Then the medium was changed twice a week and cultures examined for fibroblast outgrowth. Fibroblasts were harvested from primary culture after approximately 21 days and either subcultured immediately or cryopreserved and stored in liquid nitrogen for future experiments.

Cell labeling and animal care

Carbocyanine dye (CellTracker CM-DiI; Molecular Probes Inc, Eugene, Ore) was used to label the cultured fibroblasts according to the manufacturer's standard protocol as previously described.[184] The cells were counted and suspended in Hanks balanced salt solution (Life Technologies Inc, Rockville, Md) followed by mixing using extrusion from a syringe with hyaluronic acid non–cross-linked polymer (Hylan A gel; Genzyme Biosurgery, Cambridge, Mass) until a homogeneous mixture was obtained containing approximately 1.5 x 106/mL of cells.

A total of 9 New Zealand white adult rabbits, weighing 3 to 4 kg (Covance, Denver, Pa), were used in the experiments. All work conducted on these animals was consistent with standards approved by the Animal Research and Care Committee of Children's Hospital of Pittsburgh. The animals were housed individually in standard cages, in a room with controlled temperature and light in the Animal Facility at Rangos Research Center, Pittsburgh (Animal Welfare Assurance No. A3617-01, accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International).

The rabbits were acclimated to their environment for 5 days before biopsy specimens were obtained for primary cell culture work. They were given standard rabbit diet and water ad libitum. For both the biopsy and wounding procedures, the rabbits were anesthetized using intramuscular administration of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). For all procedures, the animals were shaved and the skin was examined to ensure integrity and lack of any infection. The experimental area was thoroughly cleansed with 10% povidone-iodine and 70% alcohol swabs before manipulation. Aseptic technique was maintained throughout all procedures. The animals were fully anesthetized throughout the entire procedure and were observed every 8 hours for the next 48 hours and on a daily basis throughout the rest of the 28-day experimental period. Post- procedure behavior patterns did not change from the pre-procedure patterns. No changes in appetite or behavior were observed at any point in the study. All animals were healthy and alive at the end of the experimental period, without weight loss. Some animal noncompliance was observed, consisting of attempts to remove the occlusive dressing covering the wounds. No injury or infection resulted from this behavior.

Wounding and cell transplantation

Full-thickness incisional wounds were created in the dorsal skin of the animals. A grid was made using a permanent marker, leaving a 2-cm margin between wounds, to prevent the risk of fibroblast migration to adjacent wounds. Each animal received 24 wounds, with 3 wounds assigned to each of the 6 experimental fibroblast lines, 3 wounds as vehicle controls containing only hyaluronic acid gel, and 3 wounds as untreated controls containing neither fibroblasts nor vehicle.

A 5-mL syringe with a 20-gauge needle was then used to place approximately 50 μL of the hyaluronic acid–fibroblast mixture to each wound. The wounds were left untouched for 5 to 10 minutes after which a coating of liquid bandage (NewSkin; Medtech Products Inc, Jackson, Wyo) was applied to every treated and control wound individually and allowed to dry for 5 minutes. The entire experimental area was then covered with a sterile transparent occlusive dressing (Tegaderm; 3M, Minneapolis, Minn) and secured with a nontoxic degradable adhesive (Mastisol; Ferndale Laboratories Inc, Ferndale, Mich).

Tissue analysis

The 9 animals were euthanized in groups of three, 7, 14, and 28 days after transplantation. The rabbits were heavily anesthetized using the ketamine-xylazine mixture, followed by intracardiac administration of euthanasia solution (pentobarbital sodium and phenytoin sodium) (1.5 mL). The dorsal skin was removed using the aseptic technique, followed by dissection of each individual wound. The samples were then placed at -80°C overnight and the next day embedded in frozen section embedding medium (Fisher Scientific, Springfield, NJ). Thin 6-μm sections were cut on a cryostat at -20°C, mounted on glass microscope slides, stored in moisture-proof slide boxes at -20°C, and protected from light until evaluation by fluorescence and light microscopy.

The wound sections were analyzed using an imaging system consisting of a microscope (Nikon Inc, Melville, NY), imaging software (Metamorph; Universal Imaging Corporation, Downington, Pa), and a printer (FujiX Pictography 3000; Fuji Photo Film, Edison, NJ). Fluorescence data were collected using a rhodamine filter (TRITC). All calculations were conducted on images collected at original magnification x10. The data were then analyzed using a computer database program (Microsoft Excel; Microsoft Corporation, Bellevue, Wash) and a statistical software package (STATISTICA; StatSoft Inc, Tulsa, Okla). The thresholding function in Metamorph was used to measure the total fluorescence area (transplanted cell density) present in a cross-sectional slide obtained at the center of each wound. The fluorescence value was then stored in a Microsoft Excel database. Time and cell-type effects were determined using statistical computer software. This statistical package was used to draw conclusions about statistically significant relationships for the entire data sample consisting of 216 wounds. Individual comparisons between the different cell types were determined using means and SEMs generated by the database.

Tensiometry data were collected using a tensiometer and data collection software (custom built). The excised wounds (approximately 1-cm wide) were trimmed of subcutaneous fascia. Each wound was cut into sections 3 to 5 mm in width. The cross-sectional area of each section was measured with calipers. Then the section was clamped in the tensiometer, and force was exerted to the breaking point. Measurements were recorded by a computer and tensile strength was calculated using the following formula: Maximum Reading (Converted to Grams) ÷ Cross-sectional Area (mm2) = Tensile Strength (g/mm2). The results for individual sections from one wound were combined for each wound specimen to determine an average tensile strength per wound. The tensile

strength per wound was tabulated for each group at each time point, and the means and SDs were determined using database software.

4.2.3 Results

Cell labeling

Previous in vitro experiments were used to determine the optimum experimental procedure described herein. Adult rabbit fibroblasts were labeled with CM-DiI dye and grown in culture. The cells were imaged at 7 and 14 days. The fluorescence persisted through day 14, although the intensity was reduced due to dilution of the label with multiple cell divisions. Imaging of these living fibroblasts revealed that the dye has a microsomal distribution pattern that excludes the nucleus. Previous in vivo experiments have shown that the dye-labeled cells are still present and detectable in unwounded skin at 28 days. A second in vitro experiment was used to test the viability of cells mixed with hyaluronic acid gel. Adult rabbit fibroblasts were labeled with CM-DiI dye and mixed into a volume of hyaluronic acid gel according to the procedure discussed herein. The cell-gel mixture was cultured for 7 days. The labeled fibroblasts grew, multiplied, and exhibited the same fluorescence pattern as labeled fibroblasts grown under standard culture conditions. The hyaluronic acid gel appeared to solubilize into the medium within 2 hours of incubation.

Cell delivery analysis

Day 7 wounds were used to determine the success rate of the cell delivery technique. This was based on the assumption that on day 7 cell presence was more indicative of survival than ability to multiply. Fluorescence measurements were used to approximate the amount of cells present in each wound. Figure 42 contains a numerical analysis of the 52 samples collected at day 7, as well as photographic companions, which can be used to determine the significance of the numerical values. As shown, the absolute success rate for the delivery technique was 92% (number obtained by counting all the samples in which cell delivery succeeded, regardless of specific amount). Fluorescent label was detected in neither the untreated nor the vehicle control wounds. Therefore, although fibroblasts did migrate into the margins of the wound, they did not migrate into adjacent wounds.

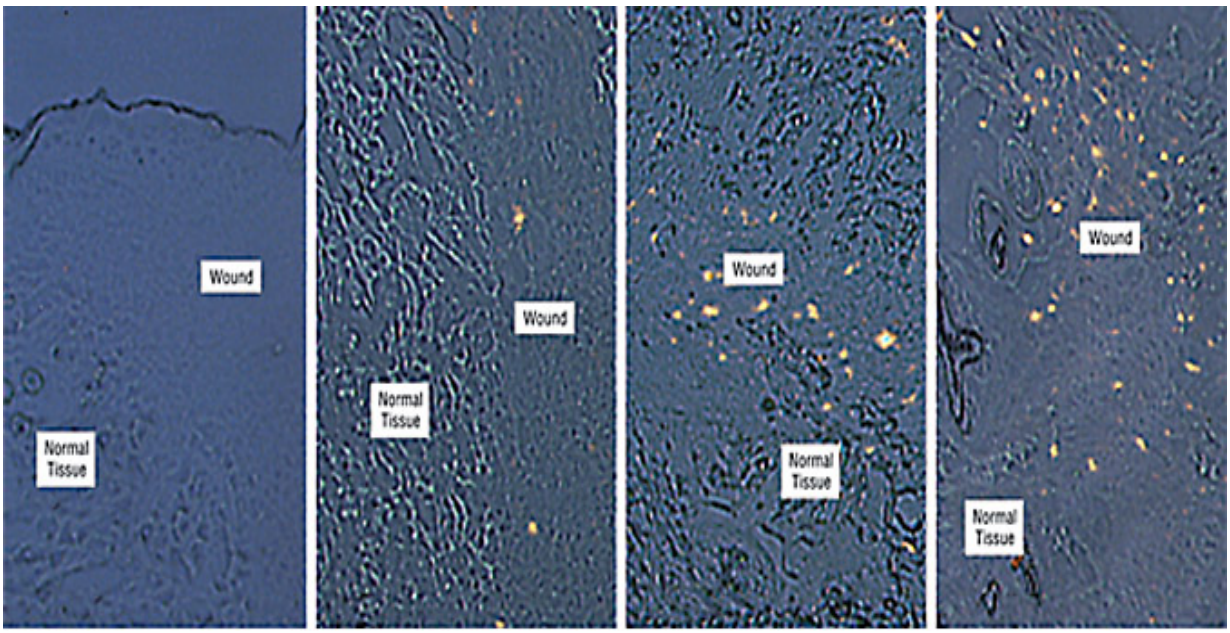


Figure 42. Day 7 images of wounds treated with labeled fibroblasts.

Day 7 images of wounds treated with labeled fibroblasts. Photomicrographs are overlays of fluorescence microscopy over bright field microscopy. Each photograph is representative of those wound samples whose fluorescent values fall within the specified range. The number of samples that fall within each range is indicated below each photograph. *i* indicates relative intensity.

Cell survival

Image analysis of all wound samples revealed several interesting trends. There was a statistically significant time effect (F test, P<.001). Figure 43 illustrates the decrease in cell number for each individual cell type from day 7 to day 28. Since only 3 time points were included, the true rate of decay cannot be determined. Statistical analysis of the fluorescence data revealed an additional cell-type effect (F test, P<.01). However, in this case, there was no clear pattern that persisted throughout the entire experimental period. The xenogenic neonatal fibroblasts seemed to exhibit optimal survival at least through the first 14 days compared with the other categories. However, the reproducibility of this result is unknown due to some variability in the delivery technique. Individual statistically significant differences are illustrated in Figure 43. Additional analysis revealed no significant differences between fetal and adult fibroblast treatment groups. There were no significant differences among autogenic, allogenic, and xenogenic fibroblasts, suggesting that fibroblasts in general are immunologically tolerated. Previously, we reported that xenogenic adult fibroblasts did not survive well in host tissue, so this cell type was excluded from further study. Histologic analysis of the various wound samples revealed additional information about the distribution of transplanted fibroblasts. As shown in Figure 42, wounds collected at days 7 and 14 revealed a cell pattern concentrated in the immediate wound area (data for day 14 not shown). A large percentage of the labeled fibroblasts were observed at the epidermal-dermal interface. However, by day 14 transplanted fibroblasts were observed migrating along the border between the dermis and the underlying fascia. As shown in Figure 44, there appears to be

a clear migration of cells from the initial wound area into the surrounding normal tissue. In addition, detailed histologic examination of all wound samples revealed no inflammation as characterized by the presence of lymphocytes and macrophages.

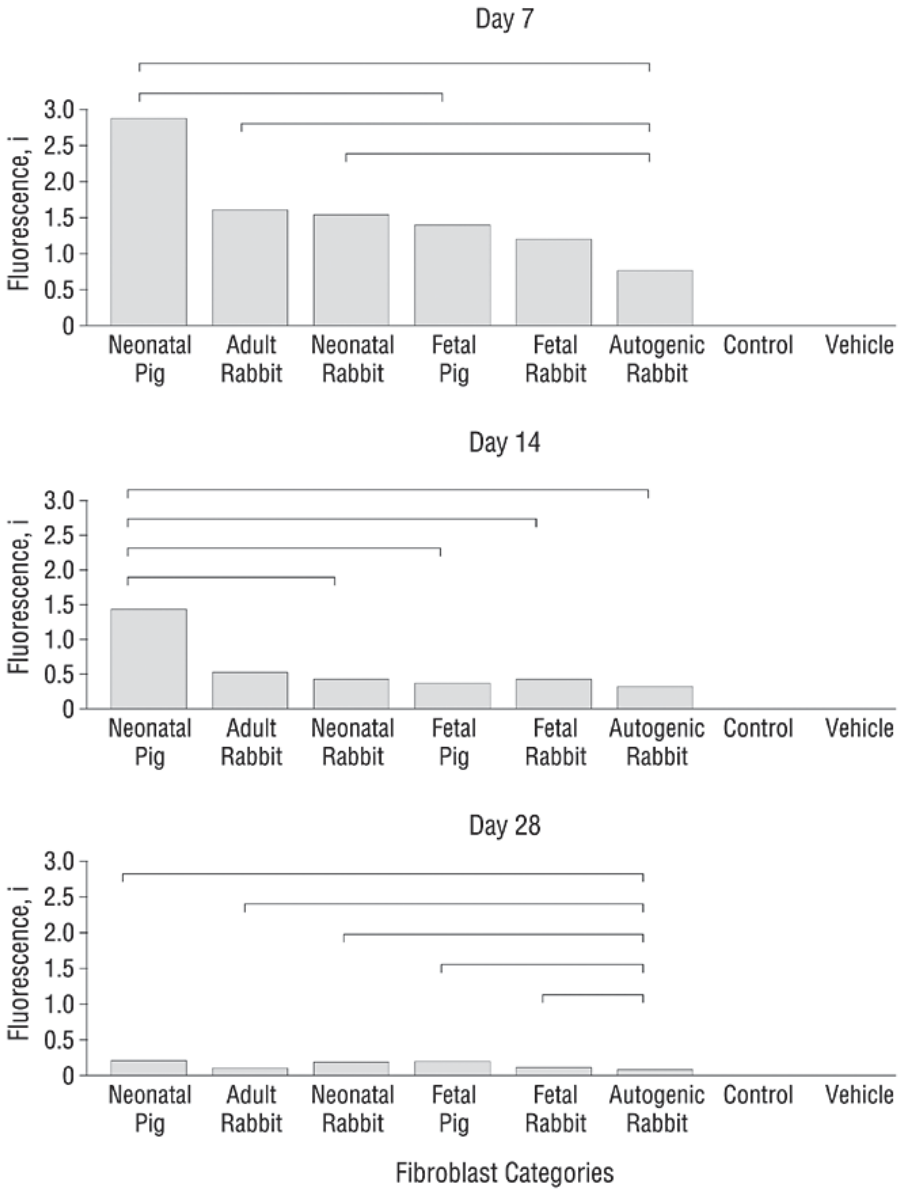


Figure 43. Histogram of fluorescence data for each cell type, vehicle, and control at 7, 14, and 28 days.

Histogram of fluorescence data for each cell type, vehicle, and control at 7, 14, and 28 days. Statistically significant relationships are indicated by the horizontal lines above each graph. *i* indicates relative intensity.

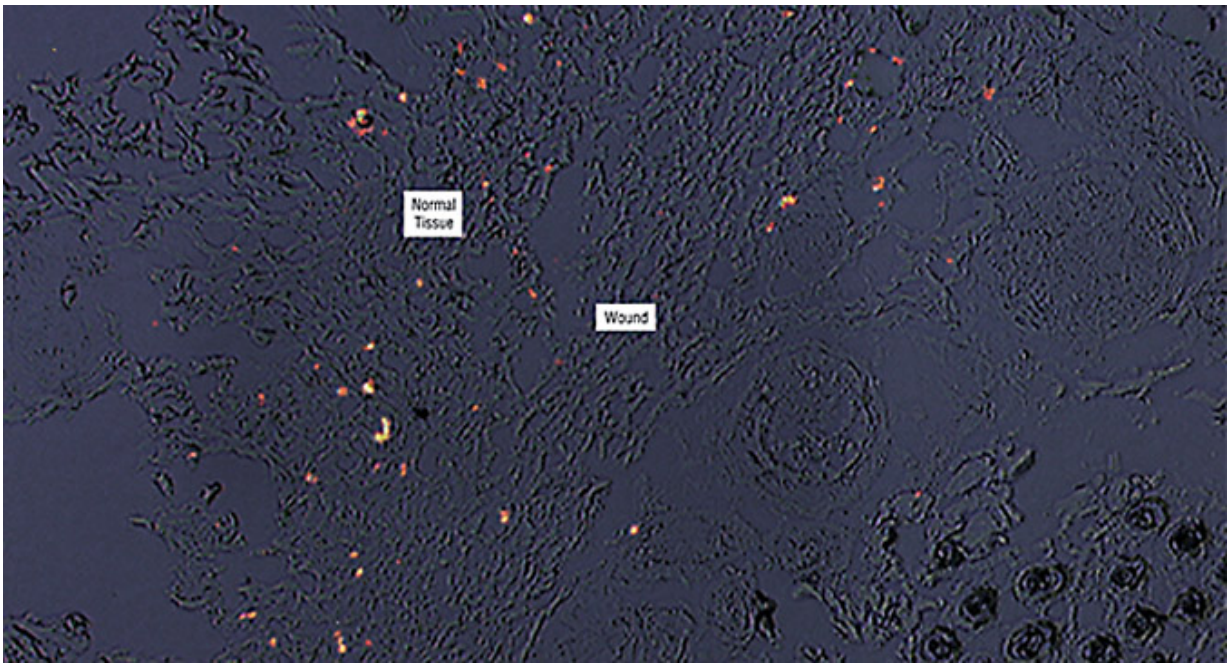


Figure 44. Photomicrograph of cutaneous wound treated with CM-DiI–labeled fibroblasts

Photomicrograph of cutaneous wound treated with CM-DiI–labeled fibroblasts (CellTracker CM-DiI; Molecular Probes Inc, Eugene, Ore) (day 14, adult rabbit fibroblast–treated wound). Cells can be seen migrating into normal wound margins as the wound begins to contract. Image was obtained at original magnification x10.

Regain of wound tensile strength

Tensiometry measurements were collected for all 3 time points. Statistical analysis revealed 2 significant effects: time (F test, P<.01) and cell type (F test, P<.001). Figure 45 illustrates the changes in tensile strength for each cell type and the vehicle and untreated control wounds throughout the 28 days of the experiment. In general, tensile strength increased with increasing time. At day 7, wounds containing xenogenic fetal fibroblasts, neonatal allogenic fibroblasts, and xenogenic neonatal fibroblasts all exhibited higher tensile strength than control wounds. At day 14, wounds containing allogenic neonatal fibroblasts and xenogenic neonatal fibroblasts still showed higher tensile strength than controls, along with wounds containing allogenic adult fibroblasts. However, by day 28, no statistically significant differences were observed between any cell type and control wounds, indicating that normal wound healing was "catching up" with cell therapy–increased healing by this time. Control and vehicle wounds yielded similar tensile strength results at all time points. Autogenic fibroblasts and allogenic fetal fibroblasts showed similar tensiometry values throughout.

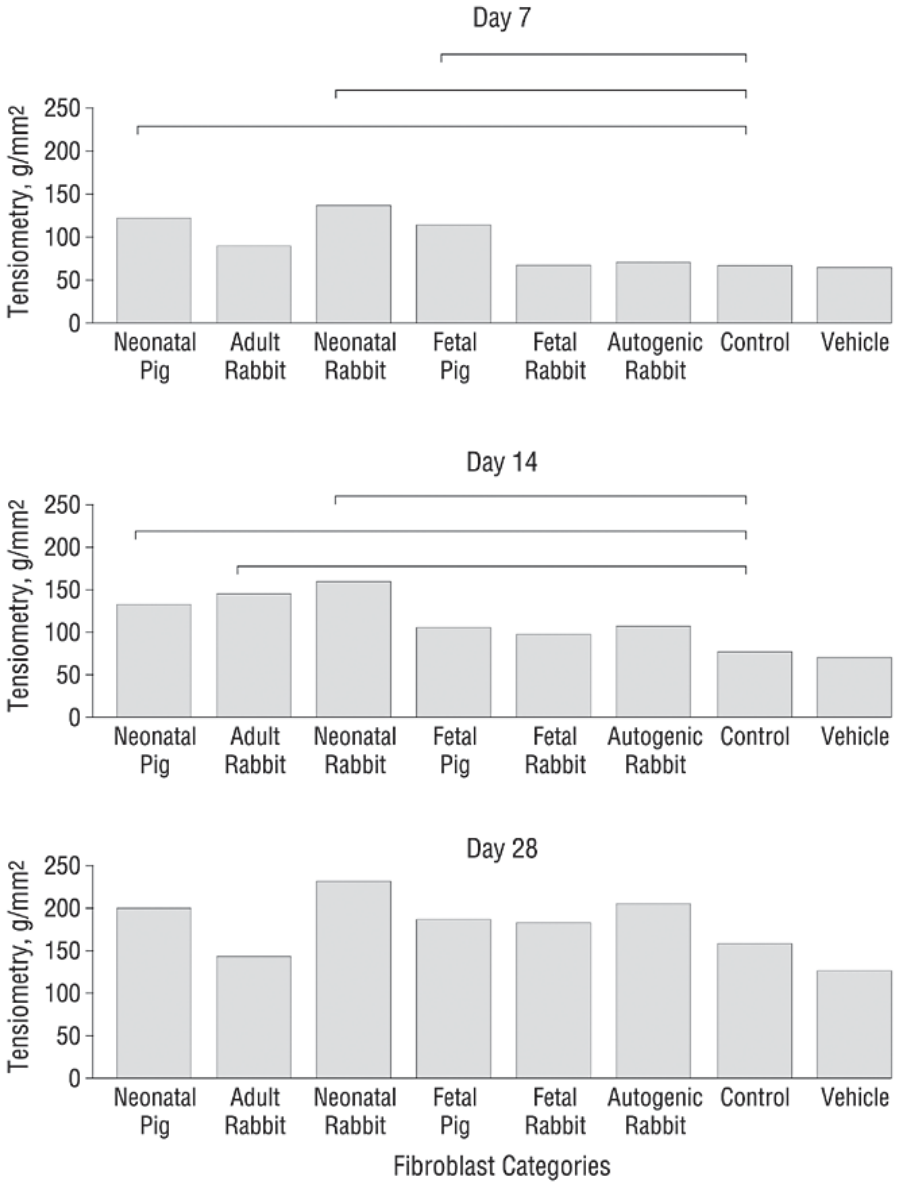


Figure 45. Histogram of tensiometry data for each cell type, vehicle, and control at 7, 14, and 28 days.

Histogram of tensiometry data for each cell type, vehicle, and control at 7, 14, and 28 days. Statistically significant relationships are indicated by the horizontal lines above each graph.

4.2.4 Discussion

We investigated a novel approach for the delivery of viable labeled fibroblasts into full-thickness cutaneous wounds. Our technique had an absolute success rate of 92%. On the basis of this study, 2 conclusions about the

viability of transplanted cell therapy for wound healing can be reached. First, transplanted fibroblasts do not appear to be immunogenic, as shown by the absence of inflammatory cell infiltrates within the wound area where donor cells were present. Second, all of the fibroblasts exhibited similar survival profiles in healing wounds throughout 28 days. The fluorescence data indicate several statistically significant differences in cell survival, most notably, the success of xenogenic neonatal fibroblasts. At day 7, these cells were present in higher numbers than most other cell types. It is interesting that xenogenic fetal and neonatal fibroblasts exhibited significantly different survival, considering the 2 cell types differ in gestational age by only about 5 weeks.

Fetal and adult fibroblasts did not exhibit significantly different survival patterns. Previous studies have shown that fetal tissue heals in a dramatically different manner from adult tissue. The importance of amniotic fluid and the sterile intrauterine environment in this process has been refuted by previous work, and many believe that it is fetal tissue and the fetal cells themselves that possess special regenerative properties. Future work will determine whether transplanted fibroblasts can regulate the qualitative outcome of healing toward regeneration, but this study demonstrates the feasibility of such an approach.

The tensiometry data suggest that understanding the dose effect of cell therapy is crucial to completely elucidating the role of exogenous fibroblasts in a wound environment. It is clear that transplanted fibroblasts can affect wound tensile strength. Several of the cell treatment groups exhibited statistically significant increased tensile strength, among them xenogenic neonatal, allogenic neonatal, and allogenic adult fibroblasts. Other groups, autogenic and allogenic fetal fibroblasts, showed a similar trend but did not achieve statistical significance due to small sample size. It is possible that by transplanting fibroblasts into the wound, normal migration processes, including fibroplasia, have been bypassed, allowing for earlier wound repopulation and matrix production, as we had hypothesized. We are currently investigating this hypothesis using immunohistochemical methods. Irrespective of the mechanism of action, the effect of the transplanted cells on the regain of wound tensile strength indicates that it is possible for transplanted cells to have an impact on the host wound environment. However, the increase in tensile strength did not precisely correlate with the number of cells present in the wound. For example, at day 7 allogenic adult fibroblasts were second highest with respect to cell number, yet this did not make a difference on the wound tensile strength. This may be an indication that both cell number and cell type are important parameters.

There are several sources of error, which if corrected should improve future studies. First, a micromixing apparatus that delivers a more precise dose of cells to each wound is under development. Second, variation in wound volume will be controlled by using an adhesive mesh grid to serve as a wound template, as well as using cautery and packing to prevent cell washout due to bleeding. Finally, animal noncompliance will be reduced by increasing the partial sedation of the animals and applying secondary dressings. These sources of error, however, are probably partially offset by the large number of wound samples (>200).

In conclusion, we confirm that fibroblasts transplanted in the wound survive and persist in a time-dependent manner and that they contribute to the wound healing process by expediting regain of tensile strength. The transplanted cells do not appear to incite inflammatory response. Both allogenic and xenogenic and both neonatal and fetal fibroblasts showed promising results that warrant further investigation.

4.3 PART II SUMMARY

Fibroblast activity in the dermal wound bed is highly regulated by exogenous cues which include soluble mediators and insoluble ECM components. The wound ECM can alter the activity of individual cells via two mechanisms: 1) interactions with bioactive ECM components can trigger intracellular signaling pathways (collagen-integrin) and 2) interactions between the cell body and surrounding environment can be altered by the mechanical properties of the environment (resistance and compliance). The experiments detailed above were designed to begin addressing how the second mechanism impacts the behavior of fetal and adult dermal fibroblasts. Results suggest that while fibroblast activity can be altered by the presence of collagen, fibroblasts retain a relatively robust phenotype with respect to morphological/cytoskeletal dynamics. This phenotype is represented by a generalized hyperactive state at the individual cell level, manifested as increased temporal transitions between various morphological states and increased rates of migration and ECM contraction at the population level. Surprisingly, population level differences are maintained both qualitatively and quantitatively despite transitions from inert to bioactive matrices, migration to contraction and 2D to 3D geometric constraints. As such it appears that the fetal and adult fibroblast phenotype is robust and might be used not only to explain tissue-wide differences between fetal and adult wound healing, but also as a tool for monitoring the activity of these two cell types under various test conditions.

While this analysis offers useful insights into the cellular mechanisms which might underlie the differences between fetal and adult dermal wound healing it is reductionist in nature and as such fails to account for various aspects of the larger wound healing response. Transplanting fetal dermal fibroblasts into the adult wound exposes these cells to all the present soluble and insoluble dermal components, with the quantitative, qualitative and temporal patterns comprising the normal repair process. In this context, fetal fibroblast activity can be monitored, and the behavior of these cells characterized in the context of the whole of the adult wound healing response.

Data presented above demonstrate the feasibility of such an approach with respect to several aspects. Fetal fibroblasts can be transplanted into the adult dermal wound and survive into the latter stages of repair which include ECM synthesis and remodeling. Surprisingly, xenogenic transplanted cells survive the repair process, indicating promise for future experiments using human derived fibroblasts in a variety of immunocompetent animal models. The activity of transplanted cells in the wound bed can be deduced from both direct and indirect observations. Specifically, fibroblast distribution throughout the wound bed and into the margins indicates invasive potential and this activity is suggestive of additional transplanted fibroblast activity in the host wound bed. In addition, tissue-wide effects, manifested as faster regain of tensile strength suggest at least some level of contribution of transplanted cells to the larger wound healing response.

In conclusion: 1) fetal and adult fibroblast behavior can be distinguished on either an inert substrate or a bioactive matrix, and in 2D and 3D constructs, 2) fetal fibroblasts can survive and maintain activity in an adult wound bed. Thus fetal fibroblast transplantation, coupled to more precise measurement techniques may be used in future experiments to more appropriately characterize the intrinsic fetal fibroblast phenotype, by observing the behavior of fetal fibroblasts in an adult wound bed and contrasting that with the behavior of normal adult fibroblasts under the same conditions.

5.0 GENERAL DISCUSSION

Wound healing is a complex process, made so not only by the multiple cells types, soluble mediators and ECM components involved in the process, but also by the quantitative and temporal components of the various sub-processes. The precise temporal sequence of events is essential to favorable wound healing outcomes and appropriate tissue repair. The underlying hypothesis driving this line of research is that early events during dermal repair largely dictate the final outcome of wound healing; more precisely, that inflammation contributes the development of scar formation/ fibrosis. In order to address this hypothesis, attention was focused on two aspects of dermal wound healing: 1) the role of PGE2 signaling in determining dermal scar formation and 2) the role of fibroblasts in regulating PGE2 signaling. The experiments detailed above were meant to address these two issues. This section is not meant to recapitulate discussion of results offered above, but rather to offer some generalized conclusion drawn from these completed studies. It also addresses the significance of subsequent studies detailed in Part II, meant to expand both understanding of fibroblast dynamics and current approaches to the study of differential fibroblast activity during different wound healing phenotypes.

5.1 FIBROBLAST PGE2 SIGNALING DURING DERMAL WOUND HEALING

Dedicated inflammatory cells (macrophages, neutrophils, lymphocytes) play an important role not only in the coordination of the overall inflammatory response to injury, but also in directing the subsequent activity of mesenchymal and epithelial cells. While fibroblasts are generally considered a target of inflammatory mediators, recent evidence indicates that dermal fibroblasts also have the ability to secrete a variety of inflammatory mediators, and that these mediators play an important role throughout the wound healing process.[13,14,116] Fibroblast derived inflammatory mediators become even more important when it is considered that inflammation is a transient wound healing subprocess, which resolves after several days. Dedicate inflammatory cells are seldom present in the dermal wound bed for more than 3-5 days post injury. In contrast, fibroblasts are an essential wound healing component for the entire duration of the repair process. For these reasons, it seems appropriate to address the role of fibroblast derived inflammatory mediators in the larger wound healing response. The analysis detailed above was designed to examine the role of PGE2 signaling in multiple fibroblast phenotypes with respect to endogenous synthesis and

production. PGE2 was chosen because: 1) it is an important inflammatory mediator and 2) the role of PGE2 in regulating dermal wound healing remains poorly understood.[35,46,47,119,121]

Data presented above suggest that PGE2 signaling is generally conserved across multiple fibroblasts phenotypes (fetal, normal/adult, keloid). Specifically, synthetic (COX-2, mPGES1) and degradative enzymes (15-PGDH) are present in all three cell types and are responsible for the synthesis and presence of substantial levels of endogenously produced PGE2. Receptor expression for all EP receptors is constitutive in all three cell types and conserved with respect to both mRNA and protein levels. These findings are consistent with existing studies. Previous studies have demonstrated that endogenous fibroblast production of soluble inflammatory mediators is generally conserved, but quantitative differences appear to be phenotypically dependent.[13,14,116] Wound healing literature in general, holds that different wound healing phenotypes rarely manifest as complete ablation of specific signaling pathways or individual genes, but rather represent phenotypes dictated by subtle alterations in the quantitative and temporal patterns of specific signaling cascades.[1,2] Data suggest that this may indeed be the case with respect to PGE2 signaling. Basal PGE2 production is indistinguishable in all three fibroblast phenotypes. Following stimulation, only keloid fibroblasts display a reduced endogenous capacity to produce PGE2, which may reflect reduced activation of COX-2 by primary stimuli such as IL-1β. This is surprising for two reasons. First, fetal fibroblast production of other secondary inflammatory mediators (IL-6, IL-8) is lower than that of adult fibroblasts under both basal and IL-1β stimulated conditions and this is consistent with previous studies.[13,14] Clearly, this phenotype is not generalizable to all secondary inflammatory mediators, or at least to PGE2. Second, diminished endogenous production of PGE2 by keloid fibroblasts, coincides with existing literature which has established that fibrotic lung fibroblasts also have impaired PGE2 endogenous production.[64,116] While this would suggest that skin and airway PGE2 signaling may coincide, it contrasts with a generalized existing tenet that PGE2 in the skin is pro-inflammatory and pro- fibrotic while in the airway it acts in a distinctly anti- fibrotic manner.[35,53,82,83,116,117] This finding, combined to other data discussed below should at least raise some questions regarding the validity of this notion. Keloid formation represents a distinct wound healing phenotype, which remains a significant clinical challenge.[32,33,34] Understanding of the cellular dynamics during keloid formation revolves around two central issues: abnormal fibroblast activity in the wound bed and an altered inflammatory state.[36,37,39,40,41] The second issue remains unclear. While other studies have demonstrated some systemic and local inflammatory abnormalities associated with keloid formation, no reasonable mechanistic explanations have yet been offered. The impaired activation of COX-2 in keloid fibroblasts may be associated with diminished production of PGE2 during the in vivo process. In the next section, a possible explanation for how diminished PGE2 may contribute to excessive dermal fibrosis and keloid formation is offered.

A second important conclusion derived from the experiments detailed above relates to the regulation of PGE2 during wound healing at the tissue and cellular level. Previous studies have demonstrated that: 1) PGE2 levels increase in the dermal wound following injury and 2) higher PGE2 levels appear to be associated with both increased inflammation and higher degrees of fibrosis in the latter stages of wound healing; the corollary of these two phenomena is that fetal wound healing exhibits diminished activation of PGE2 synthesizing pathways (COX-2).[35,46,47,119] These experiments confirm this in a new animal model (rabbit) and further demonstrate that diminished

COX-2 activation coincides with diminished IL-1β levels in the tissue, a possible explanation for lower PGE2 levels during fetal wound healing. This suggests that diminished activation of PGE2 synthesis during fetal wound healing may not reflect an intrinsic impairment in this particular pathway, but rather a generalized diminished inflammatory response, which also manifests at the level of PGE2 signaling. An even more important finding is that accompanying differential up-regulation of COX-2 levels is differential EP receptor expression. Building on previous work, multiple EP receptors are shown to be differentially regulated during fetal and adult dermal wound healing.[103] To date, a putative trigger for such differential regulation of PGE2 receptors remains elusive.

Under in vitro conditions, at the level of the fibroblast, COX-2 up-regulation occurs in a dose dependent manner when stimulated with IL-1β. This also holds true for mPGES1 and secreted PGE2 levels. In conclusion, fetal fibroblasts do not have an intrinsically altered PGE2 synthetic phenotype, but rather the tissue observed effect is likely a result of lower wound levels of IL-1β in fetal wounds. More importantly, in vitro analysis suggests an interesting discordance between regulation of PGE2 synthesis and the regulation of two other aspects of PGE2 signaling: degradation and receptor expression. Specifically, IL-1β, along with TNFα and IFNγ fail to regulate the levels of either 15-PGDH (a main degradative enzyme) or any of the EP receptors. There are two equally interesting explanations for these phenomena. First, primary inflammatory mediators regulate synthesis of PGE2, but not other aspects of PGE2 signaling. These other aspects could be regulated by other soluble mediators, such as growth factors. Implied in this dual regulation is this possibility that multiple regulatory mechanisms are coordinated in the generalized regulation of the overall PGE2 signaling pathways. Second, tissue levels changes in EP receptor expression may be indicative of a cell type other than the fibroblast, opening new avenues for investigation.

5.2 PGE2 AS A PUTATIVE ANTI- FIBROTIC AGENT IN THE DERMIS

The role of PGE2 during wound healing has been addressed by both in vivo and in vitro studies. In vivo studies to date can be divided into: 1) observational studies, which simply describe various aspects of PGE2 signaling during the normal wound healing process and 2) interventional studies which manipulate PGE2 signaling exogenously.[35,119] The totality of these studies lead to the following generalizable conclusions: 1) PGE2 regulates both inflammatory and fibroplastic activities, 2) effects on inflammation can be either pro- or anti- inflammatory, 3) PGE2 effects in the airway are putatively anti- fibrotic, while PGE2 effects in the skin are putatively pro-fibrotic.[85,86,88,94,105] These conclusions have been partially solidified by recent evidence indicating that scarless fetal wound healing, which is associated with minimal inflammation and fibrosis contains a minimal PGE2 signaling component, a finding confirmed above**.** What makes these data difficult to interpret mechanistically is the dual role of PGE2 during wound healing. As described earlier, inflammation is linked to fibrosis.[1,2] Therefore, it is unclear whether PGE2's effects on dermal fibrosis are direct or indirect. The only way to carefully explore this issue is to clarify the direct effects of PGE2 on fibroblasts, by examining fibroblast activities relevant to dermal wound healing. The series of studies described here was designed to do just that. The novelty of this line or investigation

does not lie in the mechanistic aspects of PGE2 signaling. The effects of PGE2 on fibroblast activity tested above have been previously described in other cell systems quite thoroughly.[92,93,94,105] The analysis presented above has several advantages with respect to previous studies. First, it uses human derived skin cells, which is important, given previous evidence that there is some species-dependent variability in cell behavior. Second, in contrast to transformed cell lines it uses fibroblast cultures derived from multiple tissue sites and various patient profiles. Third, it uses skin derived fibroblasts as opposed to previous studies which focused on airway, cervix or oral mucosa derived fibroblasts.[92,93,94,105] This is particularly important in that it has been demonstrated that fibroblast activity is tissue dependent in some respects, and particularly in the context of wound healing.[35,53] Fourth, it uses three well defined and different skin fibroblast phenotypes: fetal, normal/adult and keloid, three fibroblasts which are associated with varying degrees of scar formation. Specifically, keloid fibroblasts are a well defined, fibrotic fibroblast phenotype with relatively well characterized responses to various exogenous factors.[37,38,39,40,41] For these reasons, this analysis is quite important to the question at hand.

Given the data presented above, it appears that the effects of PGE2 on fibroblast activity under in vitro conditions are clearly anti- fibrotic. The three tested fibroblast activities, migration, ECM synthesis and ECM contraction are quite relevant to wound healing, but also to the development of excessive scar/fibrosis. In all three fibroblast phenotypes, PGE2 was shown to inhibit migration and contraction via alterations in the actin cytoskeleton. These findings, consistent with previous reports in other cell systems, should be relevant to the in vivo environment. Collagen production, a primary contributor to excessive scar formation and even more so in the context of keloid development was shown to be down-regulated by PGE2 following stimulation by TGF-β1, one of the main culprits in stimulating scar formation and keloid formation in particular. Data indicate that even keloid fibroblasts respond to PGE2 by down- regulating the endogenous production of collagen I and III, normally triggered by TGF-β1.

Surprisingly, the main difference in the response of these cell types to exogenous PGE2 is not between keloid fibroblast and normal fibroblasts, but rather between fetal and adult fibroblasts. It is surprising that while fetal fibroblast migration and contraction are similarly affected by PGE2, these cells appear to exhibit a quantitative insensitivity to this mediator. Specifically, inhibition of migration and contraction is diminished compared to that of adult counterparts, and is likely the result of intrinsically more robust cytoskeletal dynamics. This finding is consistent with previous reports which have documented that fetal fibroblasts respond to exogenous mediators with qualitatively and/or quantitatively different responses from their adult counterparts.[24,25,26] It is surprising, however, given that PGE2 acts as an anti- fibrotic agent. It would have been expected that keloid fibroblasts would be somewhat refractory and fetal fibroblasts have enhanced responsiveness to this soluble mediator.

The dual nature of PGE2 as an inflammatory and fibroplastic modulator remains difficult to understand in the larger wound healing process. Nevertheless, it appears that in the skin, PGE2 ‘seffects on fibroblast activity are anti- fibrotic. Therefore, PGE2 associated fibrosis during dermal wound healing may be a result, not of direct fibroplastic effects, but a result of its pro- inflammatory role in stimulating the larger inflammatory response. Specifically, PGE2 may act as intermediate activator of various pro- inflammatory cascades including cytokine and chemokine production, both by dedicated inflammatory cells and mesenchymal cells. What does differential PGE2 signaling in fetal, normal/adult and keloid fibroblasts tell us about the larger wound healing response? This analysis,

though relatively comprehensive, does not lead to a generalizable conclusion about either the role of PGE2 in regulating dermal wound healing or the link between inflammation and fibroplasia. Rather, we conclude that any of multiple aspects of signaling (synthesis, degradation, receptor expression, cell responses) may be altered either qualitatively or quantitatively in the context of various wound healing phenotypes. These experiments clarify and bring into specific light two generalized issues: 1) the dermal fibroblast phenotype appears to be relatively robust and 2) phenotypic differences are likely to be quantitative rather than qualitative and, more often than not, relatively subtle.

5.3 FIBROBLAST PHENOTYPE: HOW GENERALIZABLE IS AN IN VITRO PHENOTYPE?

In order to further address the two issues listed above, a series of experiments was designed to test the robustness of the intrinsic dermal fibroblast phenotype (fetal vs adult) under increasingly complex environmental constraints. This particular line of experimentation was undertaken for two reasons. First, to establish the relevance of in vitro findings with respect to PGE2 effects on dermal fibroblast activity. Previous studies have already shown some intrinsic differences between fetal and adult dermal fibroblasts. These differences span cell activities and environmental conditions (fetal bovine serum and/or the presence of bioactive matrix such as collagen). In order to extend this analysis a third dimension was added, to more closely mimic the wound environment. Second, to determine whether the phenotypic differences described above at the population level are reflected in individual cell activity. The underlying value of establishing this fact has been previously mentioned and will be discussed below in more detail.

These data indicate several things. First, the fetal fibroblast phenotype is relatively robust. At the population level, fetal fibroblast cytoskeletal dynamics are elevated, translating into a higher speed of migration. The presence of either exogenous stimuli (FBS) or changes in the underlying substrate from inert to bioactive do not ablate this phenotype. Interestingly, when converted from a 2D to a 3D environment, fetal fibroblasts still maintain elevated rates of invasion/ migration and ECM remodeling. This is particularly important, given that the 3D collagen construct recapitulates some aspects of the wound bed with respect to mechanical constraints as detailed above. Second, population-wide based observations of morphological activities such as migration, contraction and invasion appear to be representative of measurable characteristics of individual fibroblasts. In other words, observations of individual cells may allow us to distinguish between the fetal and adult phenotypes as well (quantitatively) as observations of larger cell populations. Most studies to date oriented toward describing various fibroblast phenotypic traits (migration, proliferation, protein synthesis) are designed to measure population wide-dynamics.[18,25,94] While these measurements are clearly useful and offer an important insight into cell behavior, it is becoming increasingly obvious that they fail to capture individual cell behavior. The analysis offered above attempts to address this by: 1) rather than measure larger population dynamics, use a more refined analysis which attempts to characterize the activity of small cell populations over time, in order to achieve almost a single cell resolution; 2) rather than contrive

the experimental design to achieve a single morphological dynamic, allowing a less controlled system which can be used to ascertain multiple morphological dynamics; 3) focus not only on differences between fetal and adult fibroblast dynamics, but also on how these dynamic processes are influenced by the matrix the cells encounter. Based on this analysis, the elevated population dynamics observed with respect to migration, contraction and invasion appear to be reflected at the individual cell level by a hyperactive fetal fibroblast state, exemplified by higher rates of transition between morphological states. Most interestingly, this hyperactive state appears to translate to contraction of collagen fibers inside of a 3D construct.

In addition to further elucidating cell dynamics, these findings may hold implications for future studies. Specifically, they identify two methodologies which may be used to ascertain the activity of fetal and adult fibroblasts under complex environmental constraints: 1) population -wide invasion of a 3D matrix and 2) individual cell remodeling of the collagen fibers. These methodologies could be used in the context of cell transplantation to create a novel and innovative approach to the study of fetal and adult wound healing.

5.4 CELL TRANSPLANTATION: CAN IT BE USED TO STUDY AND UNDERSTAND FETAL WOUND HEALING?

Wound healing involves not only multiple cell types, but a myriad of soluble mediators and ECM components. As such, there are virtually thousands of possible interactions which would require analysis. In vitro and in vivo studies using traditional approaches would require hundreds of experiments, which when completed would still fail to account for at least two crucial aspects to the entire wound healing process. First, in vitro conditions fail to take into account spatial gradients of soluble mediators and complex release of multiple mediators. Second, these experiments cannot replicate the precise temporal sequence of events which comprises the larger wound healing response. As such, it does not appear feasible to characterize fetal fibroblast contribution to the scarless wound healing response using conventional approaches. Transplantation of fetal fibroblasts in an adult wound bed could provide a useful alternative, by addressing two issues: 1) how these cells behave inside of the wound bed and 2) whether these cells can actually alter the larger wound healing response as a whole. Data presented in above indicate that: 1) fetal, neonatal and adult fibroblasts can survive in an adult dermal wound bed through the inflammatory phase and late into the remodeling stages, 2) cell transplantation into the wound bed can be accomplished with autogenic, allogenic and even xenogenic derived cells, despite the use of an immuno-competent host, 3) transplanted fibroblast appear to remain active as indicated by their invasion of the wound bed and surrounding wound margins and 4) transplanted fibroblasts appear to alter the native wound healing response by speeding up bulk regain of tissue tensile strength. Future studies should combine the approaches developed in the 3D in vitro model and those developed in the cell transplantation experiment. Specifically, fetal and adult fibroblasts could be transplanted into dermal wounds in a uniform vertical interface which would then be closed by the wound bed. From this interface, fibroblasts will be able to invade the wound bed in a measurable manner. The wound bed

and margins would then be analyzed ultrastructurally using high magnification imaging and electron microscopy to identify and characterize interactions between fetal fibroblasts with the surrounding adult wound environment. This type of analysis could potentially elucidate precisely how fetal fibroblasts behave in a dermal wound bed, and how this behavior differs from that of adult fibroblasts.

5.5 FUTURE DIRECTIONS: WOUND HEALING IN THE UPPER AIRWAY

Skin is the quintessential organ for studying wound healing and as such, wound healing is often equated with the study of dermal wound healing. Nevertheless, tissue repair processes occur all throughout the body with various outcomes and success rates**.** Fibrotic wound healing in the skin is largely a cosmetic challenge. In contrast, fibrotic wound healing in other tissue types can have profound clinical consequences. In the upper airway in particular, disruption of normal tissue structure can lead to mucosal fibrosis. In the trachea and subglottis, this can lead to localized or circumferential stenosis of the airway and obstruction of air flow.[185]

Wound healing in the upper airway parallels many of the events encountered during dermal repair, with sequential restoration of the multi- layer structure. The upper airway is a conduit for air exchange, with its primary function being facilitation of uninterrupted air flow during respiration. As such it possesses several anatomical traits: 1) semi-rigid structure to prevent collapse under pressure from fascia and muscle layers surrounding it, 2) flexibility to allow for distension during breathing, 3) smooth internal lumen to allow for undisturbed air flow lined by epithelium which can maintain a filtering and clearing function for both pathogens and foreign bodies**.**[186] The upper airway anatomy and physiology dictate that effective wound healing must achieve four key endpoints: 1) restoration and maintenance of vascularity, 2) re- epithelialization, 3) control of fibroblast activity and 4) maintenance of a semi-rigid cartilaginous structure. To achieve these goals, wound healing of the airway mucosa involves complex interactions between inflammatory cells, epithelial cells, fibroblasts and chondrocytes, all carefully choreographed by an array of cytokines, chemokines and growth factors. A consistent component of normal wound healing is the propensity of most connective tissues to heal through a reparative process which only partially approximates the original tissue structure and function and results in a thicker, denser lamina propria, that is, scar formation. Though scarring is never a desirable outcome when wounded tissue heals, the tendency for scar tissue to be excessive can be disastrous when it occurs in the airway. Stenosis of the airway, often caused by what is essentially a type of hypertrophic scarring, reduces the cross-section of the lumen through which the body's air supply flows.

The fetal airway mucosal wound healing phenotype has been described in the last few years [187,188]. Whereas adult subglottic wounds healed with scar formation and induction of chronic stenosis, fetal wounds healed in a regenerative manner. Histologic analysis of healed fetal airways reveals complete reconstitution of the trilaminar structure, with restoration of functional status. These data correlate with long standing animal studies of fetal dermal wound healing suggesting that there is a gestational transition from regenerative to reparative wound healing.

Additional work is ongoing to correlate the functional requirements of the fetal upper airway with its distinct wound healing phenotype. [189]

Cytokines and chemokines are an integral part of the inflammatory response that accompanies wound healing, and can thus function to provide an insight into the potential course of the healing process. While the inflammatory reaction to injury has been well defined in the lower airway, it remains less well characterized in the tracheal mucosa. However, it is relatively clear that inflammatory cell invasion of the airway wound bed occurs through inflammatory mediators released by the endogenous epithelial cells. [190] Studies in the upper airway indicate that PGE2 signaling may play an important role in the mucosal repair process and contribute to the overall outcome of wound healing (ongoing studies). Recent data indicate that both IL-1β and PGE2 are present at detectable levels in subglottic secretions following mucosal injury. [191] The levels of these two inflammatory mediators have a temporal component which suggests that they may be reflective of the larger inflammatory dynamic (onset and resolution). Interestingly, the precise quantitative and temporal patterns of IL-1β and PGE2 in mucosal secretions appear to be partially dictated by the degree and extent of injury (manuscript in preparation). Additional evidence indicates that the secreted levels of IL-1β and PGE2 correlate well with the activation of inflammatory genes early following injury.

Activation of IL-1β during subglottic mucosal injury is important in that IL-1β has been shown to act as an early master coordinator of inflammation with significant effects on downstream targets such as PGE2, IL-6 and IL-8.[57,61] Activation of these pathways can subsequently alter the physiological parameters of the upper airway. Specifically, IL-1β up-regulation of COX-2 activity and PGE2 secretion can increase mucin gene expression, and protein secretion by airway epithelial cells. Endogenous PGE2 production is also thought to play a role in regulating the effects of hyperoxic injury to the tracheal epithelium through mechanisms which remain unclear.[192] An additional putative source for PGE2 in mucosal secretions is the mucosal fibroblast which resides within the lamina propria layer. Upper airway mucosal derived fibroblasts have the ability to synthesize and secrete endogenous PGE2, in a manner which is highly regulated by IL-1β (manuscript in preparation) at the level of both COX-2 and mPGES1.

These in vivo and in vitro studies have been complemented by cell transplantation experiments in the subglottic mucosa in order to provide much needed insight into the cellular and molecular processes which underlie the interaction between inflammation and fibrosis in the upper airway mucosa.[193]

5.6 CONCLUSIONS

Inflammation is a crucial component of wound healing, in the dermis and other tissue types. While its contribution to the overall outcome of wound healing is undeniable, the precise cellular and molecular processes which link inflammation with subsequent wound healing steps remain unclear. Previous studies have shown that dual purpose soluble mediators can serve as both inflammatory mediators and regulators of subsequent epithelial

cell and fibroblast activity in the wound bed. Prostaglandin E2 is such a dual function soluble mediator. Activation of PGE2 synthetic enzymes following dermal injury triggers up-regulation of PGE2 levels, and results in wound bed infiltration by dedicated inflammatory cells and subsequent regulation of fibroplastic events in the latter stages of tissue repair. Experimental results presented above suggest that while the role of PGE2 early on during repair may be pro- inflammatory, its latter effects during wound healing are anti- fibrotic. As such, it suggests the possibility that alterations in the temporal and quantitative aspects of PGE2 signaling may play an important role in determining the overall outcome of wound healing and final degree of fibrosis. Differential PGE2 signaling during dermal repair may indeed be partially responsible for the varied wound healing outcomes that are encountered under specific physiologic (fetal vs adult) and pathologic (normal vs keloid) conditions.

Future studies can build upon this line of analysis through the use of more complex analytical paradigms with improved resolution for measurements of individual cell activity under a variety of 2- and 3- dimensional conditions. In conjunction with novel cell transplantation approaches, it is expected that such analyses will go a long way toward increasing understanding of the sequence of events which guides the wound healing process.

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