The altered mechanical phenotype of fetal fibroblasts hinders myofibroblast differentiation

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Manuscript received: June 20, 2018
Accepted in final form: October 6, 2018

DOI:10.1111/wrr.12677

ABSTRACT

During the dermal wound healing process, the mechanical rigidity of the newly deposited extracellular matrix and transforming growth factor-β1 promote the transition of fibroblasts into myofibroblasts. Myofibroblasts generate large cellular forces that contract and remodel the extracellular matrix leading to scar formation. In contrast, myofibroblasts are not detected in fetal dermal wounds which are more compliant and contain less transforming growth factor-β1 than adult wounds. Instead, fetal fibroblasts orchestrate scarless healing of dermal wounds resulting in healed tissues that resemble uninjured dermis. While these biomechanical differences suggest that the fetal wound environment promotes smaller cellular forces which enable regeneration, previous studies indicate that fetal fibroblasts have unique contractile properties that may facilitate scarless dermal repair. Therefore, we tested whether physiologic wound rigidities and transforming growth factor-β1 induce contractile forces and myofibroblast differentiation of fetal dermal fibroblasts. In comparison to their adult dermal counterparts, we found that fetal fibroblasts exhibit a deficient contractile response to rigid extracellular matrix and transforming growth factor-β1. Our data suggest that the contractile phenotype of fetal dermal fibroblasts limits their cellular force production and prevents their ability to differentiate into myofibroblasts.

During dermal repair, fibroblasts migrate into the wound bed and generate cellular tension via actomyosin contractility in response to increasing mechanical resistance during the development of granulation tissue.[1–3] These interactions are facilitated by focal adhesions which transmit these contractile forces to the extracellular matrix (ECM) as traction forces.[4,5] As these forces increase and focal adhesions mature in response to increasing ECM stiffness or rigidity, a mechanical threshold is reached in which fibroblasts can incorporate α-smooth muscle actin (α-SMA) into stress fibers and increase collagen production which are hallmarks of myofibroblast differentiation.[6–8] Myofibroblasts then utilize this contractile machinery rich in α-SMA to generate large traction forces which excessively contract and remodel the newly deposited, collagenous ECM leading to scarring.[8,9] In addition to mechanical stress, the cytokine transforming growth factor-β1 (TGF-β1) is also necessary to promote α-SMA expression and collagen synthesis.[8,9] Therefore, both biochemical and biomechanical factors work synergistically to regulate the transition to a myofibroblast phenotype and fibrotic healing.

In contrast to this postnatal or "adult" dermal repair, early gestational skin wounds in the mammalian fetus heal without scarring and are virtually indistinguishable from unwounded skin.[10,11] These fetal wounds are characterized by reduced levels of TGF-β1 and the absence of myofibroblasts.[11,12] In addition, fetal skin and wounds contain higher levels of type III vs. type I collagen resulting in a much more compliant mechanical tissue environment which is not as conducive to myofibroblast differentiation.[13,14] However, we previously found that mechanical stress generated by contracting fibroblasts in anchored type I collagen gels promotes the formation of stress fibers containing α-SMA in adult but not fetal fibroblasts.[15] Despite the differences in the fetal wound environment, these results suggest that fetal fibroblasts exhibit distinct responses to mechanical factors that limit their ability to differentiate into myofibroblasts.[9] Therefore, the goal of this study was to systematically test whether physiologic rigidities and TGF-β1 regulate the contractile and adhesive properties necessary for myofibroblast differentiation of fetal fibroblasts using a polyacrylamide gel (PAA) system that spans the mechanical properties reported for different wound healing stages.

MATERIALS AND METHODS

Cell culture and reagents

Three strains of primary human adult (18, 38, and 44 years from lots 6,921, 13,056, and 12,925, respectively) and fetal (18, 20, and 21 weeks from lots 5,343, 8,456, and 10,111, respectively) dermal fibroblasts were purchased from a commercial source (ScienCell, Carlsbad, CA) and cultured in Dulbecco's modified Eagle's medium supplemented with

10% fetal bovine serum (FBS; ThermoFisher, Waltham, MA).[15,16] Based on our culturing and usage, these primary cells remained proliferative for 4–5 passages over a ~10 day period before becoming senescent which was also promoted by refreezing. These observations were consistent with the manufacturer's information regarding their limited proliferation and inability to be refrozen. Therefore, pairs of adult fibroblasts and fetal fibroblasts were chosen randomly and used immediately in tandem for comparisons in multiple experiments performed in parallel as denoted before cells became senescent. Lyophilized porcine TGF-β1 (R&D Systems, Minneapolis, MN) was prepared as previously described.[17–19] To induce myofibroblast differentiation, cells were cultured for 4 days in growth medium with 10 ng/ml TGF-β1 (changed twice) prior to experimentation in which it was also included.[20] Human types I and III collagen were purchased as sterile solutions (Advanced Biomatrix, Carlsbad, CA).

Immunofluorescence

Immunostaining of vinculin and α-SMA was performed based on published protocols.[21] Briefly, vinculin was identified with a mouse monoclonal antibody (Millipore Sigma, Burlington, MA) after extraction of cell membranes and cytosolic compounds with supplemented cytoskeleton buffer and fixation with 3% paraformaldehyde. α-SMA was identified with a mouse monoclonal antibody (Agilent, Santa Clara, CA) after fixation with methanol. Vinculin and α-SMA were visualized with appropriate Alexa Fluor secondary antibodies (Life Technologies, Carlsbad, CA). Immunostaining of types I and III collagen at the surface of the rigid PAAs was performed as previously described for fibronectin.[22] Briefly, types I and III collagen were identified with rabbit polyclonal antibodies (Novus Biologicals, Littleton, CO, and ThermoFisher, respectively) after fixation with 4% paraformaldehyde. For all immunostaining, fluorescent images were captured on a Nikon Ti-E or TE2000-E inverted microscope with a Plan Fluor 40× or 60× oil immersion objective, respectively. Using vinculin staining, focal adhesion numbers and sizes were quantitated using the focal adhesion analysis server (FAAS) as previously described.[23] For α-SMA, average pixel intensity levels were quantitated from outlined areas of cells and background corrected using MetaMorph software (Molecular Devices). For types I and III collagen, average pixel intensity and standard deviation for each imaged area were quantitated using MetaMorph as previously described.[22]

PAA substrates

Soft, hard, and rigid fibronectin-conjugated PAAs with elastic moduli of 1,023, 7,307, and 22,692 Pa, respectively, were cast on activated coverslips of 35 mm MatTek dishes (MatTek, Ashland, MA) as previously described.[22–25] These PAAs were composed of 8%/0.05%, 8%/0.35%, and 12%/0.6% acrylamide/BIS ratios, respectively, as well as 0.1% *N*-hydroxysuccinimide ester which polymerizes into the polyacrylamide network and binds protein throughout the gels. We have previously verified that cells are exposed to the same relative amounts of fibronectin on the surfaces of these PAAs based on the use of increasing concentrations of fibronectin (200, 215, and 230 μg/ml, respectively).[22] For traction force assays, PAAs also contained 200 nm fluorescent beads which do not change their mechanical properties.[22,24,25] Cells were incubated on PAAs for 1 or 18 hours prior to immunostaining and/or traction force microscopy. To create rigid PAAs with either collagen at the same concentration as fibronectin (230 μg/ml) and with the same mechanical properties,[22,24] the amounts of acrylamide/BIS had to be adjusted to 10%/0.35 and 10%/0.40% for types I and III, respectively, as well as a one-fold increase in ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and exclusion of the fluorescent beads to hasten polymerization to avoid fibrillogenesis. The mechanical properties of the rigid collagen-conjugated PAAs were measured using rheometry as previously described.[22]

Traction force microscopy

As previously described,[22,24,25] cells were incubated in L-15 medium with 10% FBS for 1 hour then moved into a stage top incubator (Nikon, Melville, NY) on a Nikon inverted microscope. Images of cells and beads before and after cell removal were captured using a 40× Plan Fluor objective. Using the LIBTRC program, substrate displacements were determined based on the optical flow method, and the traction forces were calculated based on elasticity theory using the maximum likelihood method. The overall collective force exerted by a fibroblast on the PAA substrates is reported as the integral of the traction field over the cell area.

Statistics

Statistics were performed as previously described using SPSS Statistics (IBM, Armonk, NY).[22,23,25,26] Briefly, all data were evaluated for normality using the Shapiro–Wilk or Kolmogorov–Smirnov test then analyzed using either parametric or nonparametric methods with appropriate post hoc tests for groups. For normal data, a Student's *t* test was used for comparisons between two datasets, and a one-way ANOVA with multiple *t* tests and Bonferroni correction were used for pairwise comparisons within a group. For non-normal data, a Mann–Whitney test was used for comparisons between two datasets, and a Kruskal–Wallis test with Tamhane post hoc test or multiple Mann–Whitney tests with Bonferroni correction were used for pairwise comparisons within a group. A *p*-value less than 0.05 was considered significant.

RESULTS

ECM mechanical properties are sensed by integrin-based focal adhesions and cytoskeletal forces generated by actomyosin contractility.[27] We previously found that populations of human fetal dermal fibroblasts contract collagen gels less than their adult counterparts once mechanical stress develops in the gels.[15] Our work suggests that fetal fibroblasts have altered responses to ECM mechanical cues; therefore, we first tested whether mechanosensing by adult and fetal fibroblasts is differentially regulated by ECM rigidity. Since collagen gel contraction is mediated by actomyosin contractility, we performed traction force microscopy on adult and fetal fibroblasts cultured for 1 hour on soft, hard, and rigid PAAs conjugated with fibronectin to measure the early generation of contractile forces (Figure 1A). These substrates span the mechanical properties reported for different stages

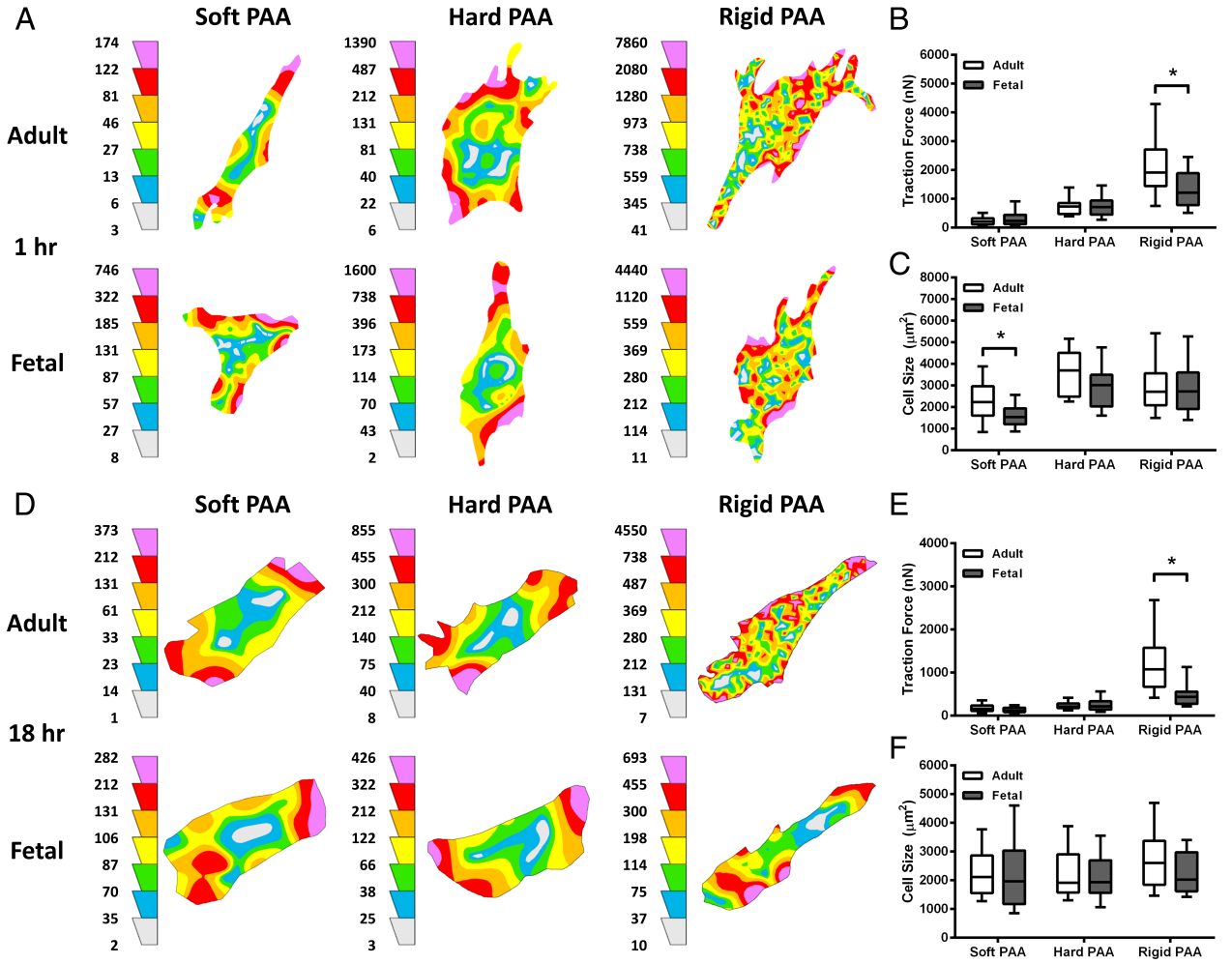

Figure 1. Fetal fibroblasts exert less contractile force than adult fibroblasts on rigid ECM. Representative traction maps with colors representing local traction stress levels of adult and fetal fibroblasts cultured for (A) 1 hour and (D) 18 hours on soft, hard, and rigid PAAs. Quantitation of the (B and E) integral of the traction field (i.e., collective force) per cell and (C and F) cell area in the traction force assays at 1 and 18 hours, respectively. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for (A–C) $n = 24–31$ cells from four independent experiments using lots 6,921 and 5,343 and (D–F) $n = 16–29$ cells from three independent experiments using lots 13,056 and 8,456.

of wound healing including the fibrin clot and the early and late phases of granulation tissue (i.e., formation and contraction) with the latter able to induce myofibroblast differentiation.[6–8,28] On soft and hard PAAs, there were no differences in force generation between adult and fetal fibroblasts (Figure 1B). However, adult fibroblasts exerted larger traction forces on rigid PAAs (Figure 1B) despite being the same size as fetal fibroblasts (Figure 1C). To determine whether these differential responses to ECM rigidity were stable over longer time periods, we repeated these experiments with adult and fetal fibroblasts that were cultured for 18 hours on the soft, hard, and rigid PAAs (different strains than Figure 1A–C). Using traction force microscopy, we once again found that adult fibroblasts exerted larger traction forces than fetal fibroblasts on rigid PAAs even though their cell sizes were the same (Figure 1D–F).

As our data indicated differences in force generation between adult and fetal fibroblasts, we also evaluated the number of adhesions formed by these cells at the same time points (Figure 2A and D). Vinculin was used as a marker for adhesions since it is present at all stages of maturation.[8] Focal adhesion sizes were divided based on area in to immature (<2 μm^2 with a minimum size of 0.5 μm^2) and mature (>2 μm^2).[29] Similar to the traction forces (same strains as Figure 1A–C), we did not observe a difference in the number or types of adhesions in either adult or fetal fibroblasts on the soft and hard PAAs at the early time point (Figure 2B and C). However, adult fibroblasts did express more mature focal adhesions on rigid PAAs (Figure 2C). Over the longer time period (same strains as Figure 1D–F), we observed that adult fibroblasts formed more focal adhesions on both hard and rigid PAAs which coincided with

increases in the number of immature and mature adhesions (Figures 2E and F). In a third strain of each cell type, we also found that traction forces and focal adhesion numbers (total, immature, and mature) were higher in adult fibroblasts when compared to fetal fibroblasts on rigid PAAs after overnight incubation (Supporting Information Figure S1 and the rigid PAAs with FN in Figure 6F–H).

TGF-β1 is a major regulator of myofibroblast differentiation and works synergistically with ECM rigidity to promote this phenotype.[6,8,9] As the rigid PAAs fall within the mechanical range for inducing myofibroblast differentiation and led to differences in force production (Figure 1B and E),[6–8,28] we treated adult and fetal fibroblasts with TGF-β1 for 4 days and once again evaluated traction force generation and focal adhesion formation on rigid PAAs (same strains as Figures 1D–F and 2D–F). TGF-β1 treatment resulted in an increase in traction forces exerted by adult fibroblasts but not fetal fibroblasts without any change in cell size (Figure 3). Furthermore, the total number of focal adhesions as well as the number of immature and mature adhesions increased only in adult fibroblasts in response to exogenous TGF-β1 (Figure 4). To verify that these changes in traction force production and adhesion formation were a result of myofibroblast differentiation, we evaluated the levels of α-SMA in adult and fetal fibroblasts treated with TGF-β1 on the rigid PAAs using quantitative immunofluorescence. In contrast to fetal fibroblasts, TGF-β1 and ECM rigidity induced the formation of stress fibers rich in α-SMA and an overall increase in α-SMA levels in adult fibroblasts (which were not present in the control case despite the use of growth medium; Figure 5).

Fetal skin and wounds are composed of more type III than type I collagen which contributes to their compliant nature.[13,14,30] In addition, these collagens also have different binding motifs,[31,32] and fetal fibroblasts have been shown to express lower levels of integrin subunits associated with collagen binding.[33] These factors can alter cellular adhesion

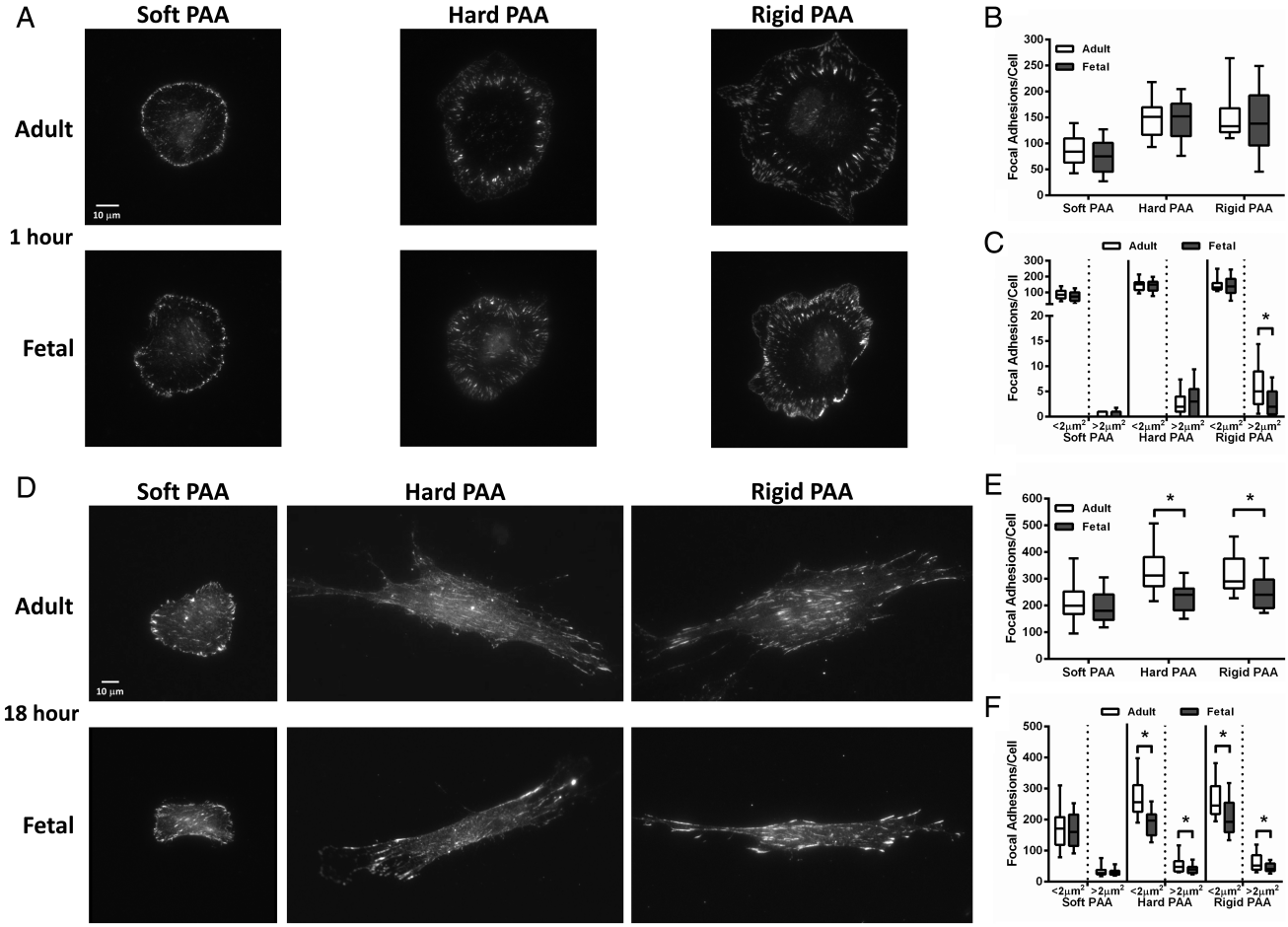

Figure 2. Focal adhesion formation and maturation in adult but not fetal fibroblasts is promoted by ECM rigidity. Representative wide-field immunofluorescence images of focal adhesions in adult and fetal fibroblasts on soft, hard, and rigid PAAs after (A) 1 hour and (D) 18 hours. Focal adhesions were identified with vinculin immunostaining. Quantitation of the (B and E) number of total focal adhesions per cell which were divided into (C and F) immature (<2 μm^2) and mature (>2 μm^2) from vinculin immunostaining at 1 and 18 hours, respectively. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for (A–C) $n = 25$ cells from two independent experiments using lots 6,921 and 5,343 and (D–F) $n = 23$–27 cells from three independent experiments using lots 13,056 and 8,456. Scale bar represents 10 μm.

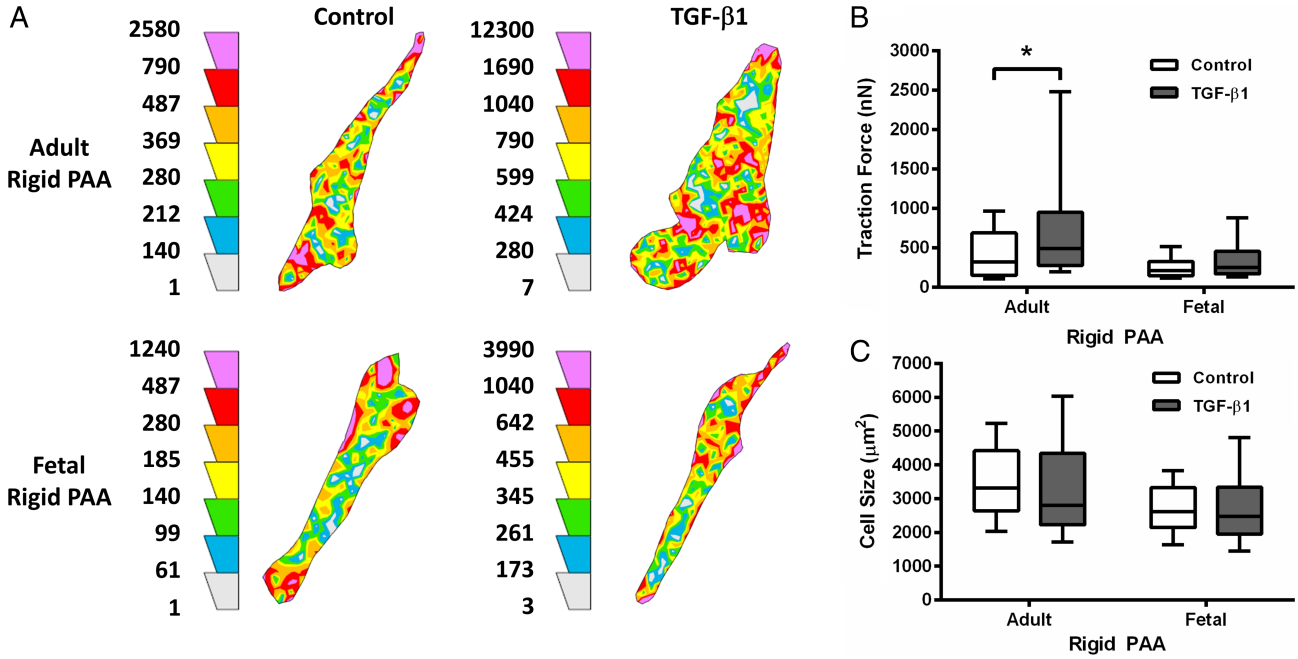

Figure 3. TGF-β1 enhances traction force generation in adult but not fetal fibroblasts. (A) Representative traction maps with colors representing local traction stress levels of adult and fetal fibroblasts cultured for 18 hours on rigid PAAs after 4 days of treatment with 4 mM HCl solution containing 0.1% bovine serum albumin (control) or 10 ng/ml TGF-β1. Quantitation of the (B) integral of the traction field (i.e., collective force) per cell and (C) cell area in the traction force assays without and with TGF-β1 treatment. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for $n = 34–49$ cells from four independent experiments using lots 13,056 and 8,456.

and contractile ability.[33,34] Therefore, we first tested whether these ECM components differentially affected the formation of focal adhesions while keeping rigidity constant. To test the role of these collagens, we modified our protocol for creating rigid PAAs with types I and III collagen instead of fibronectin but at the same concentration.[22–25] To achieve relatively uniform distributions of collagens at the surface and similar PAA mechanical properties, we had to decrease the polymerization time and adjust the amounts of PAA components (see Materials and Methods and Figure 6A–E). Using these rigid PAAs with different ECM proteins and a third strain of each cell type, adult fibroblasts formed more total, immature, and mature focal adhesions than fetal fibroblasts regardless of ECM composition (Figure 6F–H). To verify that these differences were not due to alterations in phenotype given the use of growth medium, we once again evaluated α-SMA levels with quantitative immunofluorescence. In basal conditions, α-SMA was only diffusely expressed throughout the cells as before (Figure 5A) with no differences in levels for either fibroblast type (Figure 6I).

DISCUSSION

As ECM rigidity and TGF-β1 increase in the wound bed, traction forces increase in magnitude as focal adhesions mature and actin filaments bundle into stress fibers containing α-SMA to promote myofibroblast differentiation.[3,8] Although fetal wounds are more compliant than adult wounds,[13,14] our previous data suggest that fetal fibroblasts have altered responses to ECM mechanical cues.[15] Therefore, we tested whether physiologic rigidities and TGF-β1 could induce a mechanical response from fetal fibroblasts necessary for myofibroblast differentiation using substrates with mechanical properties similar to different wound healing stages. We have found that traction forces and focal adhesion formation is inhibited in fetal fibroblasts on rigid PAAs that mimics late-stage granulation tissue. TGF-β1 treatment promoted myofibroblast differentiation of adult fibroblasts on rigid PAAs but had little effect on fetal fibroblasts. Overall, these results suggest that fetal fibroblasts have a distinct contractile phenotype that hampers myofibroblast differentiation.

We evaluated the response of adult and fetal fibroblasts at both short and long time points to assess the development and stability of their contractile phenotype. Over a short duration, we found no phenotypic differences in their ability to generate contractile forces or form focal adhesions on the soft and hard PAAs. The mechanical properties of these two substrates correspond to the initial stage of the wound healing process when clots are formed (E~100–1,000 Pa) and the early stage of granulation tissue formation in which fibroblasts have not yet converted to fully differentiated myofibroblasts containing α-SMA (E~3,000–18,000 Pa).[8,28] In contrast, fetal fibroblasts generated less force on rigid PAAs which corresponds to the mechanical properties of

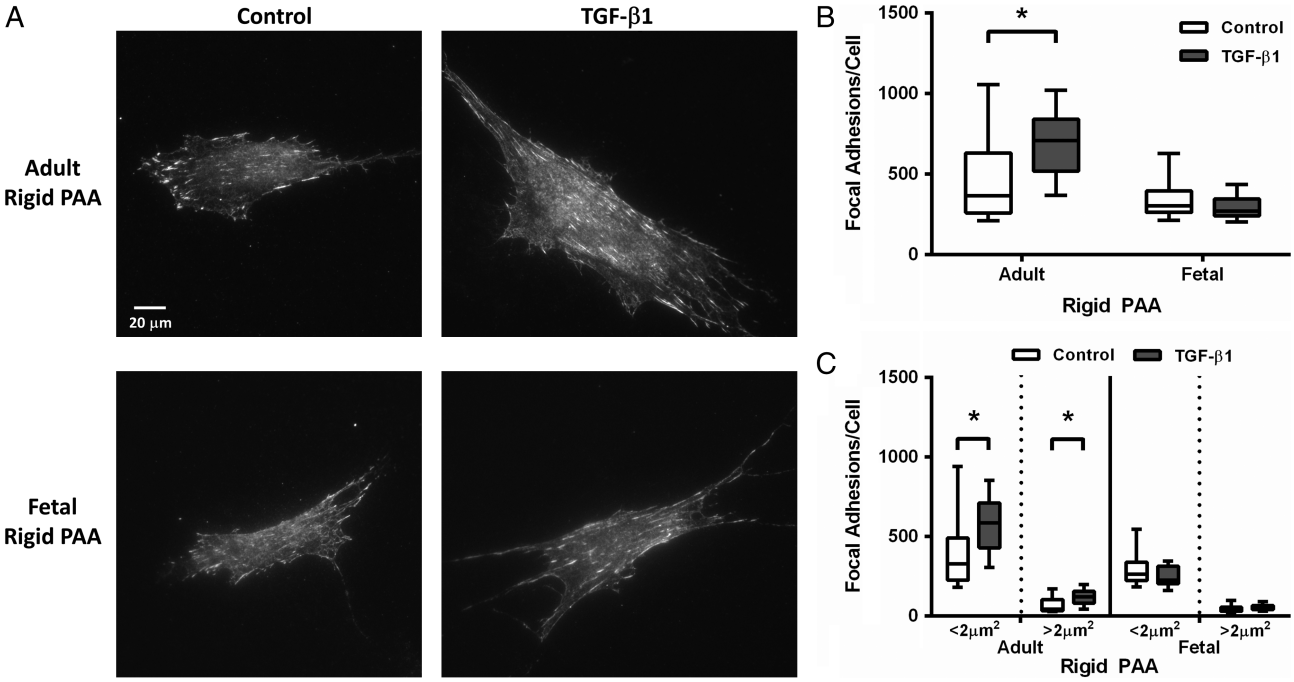

Figure 4. The number of focal adhesions increase with TGF-β1 in adult but not fetal fibroblasts. (A) Representative wide-field immunofluorescence images of focal adhesions in adult and fetal fibroblasts cultured for 18 hours on rigid PAAs after 4 days of treatment with 4 mM HCl solution containing 0.1% bovine serum albumin (control) or 10 ng/ml TGF-β1. Focal adhesions were identified with vinculin immunostaining. Quantitation of the (B) number of total focal adhesions per cell which were divided into (C) immature (<2 μm^2) and mature (>2 μm^2) from vinculin immunostaining. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for $n = 23–29$ cells from three independent experiments using lots 13,056 and 8,456. Scale bar represents 20 μm.

late-stage granulation tissue (E > 20,000 Pa) that is stiff enough to induce myofibroblast differentiation in wound healing conditions.[6–8] While we found no difference in the total number of focal adhesions on rigid PAAs, these force differences did coincide with less mature focal adhesions in fetal fibroblasts despite similar cell sizes. These results suggest that the mechanical response of fetal fibroblasts to ECM rigidity is impaired compared to adult fibroblasts

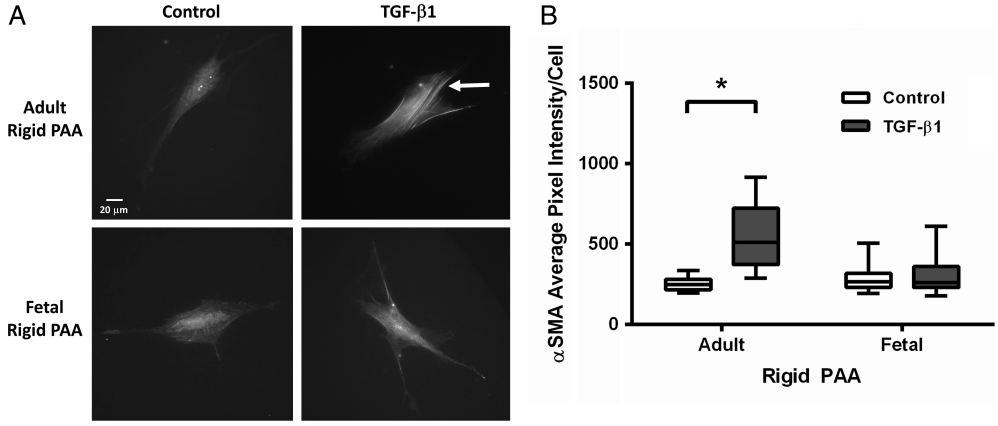

Figure 5. Levels of α-SMA increase in adult but not fetal fibroblasts in response to TGF-β1. (A) Representative wide-field immunofluorescence images of α-SMA in adult and fetal fibroblasts cultured for 18 hours on rigid PAAs after 4 days of treatment with 4 mM HCl solution containing 0.1% bovine serum albumin (control) or 10 ng/ml TGF-β1. (B) Quantitation of α-SMA average pixel intensities with and without TGF-β1 treatment. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for $n = 40–52$ cells from four independent experiments using lots 13,056 and 8,456. Scale bar represents 20 μm.

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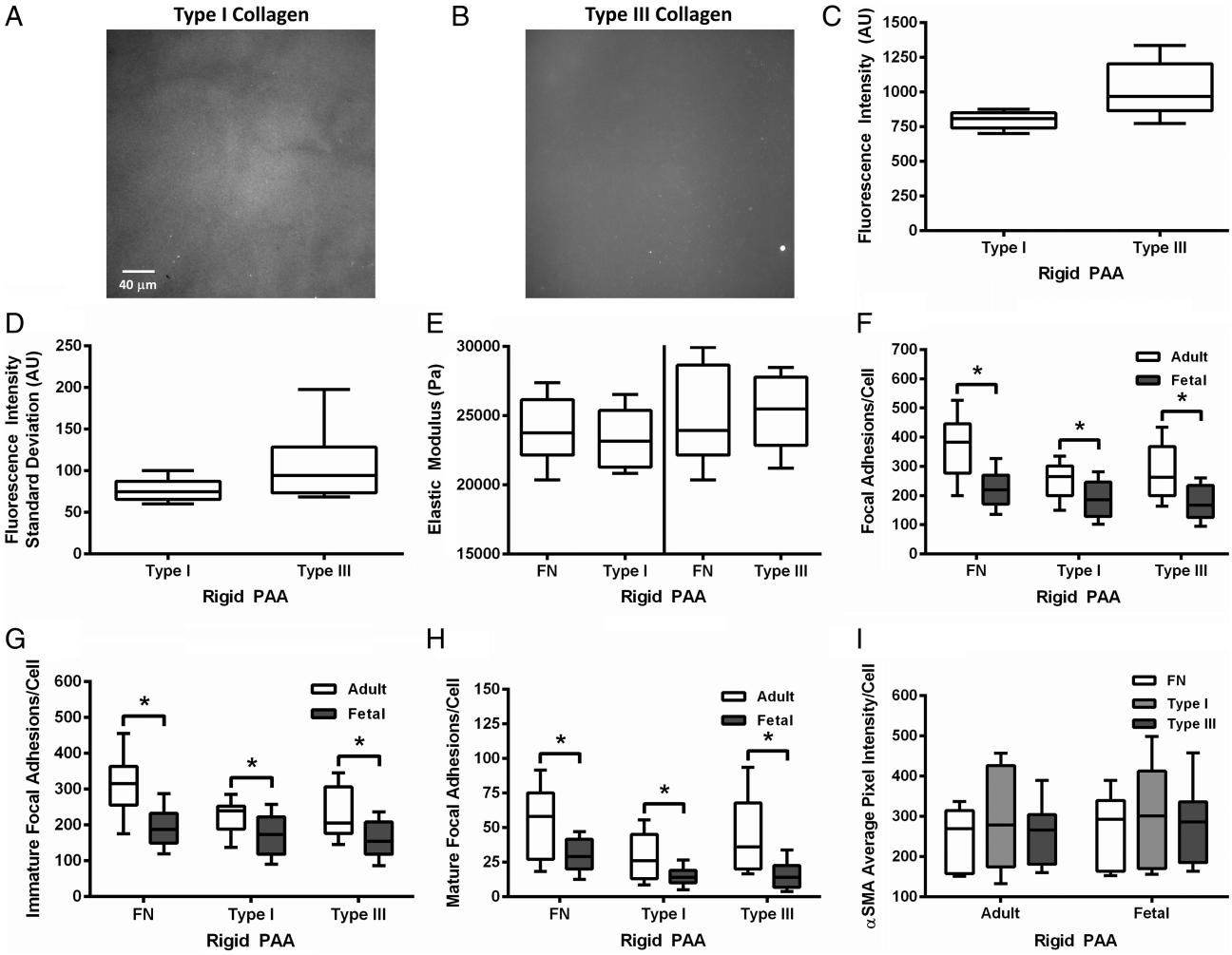

Figure 6. Fibronectin induces more focal adhesions than types I and III collagen. (A) Type I and (B) type III collagen were incorporated into rigid PAAs yielding uniform coatings on the substrate surfaces as identified by immunostaining with representative wide-field immunofluorescence images shown. Quantitation of (C) the average fluorescence intensities and (D) their standard deviations of types I and III collagen at the surfaces of rigid PAAs. (E) The elastic moduli of the rigid PAAs with types I and III collagen were similar to those with fibronectin and calculated based on measurements of the shear elastic moduli (G′) using rheometry at a constant strain of 0.05% and frequency of 1 Hz assuming E = 3G′ and a Poisson's ratio of 0.5. Quantitation of the (F) number of total focal adhesions per cell which were divided into (G) immature (<2 μm^2) and (H) mature (>2 μm^2) from vinculin immunostaining of adult and fetal fibroblasts on rigid PAAs with fibronectin, type I collagen, and type III collagen. (I) Quantitation of α-SMA average pixel intensities from α-SMA immunostaining of adult and fetal fibroblasts on rigid PAAs with fibronectin, type I collagen, and type III collagen. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for $n = 60$ areas from three independent experiments for (C) and (D), $n = 7–8$ gels from two independent experiments for (E), $n = 22–26$ cells from three independent experiments for (F), (G), and (H), and $n = 30$ cells from three independent experiments for (I) using lots 12,925 and 10,111. Scale bar represents 40 μm.

which is consistent with our previous finding in stressed collagen gels.[15]

Over a longer duration, the phenotypic differences between adult and fetal fibroblasts became even more apparent. Fetal fibroblasts formed less total and mature adhesions on both hard and rigid PAAs. While these differences did not yield changes in the traction forces generated on the hard PAA, fetal fibroblasts generated far less force on rigid PAAs despite similar cell sizes once again. While further experiments using live cell imaging would be required, these data suggest that there are potentially significant differences in adhesion maturation and turnover which are likely force dependent. Regardless, the increases in mature adhesions and traction forces suggest that adult fibroblasts have transitioned to a proto-myofibroblast phenotype.[8] This phenotype represents an intermediate phase between fibroblasts and

fully differentiated myofibroblasts that occurs on stiffer substrates (E > 3,000 Pa) that do not yet induce the cellular forces necessary for expression and incorporation of α-SMA into stress fibers.[8,28] Such a transition would be consistent with the absence of α-SMA positive stress fibers that were observed on rigid PAAs in control conditions (i.e., lacking active TGF-β1). Therefore, fetal fibroblasts appear to lack the ability to generate the mechanical stress necessary to begin the transition to a myofibroblast phenotype which occurs even before the threshold necessary for full differentiation.

In addition to ECM rigidity, TGF-β1 is also necessary to promote full myofibroblast differentiation.[6,8,9] In contrast to adult fibroblasts, fetal fibroblasts showed no changes in traction forces, focal adhesions, or α-SMA with TGF-β1 treatment on rigid PAAs. Although fetal fibroblasts have been shown to express similar levels of TGF-β receptor and Smad mRNA as adult fibroblasts, early studies also found that fetal fibroblasts do not differentiate into myofibroblasts or only briefly when treated with TGF-β1 in collagen gels or in culture.[11] These results are consistent with observations in vivo that myofibroblasts are not present or only transiently expressed during fetal dermal wound healing.[35,36] While exogenous TGF-β1 has been shown to induce α-SMA expression in cells of fetal wound tissues resulting in fibrotic healing, this increase can occur in a variety of mesenchymal cell types that can be recruited to wounds and differentiate into myofibroblasts including smooth muscle cells.[37] A recent study has shown stable expression of myofibroblast-associated genes in fetal fibroblasts seeded in collagen matrices with TGF-β1 stimulation.[38] However, the collagen matrices used in that study were sponge scaffolds composed of porcine elastin and collagen with a longitudinal pore structure that has been engineered to have higher tensile strength than other dermal equivalents for surgical wound applications suggesting relatively high mechanical properties. In contrast, we have found that TGF-β1 does not induce myofibroblast differentiation of fetal fibroblasts in a physiologically relevant mechanical environment. Furthermore, our findings with adult fibroblasts are consistent with other reports using different types of substrates that mimic the same range of mechanical properties used in our study.[6,8,28]

One noted difference in our study compared to in vivo wounds regarding the ECM is the exclusive use of fibronectin in our PAA substrates. While fibronectin is a ubiquitous protein present in wounds which engages β3 integrins,[11,34] adult and fetal skin and wounds are also characterized by different levels of types I and III collagen which affect wound mechanical properties.[13,14,30] These fibrillar collagens bind β1 integrins which are also responsible for exerting cellular forces[39]; however, they engage different integrin-binding motifs that affect fibroblast adhesion which may be important for force generation and myofibroblast differentiation.[31,32,34] As forces are transduced through and promote the maturation of focal adhesions, we tested whether these collagens affected the adhesion properties of adult and fetal fibroblasts. To incorporate types I and III collagens into the rigid PAAs with the same mechanical properties, we had to alter our protocol including a reduction in the polymerization time to inhibit fibrillogenesis to get an even molecular distribution throughout the gels and on the surface. Despite these adjustments, we were not able to include beads for traction force measurements as they promoted fibrillogenesis even during shorter polymerization times. However, we did find that adult fibroblasts formed more focal adhesions than fetal fibroblasts on the rigid PAAs regardless of the type of ECM protein suggesting that these phenotypic differences are primarily a result of ECM rigidity.

While our findings are consistent with our previous work and other studies both in vitro and in vivo, we do acknowledge some limitations. Replication of different experiments with multiple cell strains was limited based on the relatively short period in which the primary fibroblasts remained proliferative. However, several key results were confirmed in more than one strain of adult and fetal fibroblasts at multiple time points including traction force generation (rigid PAAs in Figure 1B and E and Supporting Information Figure S1A), focal adhesion formation (rigid PAAs with fibronectin in Figures 2 and 6F–H), and α-SMA levels (rigid PAAs with fibronectin in Figures 4 and 6I). Experiments using the same cell strains and conditions were performed in parallel to minimize phenotypic drift. In addition, we also focused only on individual cells to evaluate their phenotypic characteristics. Furthermore, experiments were performed in a 2D environment; however, contractility in this manner has been shown to correlated with cellular forces in 3D due to similar mechanisms required for contractile force generation.[40]

In this study, we have found that fetal fibroblasts have inherently different mechanical responses to physiologic rigidities which may be important in understanding their lack of myofibroblast differentiation and ability to promote scarless healing.[9] Further studies are required to understand the molecular mechanisms that regulate their mechanical phenotype which may yield new therapeutic targets to inhibit myofibroblast differentiation by adult fibroblasts and thus reduce scarring. Such interventions would be beneficial to a wide variety of conditions and diseases that are driven by mechanical signaling resulting in tissue fibrosis from fibroblast activity.

ACKNOWLEDGMENTS

We would like to thank Alissa Weaver and Scott Guelcher for the use of equipment in their laboratories and Boris Hinz of the University of Toronto for assistance with myofibroblast induction and staining. We would also like to thank Micah Dembo of Boston University for licensing of the LIBTRC software and his guidance for its use as well as Matthew Berginski of the University of North Carolina (Gomez laboratory) for his assistance with the FAAS.

Source of Funding: We acknowledge the support provided by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award number R03AR066875 to AP. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

Conflict of Interest: The authors declare that there is no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. ECM rigidity differentially regulates contractile force generation by an additional adult and fetal fibroblast strain on the rigid PAA. Quantitation of the (A) integral of the traction field (i.e., collective force) per cell and (B) cell area in the traction force assays of adult and fetal fibroblasts cultured for 18 hours on soft, hard, and rigid PAAs. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating $p < 0.05$ for $n = 25–27$ cells from three independent experiments using lots 12,925 and 10,111.

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