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Dermal Fibroblasts from the Red Duroc Pig Have an Inherently Fibrogenic Phenotype: An *in vitro* Model of Fibroproliferative Scarring

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Abstract

Background—The pathophysiology of hypertrophic scarring is unknown due in part to the lack of a robust animal model. Although the red Duroc pig has emerged as a promising *in vivo* model, the cellular mechanisms underlying Duroc scarring are unknown, and the size and cost of Duroc pigs are obstacles to their use. Given the central role of the dermal fibroblast in scarring, we hypothesized that dermal fibroblasts from the Duroc pig exhibit intrinsic differences in key aspects of the fibroblast response to injury compared to those from the Yorkshire pig, a same-species control that heals normally.

Methods—Duroc and Yorkshire dermal fibroblasts were isolated from uninjured dorsal skin. Actin stress-fibers and focal adhesions were visualized by immunocytochemistry and transmission electron microscopy. Cell migration was measured using a scratch wound-closure assay. Contractile function was assessed by collagen-gel contraction. Expression of scarring-related

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Author Contributions

- RFS contributed to experimental design and coordination, carried out experiments, analyzed and interpreted data, and helped to draft the manuscript.
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- MES carried out experiments, analyzed and interpreted data, and helped to draft the manuscript.
- MG carried out experiments and analyzed data.
- PS carried out experiments and analyzed data.
- AMH contributed to experimental design and coordination, carried out experiments, and analyzed and interpreted data.
- NSG conceived of the study, contributed to experimental design and coordination, and helped to draft the manuscript.
- All authors read and approved the final manuscript.

genes was determined by quantitative RT-PCR, and transforming growth factor beta 1 (TGF- β 1) protein expression was determined by Western blotting.

Results—Duroc dermal fibroblasts display increased adhesion-complex formation, impaired migration, enhanced collagen contraction, and pro-fibrotic gene- and protein-expression profiles compared to Yorkshire fibroblasts at baseline. In addition, Duroc fibroblasts over-expressed TGF- β 1 and were less responsive to exogenous TGF- β 1.

Conclusions—Duroc dermal fibroblasts have inherent myofibroblastic differentiation that may account for the pathologic scarring in these animals. Our data further validate the Duroc model and support Duroc fibroblast cell culture as a simple, inexpensive, reproducible, and biologically tractable *in vitro* model for the study of fibroproliferative scarring.

Introduction

Hypertrophic scar (HTS) forms following partial-thickness burns and other deep-dermal wounds, representing a distressing complication for patients. This highly debilitating proliferative response to injury, estimated to occur following 32-72% of burns (1), results in raised scar within the boundaries of the original wound (2). Clinically, it is associated with pruritus, pain, functional impairment, disfigurement, psychological morbidity, and decreased quality of life (3). Development of effective therapies has been limited by incomplete understanding of HTS pathophysiology (4) due in part to lack of a validated animal model (5).

During the past decade, the red Duroc pig has emerged as a promising HTS model. After deep partial-thickness wounding, Duroc pigs form thick, contracted, hyperpigmented scars resembling HTS at the gross, histologic, and molecular levels (6). In contrast, Yorkshire pigs heal normally, serving as a same-species control (7). However, the considerable size and cost (8) of pigs as well as the desire of the scientific community to minimize suffering of research animals represent obstacles to use of the Duroc porcine model. Hence, a less resource-intensive *in vitro* Duroc fibroblast model would be advantageous for studying the fibroproliferative mechanisms underlying the pathologic scarring prior to pre-clinical studies in the Duroc pig.

Current understanding of HTS pathophysiology centers on the dermal fibroblast (9). After cutaneous injury, fibroblasts migrate from adjacent dermis and are stimulated by local factors including mechanical tension to differentiate into proto-myofibroblasts (10). Transforming growth factor beta 1 (TGF- β 1), released by multiple cell types including fibroblasts (11, 12), mediates full myofibroblastic differentiation, characterized by alpha-smooth muscle actin (α -SMA) expression, elongated, “supermature” focal adhesion formation, collagen-rich extracellular matrix (ECM) deposition, and exertion of contractile force (13). We hypothesized that dermal fibroblasts from the Duroc pig would exhibit an intrinsically fibrogenic phenotype and serve as *in vitro* model for mechanistic studies of fibroproliferative scarring.

Materials and Methods

Porcine-fibroblast isolation and culture

The University of Washington Animal Care and Use Committee approved all animal procedures. Female Duroc (Stein & Stewart Genetics, Odessa, MO) and Yorkshire (Washington State University Swine Center, Pullman, WA) pigs were purchased for unrelated medical research. Porcine fibroblasts were isolated from uninjured dorsal skin of Duroc pigs at 9-13 weeks old and Yorkshire pigs at approximately 16 weeks old. Because fibroblast phenotypes vary with dermal depth (14), we isolated pooled fibroblasts from full-thickness skin samples. This approach simplifies our model and enhances reproducibility by avoiding technical variability associated with selective isolation of fibroblasts from a specific dermal layer. Excised full-thickness dorsal skin samples were washed in phosphate-buffered saline (PBS) with 1% antibiotic/antimycotic solution. Subcutaneous fat was excised, and ~2mm² skin pieces were digested in 2U/mL dispase overnight at 4°C. The dermal sheet was separated from the epidermis, minced, and digested in 200U/mL collagenase for 2 h at 37°C, passed through a 70 µm mesh cell strainer, and centrifuged. Re-suspended cells were seeded at 5×10^3 cells/cm² and grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum (Atlantic Biological, Lawrenceville, GA) and 1% antibiotic/antimycotic solution at 37°C with 5% CO₂. Media were changed every 2 days, and fibroblasts were utilized between passages 5 and 8 for all experiments. Since we and others (15) have observed poor porcine fibroblast viability in serum-free media, all experiments were performed in medium supplemented with 10% fetal bovine serum. Experiments were performed $\pm$ TGF- β 1 (10 ng/mL; PeproTech, Rocky Hill, NJ). Unless noted, cell-culture reagents were obtained from Life Technologies (Carlsbad, CA).

Cell proliferation

Duroc and Yorkshire fibroblasts were grown in 6-well plates for 72 hours, serum-starved overnight for cell-cycle synchronization, and incubated in complete medium $\pm$ TGF- β 1 for 48 hours. Viable cells were counted using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Fullerton, CA).

Cell migration

Dermal-fibroblast migration was measured using our established scratch wound closure assay (16). Briefly, cells were grown in pre-etched 6-well plates to approximately 95% confluence. The monolayer was scratched using a p200 micropipette tip, rinsed with growth medium to remove cellular debris, and incubated in complete medium $\pm$ TGF- β 1. Images were taken immediately pre- and post-scratch and at 24, 48, and 72 hours post-scratch. Two images were obtained per well, using the etched pattern to capture precisely the same areas at each time point. Scratch area was measured serially by image analysis.

Collagen contraction

Dermal fibroblasts were seeded at 5×10^5 cells/well in rat-tail collagen type I (1.86 mg/mL) in 24-well plates. Cell-collagen solutions were prepared on ice, pH-adjusted with sodium

hydroxide, and buffered with PBS in a final volume of 0.5 mL/well; experimental gels contained TGF- β 1. Plates were incubated at 37°C for 1 hour to allow gel polymerization and 1 mL of warm medium $\pm$ TGF- β 1 was added to each well. The seeded gels were incubated overnight to allow stress-fiber formation and then gently released from the edges and bottom of each well to allow contraction. Images were taken immediately pre- and post-release and at 6, 24, 48, and 72 hours post-release. Gel area was measured serially by image analysis.

Immunofluorescence microscopy

Dermal fibroblasts $\pm$ TGF- β 1 were grown on 4-well chamber slides for 4 days. Cells were fixed for 20 minutes with 4% paraformaldehyde, permeabilized for 10 minutes with 0.05% Triton X-100 in PBS, and blocked for 1 h in 1.3% normal goat serum and 0.1% bovine serum albumin in PBS. Cells were incubated overnight at 4°C with primary antibody and washed with PBS prior to a 30-minute incubation with secondary antibody. Cells were washed and incubated for 20 min with Alexa Fluor® 555 phalloidin (1:80; Life Technologies, Grand Island, NY) to stain the actin cytoskeleton. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted using ProLong Gold Antifade Reagent (Life Technologies, Grand Island, NY). Digital images were captured using a QICAM Fast 1394 digital camera (QImaging, Surrey, BC) mounted on an upright Eclipse 80i microscope (Nikon, Melville, NY) with QCapture Suite version 3.1.3.10 (QImaging, Surrey, BC). Images were unaltered other than standardized adjustment of brightness and contrast using Adobe Photoshop CS5. For focal-adhesion complexes, the primary antibody was a rabbit monoclonal anti-vinculin IgG (1:1000; Life Technologies, Grand Island, NY), and the secondary was goat anti-rabbit IgG conjugated with Alexa Fluor® 488 (1:1000; Life Technologies, Grand Island, NY). For α -SMA, the primary antibody was a mouse monoclonal anti- α -SMA IgG (1:1000; Sigma-Aldrich, St. Louis, MO), and the secondary was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA).

Image analysis

All image analysis was performed using Adobe Photoshop CS5 with the Fovea Pro plug-in (Reindeer Graphics, Asheville, NC). To measure focal adhesions, 20 $\times$ monochrome (grayscale) images of the vinculin staining were imported into Photoshop and converted to black-and-white binary images. Individual focal-adhesion length was measured and averaged for each condition. Quantification of α -SMA expression was based on 10 $\times$ images. Total FITC signal was normalized to cell number, determined by DAPI-stained nuclei.

Transmission electron microscopy

Cells cultured in 35 mm² dishes in complete medium $\pm$ TGF- β 1 for 4 days were fixed in half-strength Karnovsky fixative overnight at 4°C, washed with 0.1M cacodylate buffer, and post-fixed in a 1:1 solution of 2% osmium tetroxide and 0.2M cacodylate buffer. After another wash in buffer, cells were dehydrated with an ascending series of ethanol washes, including a propylene oxide step, and embedded in Epon 812. Inverted beam capsules were utilized in the embedding process, and 1 μ m sections on slotted grids were stained with uranyl acetate and lead citrate. Sections were viewed on a TEM 1400 transmission electron microscope (JEOL USA, Peabody, MA). Images were captured at 5000 $\times$ using

DigitalMicrograph version 2.1 (Gatan Inc., Pleasanton, CA) and presented without alteration.

Gene expression

Fibroblasts were grown in 6-well plates with complete medium $\pm$ TGF- β 1 for 4 days. RNA was isolated with Trizol (Life Technologies, Carlsbad, CA) followed by DNase digestion on a PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA). For cDNA, 500 ng RNA was reverse transcribed using an Omniscript RT Kit (Qiagen, Valencia, CA). The cDNA was subjected to quantitative real-time reverse-transcriptase-PCR (RT-PCR) in a Viia7 instrument (Applied Biosystems, Foster City, CA). Primer sequences follow:

α -SMA,

forward: 5'-GTG TGA GGA AGA GGA CAG CA-3';

reverse: 5'-TAC GTC CAG AGG CAT AGA GG-3';

alpha-1 type I collagen,

forward: 5'-GTG GCC CAG AAG AAC TGG TA-3';

reverse: 5'-CGC CAT ACT CGA ACT GGA AT-3';

decorin,

forward: 5'-TAG AAC TTG GCA CCA ACC CG-3';

reverse: 5'-AAT GTT GGT GTC AGC GAT GC-3'.

A dissociation curve was generated for each primer set to ensure amplification of a single specific product. The comparative C_t method (17) was used to quantify gene expression (with untreated Yorkshire fibroblasts as the reference) after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data are reported as mRNA fold change.

TGF- β 1 protein expression

Duroc and Yorkshire fibroblasts were grown in complete medium $\pm$ TGF- β 1 in 6-well plates for 4 days. Cell lysates were resolved by SDS-PAGE and proteins were transferred from the SDS-PAGE gel to a nitrocellulose membrane and incubated overnight with rabbit anti-TGF- β 1 antibody (1:1000; Thermo Scientific, Rockford, IL) at 4°C. The membrane was incubated with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG secondary antibody (1:2000; Santa Cruz Biotechnology, Dallas, TX). The stripped membrane was incubated with mouse anti-GAPDH primary antibody (1:5000; Millipore, Darmstadt, Germany) followed by goat-anti-mouse IgG-HRP (1:2000; Santa Cruz Biotechnology, Dallas, TX). Protein bands were detected using the ECL Western Blotting Analysis System (Sigma-Aldrich, St. Louis, MO), and TGF- β 1 levels were quantified and normalized to GAPDH using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

All experiments were repeated at least twice in cell isolates from the same Duroc and Yorkshire pigs. Proliferation, collagen contraction, and α -SMA, alpha-1 type I collagen, and

decorin gene expression were repeated in fibroblasts derived from different Duroc and Yorkshire pigs in order to confirm consistency of results across biological replicates. Data are presented as mean $\pm$ standard deviation of technical replicates within a single representative experiment. The Student's *t*-test was used to determine differences between conditions, with $P < 0.05$ considered statistically significant.

Results

Duroc dermal fibroblasts demonstrate increased adhesion and stress-fiber formation

After isolation and *in vitro* expansion, Duroc dermal fibroblasts had a flattened, spread appearance with numerous cellular processes and were resistant to trypsinization (data not shown), suggesting an adhesive phenotype. Vinculin and actin immunostaining to visualize focal-adhesions, contact points between the cellular cytoskeleton and ECM (13), demonstrated marked differences in actin stress-fiber formation between Duroc and Yorkshire fibroblasts at baseline; Duroc fibroblasts showed prominent stress fibers, which were largely absent in the Yorkshire cells (Fig. 1a). Duroc fibroblasts also had notably longer focal adhesions than Yorkshire fibroblasts (Fig. 1b). TGF- β 1 increased both stress-fiber formation and focal-adhesion length in Yorkshire fibroblasts but did not have an appreciable effect on Duroc fibroblasts. Using image analysis, we calculated the fraction of supermature ($> 6 \mu\text{m}$) focal adhesions, which increases with myofibroblastic differentiation (18). The baseline supermature focal-adhesion fraction was higher in Duroc versus Yorkshire fibroblasts, and TGF- β 1 increased the supermature focal-adhesion fraction in Yorkshire but not Duroc fibroblasts (Fig. 1c).

Transmission electron microscopy (TEM) (Fig. 2) confirmed numerous adhesion complexes in Duroc but not Yorkshire fibroblasts at baseline. These structures correspond to fibronexus adhesion complexes characteristic of myofibroblasts in uninjured tissue (19, 20) and hypertrophic scar (21). After TGF- β 1 treatment, adhesion-complex formation increased in Yorkshire but not Duroc fibroblasts. These data suggest that Duroc dermal fibroblast adhesion properties are consistent with a differentiated myofibroblast state.

Duroc dermal fibroblasts have impaired migration

Using a wound-closure assay in which fibroblasts were serially imaged as they migrated to close a scratch made in the cell monolayer, we observed significantly slower scratch closure and notably fewer individual migrating cells in the center of the scratch area in Duroc compared to Yorkshire fibroblasts (Fig. 3). TGF- β 1 slowed scratch closure in both cell types, but to a lesser degree in Duroc fibroblasts. Complete scratch closure occurred by day 3 in untreated Yorkshire fibroblasts, day 4 in TGF- β 1-treated Yorkshire fibroblasts, day 6 in untreated Duroc fibroblasts, and day 7 in TGF- β 1-treated Duroc fibroblasts.

As both cellular proliferation and migration contribute to scratch closure, we measured proliferation and did not detect a significant difference in the number of viable Duroc (1.1×10^5) versus Yorkshire fibroblasts (1.2×10^5 ; $p = 0.25$) at 48 hours. TGF- β 1 modestly decreased proliferation by a comparable degree in Duroc (22%) and Yorkshire cells (9%; $p = 0.11$). Hence, we conclude that our scratch-assay data reflect impaired Duroc fibroblast

migration rather than proliferation, consistent with increased adhesion and an intrinsically fibrogenic, contractile phenotype (22).

Duroc dermal fibroblasts are highly contractile

Using a well-established collagen-contraction assay (23), our observation that baseline three-dimensional gel contraction by Duroc fibroblasts was significantly greater than that of Yorkshire fibroblasts (Fig. 4) confirms a previous report (15). However, we also demonstrated that TGF- β 1 significantly increased contraction by Yorkshire fibroblasts but not Duroc fibroblasts. These results confirm that Duroc fibroblasts have a procontractile phenotype at baseline and again demonstrate decreased responsiveness to TGF- β 1, illustrating the inherent myofibroblast-like behavior of the Duroc fibroblasts. Our results were consistent across fibroblasts derived from multiple Duroc and Yorkshire pigs, indicating the robustness and reproducibility of this differentiated phenotype and implicating Duroc-specific genetic factors.

Duroc dermal fibroblast gene- and protein-expression reflect a fibrogenic phenotype

Using quantitative RT-PCR, we measured gene expression in porcine dermal fibroblast monolayers. Duroc fibroblasts exhibited 2.5-fold higher baseline expression of the myofibroblast marker α -SMA than Yorkshire fibroblasts, and TGF- β 1 significantly up-regulated α -SMA expression in Yorkshire but not Duroc fibroblasts (Fig. 5a). These results matched immunocytochemical protein quantification, which showed significantly higher α -SMA expression in the Duroc compared to Yorkshire fibroblasts (29.9×10^3 vs. 19.7×10^3 arbitrary units/cell, $p = 0.025$). We observed a similar pattern in α 1 type I collagen expression, with higher baseline expression in the Duroc fibroblasts and Yorkshire but not Duroc fibroblasts showing significantly increased expression after TGF- β 1 treatment (Fig. 5b). Duroc fibroblasts expressed about 25-fold lower baseline levels of decorin, a proteoglycan that binds and inhibits TGF- β 1 (24), than Yorkshire fibroblasts; TGF- β 1 treatment significantly down-regulated decorin expression in Yorkshire but not Duroc fibroblasts (Fig. 5c). Thus, compared to Yorkshire fibroblasts, Duroc fibroblast gene expression was consistent with a pro-fibrotic state independent of TGF- β 1 treatment. These results were reproducible across Duroc and Yorkshire fibroblasts isolated from different pigs, indicating that they reflect breed-specific genetic differences.

Given that the Duroc fibroblasts were less responsive than Yorkshire fibroblasts to exogenous TGF- β 1, we used Western blotting to determine that Duroc fibroblasts expressed nearly five-fold higher levels of TGF- β 1 compared to Yorkshire fibroblasts; TGF- β 1 up-regulated TGF- β 1 expression in Yorkshire but not Duroc fibroblasts (Fig. 5d and 5e). The higher endogenous Duroc TGF- β 1 levels may explain their decreased responsiveness to exogenous TGF- β 1, as autocrine TGF- β 1 may lead to saturation of TGF- β 1 signal transduction.

Discussion

Our novel observations regarding *in vitro* Duroc fibroblast phenotypic and genomic responses suggest that fibroblasts isolated from Duroc porcine skin are predisposed to

myofibroblastic differentiation and may explain the exuberant scarring seen in the Duroc pig after wounding. The data support our hypothesis that intrinsic differences in fibroblast function underlie the Duroc porcine fibroproliferative scarring response and advance our understanding of this promising animal model. Furthermore, the *in vitro* behavior of the Duroc fibroblasts closely mirrored human HTS fibroblasts under similar conditions.

Although HTS pathophysiology is incompletely understood, the myofibroblast plays an integral role by depositing ECM and mediating scar contraction (10). Two major determinants of myofibroblast differentiation are TGF- β 1 signaling and transduction of mechanical stress, via focal adhesions, which allow for transmission of contractile force to the ECM (25). Focal adhesions are pathognomonic morphological markers, as differentiated myofibroblasts express a higher fraction of supermature focal adhesions (13, 18). We have shown that cultured Duroc fibroblasts have more supermature focal adhesions than Yorkshire fibroblasts and that TGF- β 1 treatment does not affect supermature focal-adhesion formation in Duroc fibroblasts as much as in Yorkshire fibroblasts (Fig. 1). Likewise, human HTS-derived fibroblasts have a higher baseline fraction of supermature focal adhesions and an attenuated response to TGF- β 1 compared to normal dermal fibroblasts (26).

Fibroblasts migrating from the wound edge into the granulation tissue likely differentiate into myofibroblasts (27, 28), which become more stationary once they acquire a contractile phenotype. Impaired myofibroblast migration out of granulation tissue may lead to hypercellularity, excess ECM, and contraction seen in HTS (14, 29). We found that Duroc fibroblasts demonstrated impaired migration compared to Yorkshire fibroblasts in a manner that was largely independent of TGF- β 1 treatment. Since migrating cells would be expected to have fewer adhesion complexes, an adhesive phenotype likely explains the decreased Duroc fibroblast migration. Our results indicate a sedentary Duroc dermal fibroblast phenotype similar to that of human deep-dermal fibroblasts that contribute to HTS (14).

The well-validated *in vitro* fibroblast-populated collagen-matrix model (23) reproduces human HTS myofibroblast-mediated scar contraction (30-33). Our novel observation that TGF- β 1 significantly augments Yorkshire but not Duroc fibroblast collagen-contraction highlights the inherent baseline myofibroblast-like behavior of the Duroc fibroblasts and reinforces their phenotypic similarity to human HTS fibroblasts. To confirm the intrinsic *in vitro* Duroc myofibroblast phenotype, we have shown that these cells express high baseline levels of both α -SMA and type I collagen; in contrast to Yorkshire fibroblasts, this expression did not depend on exogenous TGF- β 1 (Fig. 5), matching human HTS fibroblast studies (34-36).

The proteoglycan decorin regulates collagen fibrillogenesis (37), blocks TGF- β 1 activity (24), and decreases HTS fibroblast proliferation, collagen synthesis (36), α -SMA expression, and collagen-gel contraction (32). Human HTS samples and HTS-derived fibroblasts have reduced decorin levels compared to uninjured skin (38). Thus, the significantly reduced decorin expression by Duroc fibroblasts might account for their elevated expression of both α -SMA and type I collagen compared to the Yorkshire fibroblasts. Since decorin binds and inhibits TGF- β 1, decreased Duroc decorin expression

may accentuate the fibrogenic phenotype induced by elevated autocrine TGF- β 1 levels. Abundant endogenous TGF- β 1 coupled with low decorin expression may lead to constitutive saturation of TGF- β 1 signaling mechanisms in the Duroc cells. We propose that a TGF- β 1 autocrine loop occurs in the Duroc cells, as has been previously shown to contribute to the fibrogenic phenotype of human HTS fibroblasts (26).

Our results were highly consistent across cell isolates from different Duroc and Yorkshire pigs, and our baseline collagen-gel contraction data confirm a previous report (15), illustrating the reproducibility of the model across laboratories and pigs from different suppliers. We also observed consistent results at passage numbers ranging from 5 to 8 (the highest tested). Our *in vitro* Duroc fibroblasts match the behavior of human HTS-derived fibroblasts (Table 1), suggesting that essential features of fibroproliferative pathophysiology are captured in simple monolayer culture of Duroc fibroblasts. Chun and colleagues recently confirmed major differences in key aspects of fibroblast function depending on the maturity of the scar from which it was derived (39). In contrast to studies based on human scar-derived fibroblasts, however, studies of fibroblasts from uninjured Duroc skin are not subject to variability associated with scar-specific factors such as age, sex, and race of the human subject, the body site and skin depth of origin, or the maturity of the scar. The ability to isolate cells from uninjured skin or wounds from one Duroc pig and expand them to a high passage number without loss of fibrogenic phenotype increases the utility of *in vitro* Duroc fibroblasts for studying mechanistic approaches to fibroproliferative biologic research. Since the size and cost of the Duroc pigs are drawbacks to adoption of the *in vivo* fibroproliferative model (6, 8), our *in vitro* Duroc fibroblast model potentially minimizes both cost and animal suffering.

Although the *in vitro* Duroc fibroblast model is advantageous given its low cost, ease of use, and robust reproducibility, its simplicity also imposes certain limitations. Since the model includes only fibroblasts, it does not capture the potential influence of other cell types on fibrinogenesis, including other skin cells, such as keratinocytes, Langerhans cells, and endothelial cells, as well as a variety of inflammatory cells recruited to cutaneous wounds, such as neutrophils, macrophages, T-lymphocytes, mast cells, and platelets, all of which have been implicated in HTS formation (11). Similarly, *in vitro* models do not allow for the study of systemic effects (e.g., the influence of circulating cytokines or hormones) on local cellular responses. In addition, neither 2D monolayer cell culture nor fibroblast-populated 3D collagen gels reproduce the complex 3D environment of a cutaneous wound that includes not only interactions between multiple cell types, but also dynamic cell-ECM interactions influencing mechanical loading, which is thought to be an important determinant of scar biology (13). Whereas these limitations are present to varying degrees in all currently described *in vitro* models of scarring, including more sophisticated tissue-engineered models (40), novel mechanistic hypotheses or therapeutics developed in the *in vitro* Duroc fibroblast model can be rapidly translated to the syngeneic *in vivo* Duroc scar model, which closely mimics human HTS formation and overcomes the limitations inherent to *in vitro* models.

In summary, the *in vitro* Duroc fibroblast model of fibroproliferative biology offers reproducibility coupled with low cost and easy biological tractability, allowing for

hypotheses to be tested extensively *in vitro* prior to translation to the pre-clinical porcine scar model, decreasing the overall resource burden associated with the *in vivo* model.

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Figure 1. Immunofluorescence microscopy of porcine dermal fibroblasts

Immunocytochemistry of Duroc and Yorkshire fibroblasts grown $\pm$ exogenous TGF- β 1 (n = 4 technical replicates per condition) suggests an adhesive phenotype in the Duroc compared to Yorkshire cells. For all conditions, DAPI staining (blue) was used to identify nuclei. (a) Phalloidin (red) staining was used to visualize actin stress fibers; magnification bar = 50 μ m. (b) Immunostaining for vinculin (green) was performed to visualize focal adhesions (arrowheads); magnification bar = 25 μ m. (c) Proportion of supermature (6 μ m) focal adhesions (n = 4 photos per condition). Data are presented as mean $\pm$ standard deviation. * p <0.05.

Figure 2. Transmission electron microscopy of Duroc and Yorkshire dermal fibroblasts *in vitro*
Transmission electron microscope confirmed increased numbers of adhesion complexes in the Duroc fibroblasts; magnification bar = 1 μ m. Arrowheads indicate adhesion complexes.

Figure 3. Porcine dermal fibroblast scratch wound-closure assay

(a) Duroc and Yorkshire fibroblast monolayer scratch assays were serially imaged (magnification bar = 250 μ m). (b) Scratch area was determined by image analysis. Data are reported as mean $\pm$ standard deviation (n = 6 technical replicates per condition). * p < 0.05 comparing Duroc to Yorkshire in the absence of TGF- β 1 treatment; # p < 0.05 comparing TGF- β 1 treated Duroc or Yorkshire to its respective untreated control. Three independent experiments were performed in the same Duroc and Yorkshire cell isolates (at passage 5-8) with consistent results; the presented data are from a representative experiment.

Figure 4. Contraction of 3D collagen-gel matrices by Duroc and Yorkshire dermal fibroblasts
 Porcine dermal fibroblast-populated collagen gels $\pm$ TGF- β 1 were measured by image analysis ($n = 6$ technical replicates per condition). Data are reported as mean $\pm$ standard deviation. * $p < 0.05$ comparing Duroc to Yorkshire in the absence of TGF- β 1 treatment; # $p < 0.05$ comparing TGF- β 1 treated Yorkshire-seeded gels to control Yorkshire-seeded gels. Four independent experiments were performed using fibroblasts (at passage 6-8) from two different Duroc and Yorkshire pigs with consistent results; the presented data are from a representative experiment.

Figure 5. Porcine dermal fibroblast gene and protein expression in cell culture

Cells were grown in the presence or absence of TGF- β 1 for 4 days. Gene expression was measured by quantitative RT-PCR (n = 6 technical replicates per condition): (a) α -SMA, (b) alpha-1 type I collagen, (c) decorin. Three independent experiments were performed using fibroblasts from two different Duroc and Yorkshire pigs with consistent results; data are from a representative experiment. TGF- β 1 protein expression was measured by western

blotting (n = 2 technical replicates) (**d**) and quantified by densitometry (**e**). Data are mean $\pm$ standard deviation. * $p < 0.05$ and ** $p < 0.01$.

Table 1

Comparison of dermal fibroblasts from the Duroc pig to human hypertrophic-scar-derived fibroblasts in key components of the fibroblast response to injury.

Injury Response	Duroc (vs. Yorkshire)	Human HTS Fibroblast (vs. Normal)*	*Reference(s)
Adhesion	↑	↑	Dabiri et al., 2006
Migration	↓	↓	Honardoust et al., 2012
Collagen Contraction	↑	↑	Dabiri et al., 2008; Garner et al., 1995; Zhang et al., 2009
α-SMA Expression	↑	↑	Wang et al., 2008
Type I Collagen Expression	↑	↑	Ray et al., 2013; Zhang et al., 2007
Decorin Expression	↓	↓	Scott et al., 1998
TGF-β1 Expression	↑	↑	Dabiri et al., 2006