INTRODUCTION

Keloids are disfiguring, scar-like lesions that are challenging to treat, with low response rates to current interventions and frequent recurrence. It has been widely reported that keloids are characterized by myofibroblasts, specialized contractile fibroblasts that express alpha-smooth muscle actin (α-SMA). However, evidence supporting a role for myofibroblasts in keloid pathology is inconclusive, with conflicting reports in the literature. This complicates development of more effective therapies, as the benefit of interventions targeting myofibroblasts is unclear. This study was undertaken to determine whether myofibroblasts can be considered characteristic of keloids.

Methods: Myofibroblasts in tissue sections from keloids, hypertrophic scars (HTSs), and normal skin were localized by α-SMA immunostaining. Expression of α-SMA mRNA (ACTA2 gene) in normal skin and keloid tissue, and in fibroblasts from normal skin, keloid, and HTSs, was measured using quantitative polymerase chain reaction.

Results: Normal skin did not exhibit α-SMA-expressing myofibroblasts, but myofibroblasts were identified in 50% of keloids and 60% of HTSs. No significant differences in ACTA2 expression between keloid and normal skin tissue were observed. Mean ACTA2 expression was higher in HTS (2.54-fold, \( P = 0.005 \)) and keloid fibroblasts (1.75-fold, \( P = 0.046 \)) versus normal fibroblasts in vitro. However, α-SMA expression in keloids in vivo was not associated with elevated ACTA2 in keloid fibroblasts in vitro.

Conclusions: Despite elevated ACTA2 in cultured keloid fibroblasts, myofibroblast presence is not a consistent feature of keloids. Therefore, therapies that target myofibroblasts may not be effective for all keloids. Further research is required to define the mechanisms driving keloid formation for development of more effective therapies. (Plast Reconstr Surg Glob Open 2022;10:e4680; doi: 10.1097/GOX.0000000000004680; Published online 28 November 2022.)
of more effective strategies for treatment and prevention of keloids. However, the underlying mechanisms that promote keloid formation remain incompletely understood, hindering the development of more targeted therapies.

Keloids are up to 15 times more common in populations with African ancestry compared with White populations, with slightly elevated prevalence in Asian populations, and their occurrence is often clustered in families, strongly suggesting a link with darker skin pigmentation and a genetic contribution. Although multiple genetic loci have been implicated in keloid formation, with polymorphisms linked to increased keloid predisposition, to date there have been no specific causative mutations identified, and it is likely that multiple genetic factors are involved. HTSs have a weaker association with dark skin pigmentation than keloids, no familial predisposition, and thus far no strong genetic component. Formation of HTSs after skin injury is associated with injuries to the deep dermis, whereas keloids can form with even small inciting injuries in susceptible individuals. Keloids are only observed in humans; thus, there are currently no adequate animal models that recapitulate the pathology of keloids. As a result, most keloid research to date has involved excised scar tissue and keloid-derived fibroblasts. Numerous cellular abnormalities have been documented in keloid-derived fibroblasts (recently reviewed in Limandjaja et al.), including abnormalities in transforming growth factor beta (TGF-β) and interacting signaling pathways. Indeed, keloid fibroblasts have been observed to exhibit heightened sensitivity in vitro to stimulation by TGF-β1 and other profibrotic and proinflammatory stimuli, although the underlying mechanisms remain poorly understood.

Myofibroblasts are specialized, contractile fibroblasts that play important roles during wound healing and tissue remodeling. They are absent in most normal, uninjured tissues but are activated during wound healing in numerous tissue types. During skin repair, myofibroblasts are first observed in granulation tissue and are believed to arise primarily from activation of resident fibroblasts, although other cell types may also contribute to myofibroblast formation. Myofibroblasts secrete extracellular matrix molecules, including collagen and hyaluronan, and are characterized by expression of alpha-smooth muscle actin (α-SMA), which contributes to their contractile activity. Myofibroblasts are believed to disappear following wound closure and remodeling via apoptosis; aberrant persistence of activated myofibroblasts can lead to dermal fibrosis. Because of their role in dermal fibrosis, interventions that target α-SMA-expressing fibroblasts are considered candidate therapies for suppression of abnormal scarring.

HTSs exhibit nodules containing α-SMA-expressing myofibroblasts; thus, the presence of myofibroblasts was once considered a feature that distinguished HTS from keloids. In 2004, Lee et al. reported that myofibroblasts were observed in about 70% of HTSs and 45% of keloids and, therefore, do not represent a marker that can distinguish these two abnormal scar types. Nevertheless, downregulation of α-SMA continues to be frequently utilized as a surrogate for antifibrotic activity in analysis of novel keloid therapies. Given the emphasis on reduction of α-SMA-expressing myofibroblasts as a therapeutic endpoint, it is important to critically examine the role and presence of myofibroblasts in keloid pathology. We previously performed gene expression profiling of keloid fibroblasts and failed to detect increased expression of ACTA2, the gene encoding α-SMA, compared with normal fibroblasts. Recently, Bell and Shaw summarized previous studies that failed to demonstrate increased numbers of α-SMA-positive myofibroblasts in keloid lesions using immunohistochemistry (IHC). In addition, they examined previously published gene expression data from keloid tissues and failed to identify increased ACTA2 expression in keloids compared with nonlesional skin tissue. In the current study, we extend those findings by examining expression of α-SMA in 20 keloid lesions of 17 subjects to determine whether α-SMA expression can be considered a characteristic feature of keloids. In addition, we analyzed expression of ACTA2 in keloid tissue samples compared with normal skin, and in keloid versus normal dermal fibroblasts. The results indicate that α-SMA-expressing myofibroblasts are not observed in all keloids and demonstrate that elevated ACTA2 expression in cultured keloid-derived fibroblasts does not correlate with the presence of myofibroblasts in the keloid lesions. Thus, the results are consistent with previous reports asserting that myofibroblast presence cannot be considered a characteristic feature of keloid lesions.

**METHODS**

**Tissue Samples**

All tissue samples were obtained with University of Cincinnati institutional review board (IRB) approval. Donor demographic information for all tissue samples is presented in table, Supplemental Digital Content 1, which lists donor demographic information (http://links.lww.com/PRS/GO/C284). Note that patient race/ethnicity was self-reported, and skin pigmentation levels.
were not measured. Diagnoses of keloids or HTSs were determined based on clinical evaluations by experienced burn surgeons or plastic and reconstructive surgeons and were confirmed by histological analyses of tissue sections. Most keloid and all HTS samples were obtained under an IRB-approved protocol and with informed consent of patients and/or legally appointed guardians. The normal skin samples and some keloid samples were obtained from deidentified discard skin from plastic surgery procedures. The University of Cincinnati’s IRB determined that the use of deidentified tissue samples in these studies does not constitute human subjects research, and therefore, informed consent was not required.

Subcutaneous tissues were trimmed, and full-thickness skin or scar tissue was used for isolation of cells for culture (see below). In addition, biopsies were embedded frozen in optimal cutting temperature compound (Fisher Scientific, Pittsburgh, Pa.) for IHC. Additional biopsies were frozen in liquid nitrogen or stored in RNA later (Invitrogen/Fisher Scientific) at –80 °C for subsequent isolation of total RNA.

### Cell Isolation and Culture

Fibroblasts were isolated and cultured as detailed elsewhere. Briefly, decontaminated full-thickness skin samples were treated with Dispase II (MilliporeSigma, St. Louis, Mo.) to separate the epidermis from dermis. The dermal tissue was finely minced and digested with collagenase (Worthington Biochemical Corp., Lakewood, N.J.) to release fibroblasts, which were inoculated into flasks in fibroblast growth medium consisting of Dulbecco’s Modified Eagle’s medium (DMEM; ThermoFisher Scientific), and 1× Antibiotic-Antimycotic (ThermoFisher Scientific), and 1× Antibiotic-Antimycotic Solution (ThermoFisher Scientific). Fibroblasts were harvested and cryopreserved in liquid nitrogen before subculture and were thawed and passaged once before preparation of RNA.

### Immunohistochemistry

Localization of α-SMA protein in tissue sections was performed by IHC using routine methods. Sections were incubated with primary anti-α-SMA antibody (1:250, catalog 14-1960-81, ThermoFisher Scientific) at room temperature for 1 hour, followed by secondary anti-mouse antibody (1:400, catalog A21205, ThermoFisher Scientific) at room temperature for 1 hour. Collagen IV IHC was used to distinguish α-SMA staining localized to pericytes of blood vessels. Sections were incubated with anticalcogen IV antibody (1:50, catalog 507, Yo Proteins, Rönninge, Sweden) at room temperature for 1 hour, followed by secondary antirabbit antibody (1:400, A21441, ThermoFisher Scientific) at room temperature for 1 hour. Slides were coverslipped using mounting media containing 4’,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, Calif.). Sections were viewed and photographed with an Eclipse 90i microscope equipped with a DS-Ri1 Digital Microscope Camera (Nikon Instruments Inc., Melville, NY.). Three nonoverlapping microscopic fields (10×) per sample were captured. Samples were considered positive for myofibroblasts if any level of α-SMA staining (not colocalized with collagen IV staining) was observed in any of the three microscopic fields.

### Analysis of Gene Expression

Expression of ACTA2 was measured using real-time quantitative polymerase chain reaction (qPCR). Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen Inc., Germantown, Md.), and total RNA was treated with DNase I (Qiagen). cDNA was prepared using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific). PCR amplification was performed with ACTA2 gene-specific primers (product PPH01300B-200, Qiagen) and Power SYBR Green qPCR Mastermix (ThermoFisher Scientific) using the StepOne Plus Real-Time PCR System (ThermoFisher Scientific). Expression levels were referenced to the glyceraldehyde 3-phosphate dehydrogenase gene (product PPH00150F-200, Qiagen) using the comparative 2−∆∆Ct method, and relative expression levels were determined by normalizing to mean expression in normal skin or normal fibroblasts. Technical triplicates of the qPCR amplification reactions were performed in addition to biological replicates. Statistical analyses were performed and plots generated using SigmaPlot 14.5 (Systat Software, San Jose, Calif.). Analysis of two groups was performed via t test, and differences among three groups were analyzed by one way analysis of variance and posthoc Tukey test. Differences were considered statistically significant at P values less than 0.05.

### RESULTS

#### Myofibroblasts and α-SMA Localization in Skin and Scar Tissue

In skin and scar tissue, α-SMA expression is not limited to myofibroblasts; it is also expressed by pericytes, which are found in the walls of blood vessels. To distinguish α-SMA expression in pericytes from expression in myofibroblasts, blood vessel basement membranes were stained using an antibody to collagen IV. Cells that stained positive for α-SMA but were not colocalized with collagen IV staining were considered myofibroblasts. There were no myofibroblasts observed in any of the normal human skin samples examined by IHC (N = 8; Fig. 1A, B). In contrast, ten out of 20 keloid samples displayed positive α-SMA staining in myofibroblasts (Fig. 1C–F). Figure 1 illustrates examples of keloids that were negative for α-SMA-expressing myofibroblasts and keloids that were positive for myofibroblast staining. The density and distribution of myofibroblasts in keloid samples were highly variable, with α-SMA-positive cells often observed in deeper regions of the keloid and found in clusters or scattered throughout the tissue. Myofibroblasts were also observed in three out of five HTS samples examined (Fig. 1G–H), and the α-SMA-positive cells were frequently found in clusters...
Fig. 1. Localization of myofibroblasts in normal human skin, keloids, and HTSs. Shown are representative sections of normal human skin (A, B), keloids (C, F), and HTSs (G, H) stained for α-SMA (red) and collagen IV (green). Nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (blue). Examples of myofibroblasts staining positive for α-SMA and not colocalized with collagen IV are indicated by white arrows (E–H). The scale bar in A is the same for all panels (200 μm).
throughout the dermal tissue compartments. The presence of myofibroblasts in keloids was not significantly correlated with keloid body site, donor age, or donor sex. (See table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/C284.) Although the keloids that were positive for α-SMA-expressing myofibroblasts were from a more ethnically diverse group of patients compared with keloids that were negative for myofibroblasts, the sample size for the study was not large enough to determine whether myofibroblasts are more common in keloids of any particular racial or ethnic group.

**ACTA2 Expression in Tissue and Cultured Fibroblasts**

Expression of the ACTA2 gene was analyzed in RNA isolated from lesional keloid tissue compared with normal skin tissue (N = 3 each). No differences in expression level were observed (Fig. 2). Expression of ACTA2 was also measured in human fibroblasts cultured from normal skin (N = 11), keloids (N = 12), and HTSs (N = 3). No significant difference in expression between keloid and HTS fibroblasts was observed (Fig. 3). However, both keloid fibroblasts and HTS fibroblasts exhibited significantly elevated expression compared with fibroblasts from normal skin (Fig. 3). To determine whether increased ACTA2 expression is correlated with α-SMA-positive myofibroblasts in keloid tissue samples, we compared ACTA2 levels in fibroblasts derived from α-SMA-positive keloids (N = 4; see table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/C284) with fibroblasts from α-SMA-negative keloids (N = 7; see table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/C284). There was no significant difference between the two groups (Fig. 4).

**DISCUSSION**

The results demonstrate that although some keloid lesions display α-SMA-expressing myofibroblasts, they are not observed in all keloid lesions. This is consistent with previous reports that also revealed variability in myofibroblasts frequency in keloids, with relatively high proportions of keloids devoid of myofibroblasts. Yet the notion that myofibroblasts have a central role in keloid pathology, and therefore, represent an appropriate cell type to target for keloid suppression, is still widely held in the research community. Bell and Shaw suggested that the persistence of this misconception may be due, in part, to the reliance on cell culture for most keloid research. Keloids...
do not occur in animals, complicating preclinical research due to lack of adequate animal models of keloid pathology.36 Thus, most keloid research to date has utilized cultured fibroblasts isolated from keloids. Gene expression in cultured human fibroblasts can be significantly influenced by culture conditions, and expression differences between keloid and normal fibroblasts may be amplified or even reversed depending on the media and supplements used for culture and the density of cells at the time of analysis.37 Indeed, culture medium containing 10% FBS is commonly used for culture of human dermal fibroblasts; FBS may contain high levels of TGF-β1, which promotes activation of dermal fibroblasts to myofibroblasts.38,39 Thus, the phenotype of keloid fibroblasts in vitro may not accurately reflect the phenotype of these cells in vivo. Because keloid-derived cells may exhibit exaggerated responses to TGF-β1 compared with cells from normal skin,16,17 experiments comparing keloid and normal fibroblasts in vitro may be influenced by culture conditions to exhibit differences that do not reflect the native tissues in vivo. This was observed in the current study, where fibroblasts cultured from α-SMA-negative keloid tissue expressed similar levels of ACTA2 on average compared with fibroblasts cultured from α-SMA-positive keloids. Additionally, culture of cells on tissue culture plastic increases the stiffness of the mechanical environment to magnitudes far greater than experienced in vivo. Keloid fibroblasts were previously shown to exhibit an amplified response to mechanical strain compared with normal fibroblasts, and their altered mechanotransduction has been linked with expression of α-SMA in vitro.16,40 This may contribute to the increased average expression of ACTA2 in keloid versus normal fibroblasts in vitro that was observed in the current study. Taken together, the data suggest that α-SMA-negative keloid fibroblasts may adopt a myofibroblast-like phenotype when placed in in-vitro culture. These observations are consistent with the reported intrinsic abnormalities in responses to growth factors and mechanical signals in vitro that have been previously reported in keloid-derived fibroblasts,16,40 and serve as an important reminder that gene expression in fibroblasts in vitro does not necessarily recapitulate gene expression in tissue in vivo.

In our study and in previous reports,21,33 approximately half of keloids displayed evidence of α-SMA-expressing myofibroblasts. This indicates that myofibroblasts are not a characteristic feature of keloid disorder; although they do seem to be a feature of some keloids. There can be tremendous variability among keloids from different donors, which complicates research aimed at deciphering the underlying disease mechanisms. The genetics of keloid disorder is complex, and although numerous loci have been implicated in keloid pathology, no single causative gene has yet been identified, and keloid is likely a multigenic disorder.1,41 It may be that myofibroblasts play a role in keloid development in some patients, but not others; to our knowledge, the presence of myofibroblasts has not yet been correlated with keloids that occur in any specific population or associated with a particular genotype. There are numerous factors that contribute to variability among keloids from different patients, including patient demographic factors (eg, age, race, and sex), body site, patient health status, time from initiating injury, type of injury, and prior treatments. These sources of variability must be accounted for in research design; if an insufficient number of distinct tissue donors are used, the results may not be applicable to all keloids. Importantly, this variability indicates that no single keloid fibroblast strain should be considered representative of all keloids.

Body site may affect gene expression in keloids for different reasons. There are some genes that are differentially expressed along different body axes; for example, homeobox genes are differentially expressed along the anterior–posterior body axis, and homeobox expression patterns are maintained in fibroblasts cultured from skin of different regions.42 In addition, skin of different body regions experiences different levels of mechanical tension. Mechanical tension is believed to play a role in keloid formation, and it has been proposed that keloids are more likely to form in regions where skin is subjected to greater mechanical force.43 Although it is true that keloids are more commonly observed in some body regions compared with others, they are observed in regions that do not experience much skin tension, such as the ears, in

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**Fig. 4.** Mean ACTA2 expression in keloid fibroblasts in vitro. Expression of ACTA2 in fibroblasts cultured from keloids containing α-SMA-expressing myofibroblasts (black bar; N = 4) was compared with fibroblasts cultured from keloids devoid of α-SMA-expressing myofibroblasts (red bar; N = 7). No significant difference in α-SMA expression between these two keloid fibroblast populations was observed. Plotted are mean normalized expression levels ± SDs. SD indicates standard deviation.

| Normalized Expression (Mean + SD) | αSMA+ Keloid | αSMA- Keloid |
|----------------------------------|-------------|-------------|
| 0.0                              | 1.0         | 1.5         |
| 1.0                              | 2.0         | 2.5         |
| 1.5                              | 3.0         | 3.5         |

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addition to regions under greater tension levels, such as the chest. In the current study, there did not seem to be any correlation between myofibroblast presence and body site, and keloids that were positive or negative for myofibroblasts were found both at sites that experience greater levels of tension (eg, neck, back, and arm) and lower levels of tension (eg, ear).

A limitation of the current study is that our sample set was incomplete, and thus, we were not able to directly compare all three sample types (protein in tissue sections, mRNA in tissue, and mRNA in fibroblasts) from all donors. Another limitation, which is common to many previous studies as well, is that the tissue sources were keloids that were surgically excised during elective surgery procedures. Because of this, the keloids were relatively mature, even though they may have been actively growing at the time of excision. Thus, we cannot rule out a role for myofibroblasts in the early events in keloid development. For one subject, two keloid samples (donor ID nos. 818 and 992; see table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/C284) were collected from different times after injury: the first sample was positive for α-SMA-expressing myofibroblasts, while the second, collected 8 years later, was negative. For another subject, three keloids (donor ID nos. 988, 990, and 997; see table, Supplemental Digital Content 1, http://links.lww.com/PRSGO/C284) were collected within a year of each other, and all contained α-SMA-expressing myofibroblasts. These observations are consistent with a time-dependent role for myofibroblasts in keloid pathology, although the sample numbers in the current study were much too low to draw definitive conclusions. Analysis of dynamic changes in myofibroblast presence will require collection of multiple biopsies from the same keloids over time, which is ethically challenging in a patient population in which any skin injury, including biopsy collection, can exacerbate keloid severity.

A further limitation of this study is that each biopsy examined by IHC for the presence of myofibroblasts was only a small portion of the entire keloid lesion. Analyzing sections throughout the entire mass of each lesion would be impractical, but it is possible that regional variability— a known feature of keloid lesions—may complicate identification of α-SMA-positive myofibroblasts when only a relatively small area of the tissue is assessed. Keloids can increase in size indefinitely, and it is thought that the edges of the keloid tend to be more active compared with the central region. Future studies comparing localization of α-SMA-expression myofibroblasts in different regions of keloids may reveal whether myofibroblasts are more prevalent in certain regions compared with others. Finally, although no significant differences in ACTA2 mRNA expression were observed between normal skin and keloid scar tissue, the expression of ACTA2 in pericytes of blood vessels may confound these results. However, the lack of differential ACTA2 expression in tissue samples is consistent with the absence of α-SMA-positive myofibroblasts in approximately half of keloid tissues, suggesting that the presence of α-SMA-expressing pericytes did not alter the results of this comparative analysis.

CONCLUSIONS
The results indicate that myofibroblasts cannot be considered characteristic features of keloids, and their presence cannot be reliably used to distinguish keloids from HTS. Additionally, elevated ACTA2 expression in keloid fibroblasts in vitro does not consistently correlate with myofibroblast presence in keloid lesions in vivo. Further research is needed to identify the cell types and molecular mechanisms driving keloid pathology, which can guide development of more effective, targeted therapies.

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AVAILABILITY OF DATA AND MATERIALS
Raw data will be made available upon request from the corresponding author. Materials such as cells or tissue sections may be made available on a collaborative basis, if feasible.

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