Keloid tissue analysis discredits a role for myofibroblasts in disease pathogenesis

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Abstract
Myofibroblasts, renowned for their contractility and extracellular matrix production, are widely considered the key effector cells for nearly all scars resulting from tissue repair processes, ranging from normal scars to extreme fibrosis. For example, it is often assumed that myofibroblasts underpin the characteristics of keloid scars, which are debilitating pathological skin scars lacking effective treatments because of a poor understanding of the disease mechanisms. Here, we present primary and published transcriptional and histological evidence that myofibroblasts are not consistently present in primary keloid lesions, and when alpha-smooth muscle actin (αSMA)-positive cells are detected, they are not greater in number or expressing more αSMA than in normal or hypertrophic scars. In conclusion, keloid scars do not appear to require αSMA-positive myofibroblasts; continuing to consider keloids on a quantitative spectrum with normal or hypertrophic scars, with αSMA serving as a biomarker of disease severity, is hindering advancement of understanding and therapy development.

Keywords
keloids, myofibroblast, skin, smooth muscle actin, wound

1 | INTRODUCTION
Keloids are pathological scars that can grow very large and beyond the original wound margin. This is an important medical condition due to their prevalence (up to 16% of certain ethnic populations including Afro-Caribbean and Japanese), their painful nature, lack of understanding about their aetiology and the paucity of effective treatment options.

Although keloids can develop spontaneously without any obvious tissue injury, generally they develop after an insult or wound to the skin. During normal skin wound repair, local fibroblasts and potentially other cell populations respond to the plethora of biochemical and mechanical cues (e.g., growth factors such as TGFβ1 and change in tissue tension), which trigger them to migrate into the wound bed and differentiate into myofibroblasts. Myofibroblasts are renowned for their contractility (due to alpha-smooth muscle actin (αSMA) expression; gene: ACTA2) and extracellular matrix (ECM) production; with these functions, they make important contributions to the repair process, but are also thought to be the cell type responsible for scarring. Consistent with this, there is a near-universal assumption that myofibroblasts are the culprit cells causing keloid development and growth. However, it is still unclear whether keloids fall on a quantitative spectrum with normal or hypertrophic scars, with the same wound-associated myofibroblasts persisting and causing the pathology. Confusingly, the fibroblasts in keloids have been described as everything from de-differentiated myofibroblasts to “end-stage” myofibroblasts. Despite this, the keloid cell behaviours deemed pathological in in vitro studies are widely described as myofibroblastic, with αSMA ubiquitously used as a biomarker, along with Collagen I and Fibronectin expression. Nearly all cell-based research, admirably striving to develop novel treatment strategies for this disease, interpret a reduction in αSMA expression as a readout of success, but...
unfortunately this has never translated into a successful clinical therapy. Thus, it is imperative to revisit the tissue-based evidence about whether myofibroblasts are effectors of keloid scarring. This study interrogates published as well as new transcriptional and histological data for a myofibroblast presence in keloid scars. We report that a significant proportion of keloids are devoid of \(\alpha\)SMA expression, and when present, its intensity is less than other scar types (e.g., hypertrophic scars) and therefore it does not distinguish this unique fibrotic condition.

2 | MATERIALS AND METHODS

2.1 | Searches

Literature searches were performed in PubMed and Google Scholar for “keloid AND myofibroblast” as well as “keloid AND smooth muscle actin (SMA)”. NBCI Gene Expression Omnibus (GEO) was used to find RNA analyses of keloid tissue. The following data sets were interrogated: GSE2945, GSE90051, GSE92566 and GSE158395. Graphs were created and statistical analysis was performed using GraphPad Prism 8.

2.2 | RNA extraction and real-time quantitative reverse transcription PCR analysis

With informed consent, anonymous surplus skin and keloid tissue were collected from plastic surgery procedures (UK Ethical Approval Reference: IRAS REC: NS/14/1073). Samples included in this analysis were not controlled for sex, race, age of the patient or scar, or anatomical site, although both sexes and a range of ages and body sites were represented for both tissue types. Samples were transported and stored in RNAlater solution at 4 \(^\circ\)C until RNA extraction was performed using the Qiagen RNeasy Fibrous Tissue Mini Kit, with the samples homogenised directly in the lysis buffer provided. Equal quantities of RNA (100 ng) were treated with DNase I (Thermo Scientific) and reverse transcribed using Random Primers and RevertAid Reverse Transcriptase (both Thermo Scientific) according to the supplier’s instructions.

Quantitative PCR reactions were conducted using Bioline SensiMix™ SYBR No-Rox according to instructions. The reactions were performed using the Qiagen Roto-Gene-Q qPCR machine with the following protocol: pre-incubation at 95 \(^\circ\)C for 10 min, the 45 cycle run (95 \(^\circ\)C for 15 s, 60 \(^\circ\)C for 30 s, 72 \(^\circ\)C for 30 s) to amplify target cDNA, followed by a ramp from 72 \(^\circ\)C to 95 \(^\circ\)C (melt curve) to examine primer specificity. Relative expression levels (determined against a standard curve) were normalised against the expression levels of a reference gene (P4HB). Primer sequences (designed using NCBI PrimerBlast; supplied by Sigma Aldrich) were as follows: ACTA2 (forward): CCGACCGAATGCAGAAGGA; ACTA2 (reverse): ACAGAGTATTTGCGCTCCGAA; P4HB (forward): TTCAGCCAGTTCACGATCTC. Primers were confirmed to create a single amplicon and fit to a standard curve (1:5 dilution series) with a coefficient \((R^2)\) >0.98. Graphs were created and statistical analysis was performed using GraphPad Prism 8.

3 | RESULTS

3.1 | \(\alpha\)SMA-positive myofibroblasts not consistently detected in keloid tissue

The scientific literature was searched for all papers reporting on the presence and/or abundance of myofibroblasts in keloid tissue (summarised in Table 1). The largest study used immunohistochemistry to investigate \(\alpha\)SMA protein expression in 40 keloid patient samples; 45% (18 of 45) were positive. Kamath et al had similar statistics, detecting \(\alpha\)SMA expression in 7 of 12 keloids analysed, and when it was present, the intensity of staining was comparable to hypertrophic scar examples. In further support of keloids including myofibroblasts, Hietanen et al recently reported that all 22 keloids in their study were positive for \(\alpha\)SMA, and similarly, all of the keloids

| Citation | Assay | # Keloids analysed | \(\alpha\)SMA/ACTA2 findings |
|----------|-------|--------------------|----------------------------|
| Lee et al | IHC | 40 | 45% \(\alpha\)SMA+ |
| Kamath et al | IHC | 12 | 58% \(\alpha\)SMA+ |
| Hietanen et al | IHC | 22 | 100% \(\alpha\)SMA+ |
| Limandjaja et al | IHC | 5 | 100% \(\alpha\)SMA+ |
| Ehrlich et al | IHC | 17 | 12% \(\alpha\)SMA+ |
| Liu et al | IHC | 14 | 0% \(\alpha\)SMA+ |
| Naitoh et al (GSE2945) | Microarray | 1 L and NL pair | Ratio (L/NL) = 0.77* |
| Hsu et al (GSE90051) | Microarray | 7 L and NL pairs | Average ratio (L/NL) = 0.88 |
| Fuentes-Duculan et al (GSE92566) | Microarray | 3 L and NL pairs | Average ratio (L/NL) = 0.94* |
| Wu et al (GSE158395) | RNAseq | 4 L and NL pairs | Average ratio (L/NL) = 0.83 |

Abbreviations: IHC, immunohistochemistry; L, lesional; NL, non-lesional.

*Average of two microarray features representing ACTA2.
had expression that was higher than that in adjacent normal skin. However, these elevated \( \alpha \)-SMA levels in keloids were equal to a normal scar, and not as high as in hypertrophic scars. At the other end of the spectrum, Ehrlich et al.\(^\text{12}\) found that only 2 of 17 keloids had \( \alpha \)-SMA-positive myofibroblasts, and similarly, Liu et al.\(^\text{13}\) reported that none of the 14 keloids they analysed were positive for \( \alpha \)-SMA.

Myofibroblasts can also be identified by ultrastructural features observed by electron microscopy (e.g., prominent rough endoplasmic reticulum, stress fibres). Although these cellular features have been observed in keloid tissue,\(^\text{14}\) they were less notable in keloids than many other scar types.\(^\text{15}\)

To summarise, although myofibroblast presence (based on \( \alpha \)-SMA positivity) has been reported in numerous keloid scar studies, there is no evidence that there is a greater abundance of these cells or a more extreme cellular phenotype in this pathological context compared to other scar types (i.e., normotrophic, hypertrophic). In fact, there are abundant data that many keloid lesions are entirely devoid of myofibroblasts.

### 3.2 Transcriptional profiling of keloid tissue does not endorse a myofibroblast composition

Our second approach to evaluate whether myofibroblasts are a key cell type constituting keloid scars was to mine publicly available transcriptional profiling data of keloid tissue. There were four studies that compared gene expression between lesional and patient-matched non-lesional skin whose results were interrogated specifically for ACTA2 gene expression (three microarray and one RNAseq; Table 1).\(^\text{4-7}\) There were remarkably small differences in ACTA2 levels between control and keloid tissue in all four studies and any marginal difference was in fact towards less expression in the lesional tissue compared to adjacent normal skin (range 0.77–0.94; ratio of lesional/non-lesional expression values; Figure 1(A)).

The above-mentioned studies had statistical strength in that patient-matched non-lesional skin served as the control against which the gene expression within the keloid lesion was compared; however, this raised the question of how expression in keloid-prone or lesional skin compares to normal/healthy control skin. Wu et al.\(^\text{7}\) also had normal skin samples included in their analysis, and in terms of ACTA2 expression, these were very similar to both non-lesional skin (non-lesional/normal: 1.02) and keloid samples (keloid/normal: 0.85; Figure 1(B)). We performed our own studies comparing ACTA2 gene expression in keloid tissue versus normal skin (four unrelated biological replicates) using quantitative (q)RT-PCR, and this revealed significantly less expression in the keloid tissue (keloid/normal: 0.27; Figure 1(C)). In summary, none of the gene expression studies on keloid tissue indicate that ACTA2 expression, the main identifier of myofibroblasts, is elevated in keloid lesions.

### 4 DISCUSSION

The keloid research community has extrapolated the understanding that myofibroblasts are key effector cells in skin wound repair and scarring,\(^\text{1}\) as well as in fibrosis of many organ systems, and assumed that myofibroblasts are also likely the causative cell driving keloid development, growth and persistence. However, with few publications on the keloid scar tissue itself, and most research relying on cell culture techniques known to trigger myofibroblast differentiation, it was important to revisit the evidence.

We first considered published histological analyses of keloid tissue for \( \alpha \)-SMA-positivity\(^\text{8-13}\); although the findings were highly

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**FIGURE 1** RNA analysis of keloid tissue discounts significant myofibroblast composition. (A) Publicly available microarray and RNA-sequencing data (GSE2945,\(^\text{4}\) GSE90051,\(^\text{5}\) GSE92566\(^\text{6}\) and GSE158395\(^\text{7}\)) were mined for ACTA2 expression. The differential expression values (log2 fold-change, lesional/non-lesional) are plotted (mean ± SD). (B) ACTA2 expression was compared between keloid and normal skin tissue in GSE158395.\(^\text{7}\) Normalised expression values (FPKM) are shown. (C) ACTA2 expression was analysed by qRT-PCR in 4 normal skin and keloid scar tissue samples (in technical duplicate); expression values were established against a standard curve and normalised to a reference gene (P4HB). Mean ± SD is plotted; *\( p < 0.05\), **\( p < 0.01\) by non-parametric Mann–Whitney test.
variable, from 0 to 100%, the results demonstrate that myofibroblast presence is not a requirement of disease. Also, when αSMA was detected in keloids, its intensity was not greater than other scar types, reinforcing that this is not a distinguishing feature of keloids. Publications with transcriptional profiling of keloid tissue were also examined for ACTA2 gene expression. The data were consistent in revealing very little difference in ACTA2 transcript abundance in keloid lesions compared to non-lesional tissue, and indeed slightly less expression in all cases. In line with this, Wu et al and our own real-time qRT-PCR analysis detected less expression in keloids compared to normal healthy skin. There are a number of variables that may influence these results, one being anatomical location. Although site was not controlled for in our samples, in the Wu et al's study, all tissues were trunk-derived. The age or maturity of the scar may also be a relevant variable; most keloids being surgically removed and studied experimentally (and indeed those in our analysis) would be long established and relatively mature. Interestingly however, RNA-seq investigations comparing the early scar response (6 weeks after wounding) of keloid-prone individuals versus non-prone controls also did not show elevated ACTA2, countering the idea that keloid fibroblasts may initially have a myofibroblast phenotype, which then progresses, regresses, or changes entirely.

Collectively, the published work regarding αSMA-positive myofibroblasts in keloids demonstrates that this is not an overabundant or overzealous cell type in these lesions. This is consistent with the clinical observations that keloids are generally not associated with contractures, whereas hypertrophic scars are. Distinct constituent cells between the two scar types may also account for the histological difference (e.g., the ECM). These observations highlight numerous unanswered questions warranting additional research:

- Are αSMA-positive and αSMA-negative keloids distinct? Could and should they be stratified on this feature?
- If myofibroblasts are not forming keloids, what is the identity of the cells that are and where do they fall on a differentiation spectrum (from undifferentiated and potentially plastic through to highly differentiated and stable)?
- TGFβ1, its regulators and its downstream signalling pathways have been widely implicated in keloid pathogenesis; what are their roles in this context, if not triggering myofibroblast differentiation?
- Is contractility a functionally significant feature of keloid cells? If yes, what machinery is driving this, if not αSMA?

Considering the tissue-based evidence presented herein that keloids are not masses of overactive contractile myofibroblasts, it is essential to recognise that keloids may not fall on a quantitative spectrum with normal or hypertrophic scarring but rather could be qualitatively distinct. Appropriate biomarkers of keloid disease are urgently needed, as is sufficient understanding of the disease processes in order to establish the pathological cellular features important to target clinically.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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