TO THE EDITOR

Keloidal scarring is a common and disfiguring skin problem, yet its pathobiology is only partially understood, and treatments remain sub-optimal (Glass, 2017). To date, most investigative studies have focused on established keloid lesions and the surrounding extracellular matrix (He et al., 2017). In contrast, we explored transcriptomic alterations at an earlier time point—during keloid formation. We studied keloid-prone individuals from pedigrees with an autosomal dominant history of keloids, as well as unaffected family members and healthy matched control subjects without any tendency to form keloids (see Supplementary Figure S1 online). All subjects were Taiwanese.

Following institutional ethics approval (IRB National Cheng Kung University Hospital; project A-BR-104-011) and written informed consent, we performed 3-mm punch biopsies of non-lesional upper outer buttock skin, followed by an additional 4-mm punch biopsy of the same site 6 weeks later (see Supplementary Table S1 online and Supplementary Figure S2 online). For the study, biopsying buttock skin was deemed acceptable by both the participants and the ethics committee (see Supplementary Materials online for further discussion). The 6-week time point was chosen based on feedback from the keloid-prone individuals as to when they were normally first aware that a keloid scar was developing. We undertook an integrative approach of RNA-seq (RNA-Seq) and microRNA (miRNA) expression analysis based on the 2 sets of skin biopsies (baseline and 6 weeks later).

The study involved 8 keloid-prone subjects and 6 healthy matched individuals. Each skin biopsy was immediately immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA) and total RNA was isolated using the RNeasy Plus Universal kit (Qiagen, Hilden, Germany), retaining miRNAs according to the manufacturer’s protocol. RNA samples were subjected to microarray analysis on Affymetrix GeneChip miRNA 4.0 arrays and total RNA-Seq analysis on Illumina pair-end sequencing (see Supplementary Materials). The RNA-Seq raw data files and metadata have been deposited in the Sequence Read Archive (SRA ID: SRP137071) and the miRNA raw data and metadata in Gene Expression Omnibus (GEO ID: GSE113621).

A stepwise bioinformatics strategy was followed to identify differentially expressed miRNAs that may contribute to keloid pathogenesis (see Supplementary Materials and Supplementary Figure S3 online). This analysis highlighted 37 miRNAs that were differentially expressed in the keloid-prone subjects. Hierarchical clustering revealed 2 clusters that were upregulated 6 weeks after wounding (see Supplementary Figure S4 online).

In parallel, differential expression analysis was applied to the RNA-Seq data between keloid-prone and healthy subjects, which identified 8 genes at baseline and 47 genes at 6 weeks after wounding that were differentially expressed (adjusted P < 0.05; see Supplementary Materials). Comparing healthy controls before and after wounding identified 2,215 differentially expressed genes, whereas the same analysis in the keloid-prone individuals identified 3,161 differentially expressed genes (see Supplementary Figure S5a online). Of those genes, there were 513 genes specific to the healthy individuals and 1,449 genes specific to the keloid phenotype (see Supplementary Figure S5b). Hierarchical clustering of the differentially expressed genes specific to the keloid phenotype exhibited 2 distinct clusters showing changes in expression between baseline and 6 weeks after wounding (see Supplementary Figure S6 online).

We further assessed pathway enrichment in the RNA-Seq data using the Gene Set Variation Analysis package.
For genes specific to the keloid phenotype, there were 101 differentially activated pathways between baseline and 6 weeks after wounding, while 24 pathways were found to be differentially activated for the genes that were specific to the healthy individuals (Figure 1, and see Supplementary Tables S2, S3 online). Of these, 22 pathways that were specific to the keloid-prone individuals were present on the KEGG and Reactome pathway databases, which are manually curated and well annotated. Of note, NOTCH signaling, mitogen-activated protein kinase signaling, and Toll-like receptor pathways were found to be altered in keloid-prone individuals after wounding with a decrease in pathway activity. These pathways have already been suggested to play a role in keloid disease, and our analysis provides further evidence to support their involvement (Bagabir et al., 2011; Syed and Bayat, 2012; Wu et al., 2017). Moreover, DNA repair and p53 signaling pathways were also highlighted (Yamauchi et al., 2018). In addition, the analysis also identified altered regulation of insulin secretion and metabolic pathways (RNA, protein, fructose, mannose, and glycerophospholipid metabolism) in keloid pathobiology. Of note, recent work has shown increased glycolytic metabolism in keloid fibroblasts, suggesting that dysregulation of metabolic pathways such as glucose metabolism can contribute to keloid formation (Li et al., 2018).

To identify the targetome of the differentially expressed miRNAs for each of the 2 clusters of the differentially expressed genes in keloid-prone individuals (see Supplementary Figures S4, S6 online), we intersected the 37 miRNAs with the 1,449 genes that were specific to the keloid phenotype and that were identified from the analyses described here. As a result, there were 403 overexpressed mRNA–miRNA interactions for 24 differentially expressed miRNAs and 635 downregulated mRNA–miRNA interactions for 29 differentially expressed miRNAs. Figure 2a visualizes the networks derived from both up- and downregulated putative targets that are specific to the keloid phenotype 6 weeks after wounding.
Figure 2. miRNA–mRNA targetome and gene association network analysis during keloid formation. (a) Targetome of differentially expressed miRNAs. Size of gray nodes indicates the P-adjusted value for the differentially expressed miRNAs; the larger the node the more biologically significant the miRNA is likely to be.
Next, to investigate the functional dynamic changes of the 1,449 differentially expressed genes that were involved in the targetome, we conducted gene set enrichment analysis using the R package GAGE (Luo et al., 2009). This analysis identified the mitogen-activated protein kinase signaling pathway as the only gene set to be significantly dysregulated in the keloid prone subjects 6 weeks after wounding (see Supplementary Tables S4, S5 online). Notably, other published data have shown that inhibition of mitogen-activated protein kinase hinders invasive growth of keloid fibroblasts (Wu et al., 2017).

Gene association network analysis was also performed to further classify the differentially expressed genes from the RNA-Seq data set according to Reactome pathway terms and correlated expression values among them (Figure 2b, and see Supplementary Materials). This analysis demonstrated a divergent average expression profile of cytokine signaling genes between keloid-prone and healthy individuals during wound healing. Of note, IL-1α, IL-1β, IL-6, and TNF-α proinflammatory cytokines have been shown to be upregulated in keloid tissue (Ogawa, 2017). Differences in organelle biogenesis and metabolism were also highlighted, providing further support that dysregulation of metabolic pathways may contribute to keloid formation.

In summary, our study provides a comprehensive and integrative analysis of the keloid transcriptome and miRNAome and highlights biological pathways that feature during keloid formation. Functional validation will be required to confirm these findings and determine mechanistic and potential therapeutic relevance. Similar studies at earlier time points after wounding are also likely to add further insight to keloid biogenesis.

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**REFERENCES**
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**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.05.017.

Red nodes indicate upregulated genes, while green nodes indicate downregulated genes in keloid subjects during keloid formation. (a) Gene association network during keloid formation (top panel). Genes are represented by colored nodes and are split into 27 clusters, sized by the number of genes in them. Genes in the same cluster are connected with black edges and genes that are connected across clusters with red edges. Mean expression change in each cluster of the gene association network in keloid-prone and healthy individuals between baseline and 6 weeks after wounding (bottom panel). Each cluster is referenced by the x, y coordinate system. The x-axis represents time points, while the y-axis represents the average expression in each cluster. All expression levels are standardized and centered for plotting purposes.