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ORIGINAL ARTICLE

Mapping cellular senescence networks in human diabetic foot ulcers

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Abstract Cellular senescence, a cell fate defined by irreversible cell cycle arrest, has been observed to contribute to chronic age-related conditions including non-healing wounds, such as diabetic foot ulcers. However, the role of cellular senescence in the pathogenesis of diabetic foot ulcers remains unclear. To examine the contribution of senescent phenotypes to these chronic wounds, differential gene and network analyses were performed on publicly available bulk RNA sequencing of whole skin biopsies of wound edge diabetic foot ulcers and uninvolved diabetic foot skin. Wald tests with Benjamini–Hochberg correction were used to evaluate differential gene expression. Results showed that cellular senescence markers, *CDKN1A*, *CXCL8*, *IGFBP2*, *IL1A*, *MMP10*, *SERPINE1*, and *TGFA*, were upregulated, while *TP53* was downregulated in diabetic foot ulcers compared to uninvolved diabetic foot skin. NetDecoder was then used to identify and compare context-specific protein–protein interaction networks using known cellular senescence markers as pathway sources. The diabetic foot ulcer protein–protein interaction network demonstrated significant perturbations with decreased inhibitory interactions and increased senescence markers compared to uninvolved diabetic foot skin. Indeed, *TP53* (p53) and *CDKN1A* (p21) appeared to be key regulators in diabetic foot ulcer formation. These findings suggest that cellular senescence is an important mediator of diabetic foot ulcer pathogenesis.

Keywords Wound healing · Diabetic foot ulcer · Cellular senescence · Skin aging · Network analysis

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Introduction

Wound healing is a complex and dynamic phenomenon entailing tightly regulated intracellular and extracellular signals that coordinate to clear damage and regenerate tissue [1, 2]. The diabetic wound bed represents a chronic stalled wound state that affects 1 to 3.5 million persons in the USA with diabetic foot ulcers, resulting in frequent clinic visits, poor quality of life, and significant healthcare burden [3]. Cell signaling impairments in diabetic patients, including deregulated inflammation, epidermal hyperproliferation, reduced angiogenesis, and abnormal stem cell function, predispose them to non-healing vascular wounds [4–6]. As a result of these dysfunctional processes, diabetic foot ulcers are often chronic and recurrent, leading to significant morbidity, mortality, and healthcare burden [7–10]. Notably, the genetic pathways underlying diabetic foot ulcers, including those associated with cellular senescence, are poorly understood.

Cellular senescence is a cell fate characterized by essentially irreversible growth arrest, resistance to apoptosis, and a senescence-associated secretory phenotype (SASP) [11, 12]. It is triggered as a defense mechanism by intrinsic or extrinsic stresses, such as DNA damage, lipid-based signaling, mitochondrial dysfunction, aggregates of abnormal proteins, inflammation, and danger signals [13]. Senescent cells are not only byproducts of aging and disease processes, but they have been demonstrated to play active roles in mediating age-related skin dysfunction [14–16]. SASP factors, which include pro-inflammatory cytokines, matrix metalloproteinases, and growth factors, can modulate the local microenvironment and disrupt neighboring cells [17, 18]. Accumulation of senescent cells could thereby contribute to skin deterioration by disrupting physiological functions, including epidermal stem cell renewal [19] and extracellular matrix deposition [20].

Cellular senescence is implicated in both normal and impaired wound healing [1, 12, 21]. Early senescence may promote regeneration in early or acute wound healing [2, 22] and protect against cancer cell proliferation [16]. Acute SASP (i.e., *CCL2*, *CCL5*, *PAI-1*, *PDGFα*) is postulated to benefit the pro-inflammatory wound cascade [1, 2]. However, gene expression and secretory signals are observed to change when cellular senescence progresses from early to late phases [23]. Not only do their phenotypes change, but late senescent cells have been shown to be detrimental to tissue function and health [24]. Elevated or persistent senescence and a chronic SASP (i.e., *CXCL1*, *CXCL2*, *IL-1Ra*, *IL-6*, *RANTES*, *TIMP1*, *TNFα*) are associated with impaired and delayed healing, as well as chronic non-healed wound beds [25, 26].

Increased senescent cell burden has been observed in chronic diabetic wounds, but the contribution of senescence to the pathophysiology of diabetic foot ulcers remains unclear [12, 26]. Herein, we delineate the role of senescence-associated genes and associated protein–protein interaction networks in diabetic foot ulcers. Gene expression profiles in diabetic foot ulcers and uninvolved diabetic foot skin were compared to contrast their wound healing cascades. Protein–protein interaction networks were identified to discern pathways and key regulators differentiating wounded skin. Senescence-associated genes were specifically highlighted in expression and network analyses to elucidate the role of cellular senescence in diabetic foot ulcers.

Methods

Datasets and preprocessing

Bulk RNA sequencing data were retrieved from gene expression values (RPKM, reads per kilobase exon per million reads) generated by deep sequencing (Illumina NextSeq500) using publicly available data from the Gene Expression Omnibus database, under accession number GSE134431 (https://www.ncbi.nlm.nih.gov/geo/) [6]. These data were obtained from sequencing full-thickness skin biopsies of wound edges of diabetic foot ulcers (DFUs) from 13 patients (age 56 ± 13 years old; 13 males). For comparison, skin samples were obtained from uninvolved diabetic foot skin (DFS) without ulcers from 8 patients (ages 66 ± 13 years old; 7 males and 1 female). All samples were obtained from patients receiving care at the University of Miami Hospital Wound Care, after receiving written informed consent.

Patients included in the DFU group met inclusion criteria for diabetes mellitus type II, ulcer on plantar aspect of foot at least 0.5 cm^2 in size, peripheral neuropathy, at least 21 years old, wound duration of at

least 4 weeks, and HbA1c ≤ 13.0%. Exclusion criteria for the DFU group included active cellulitis, osteomyelitis, gangrene, vascular insufficiency, measured by ABI < 0.7 or ABI > 1.3 with revascularization in the last 6 weeks, and experimental drugs taken in the preceding 4 weeks.

Reads were aligned, and missing values were handled as previously described [6]. They were preprocessed and analyzed using R (version 4.2). Genes with reads of less than 1 RPKM were pre-filtered to increase efficiency and clarity of visualization. Regularized logarithmic transformation to $\log_2$ scale was performed on the reads to obtain log fold changes for DESeq2.

Differential gene expression analysis

The data were processed and normalized using the DESeq2 package (release 3.16) in R (version 4.2) [27]. This package allowed for assessment of similarity between samples using hierarchical clustering of sample distances and principal component analysis to determine whether DFU and uninvolved DFS groups had significantly different overall gene expression. Additionally, heat maps of the count matrix and sample-to-sample distances were obtained to evaluate the detection of differentially expressed genes across the two groups of samples. Next, senescence-associated markers, including SASP genes, were compared between DFU and uninvolved DFS groups. After reviewing literature for senescence-associated markers, senescence profiling was performed with intrinsic cellular senescence genes *CDKN1A*, *CDKN2A*, and *TP53* [1]; skin-specific proliferation and differentiation-related genes *KRT14*, *MITF*, and *TGFα* [28–30]; and SASP genes *CCL2*, *CXCL1*, *CXCL8*, *CXCR2*, *CYR61*, *IGFBP2*, *IGFBP4*, *IL-1α*, *IL-6*, *INHBA*, *MMP2*, *MMP3*, *MMP9*, *MMP10*, *MMP12*, *PAPPA*, *PLAT*, *PDGFα*, *SERPINE1*, *SPP1*, and *VCAM1* [2, 18, 31–36]. In addition, the gene set SenMayo was evaluated to corroborate the findings with results validated in human studies [35].

Statistical analysis

Statistical tests were performed on differential gene expression using the DESeq2 package (release 3.16) in R (version 4.2) [27]. Wald tests were performed on each gene to compare DFU and uninvolved DFS groups, and Benjamini–Hochberg adjusted *p*-values were reported to reduce false discovery rates. Differences between groups with adjusted *p*-values less than 0.05 were deemed significant.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed with default settings, specifically, 1000 permutations with no collapse [37, 38]. DFS and DFU transcriptomes were compared in unbiased analysis of the human hallmark (H), curated (C2), ontology (C5), and SenMayo gene sets [35, 37, 39].

Network analysis

NetDecoder (https://github.com/HuLiSyspharm/NetDecoder) was used to analyze protein–protein interaction networks, comparing the DFU group with the DFS group as a control [40]. Co-expression networks were derived for each phenotype from the bulk RNA-seq (Illumina NextSeq 500) global transcriptome, and Pearson's correlation coefficient matrices were used to produce their respective edge-weighted interactomes.

For the first set of network analyses, the source gene list was comprised of known cellular senescence markers listed above. NetDecoder default parameters were used, specifically, a size of functional neighborhood (SNF) of 0.95, threshold of flow ratios between phenotypes (ratioThreshold) of 5, and flow threshold (corThreshold) of 0.5.

For the second set of network analyses, the source gene list was comprised of the top 20 genes in human SenMayo-enriched cells [35]. To allow for adequate visualization, NetDecoder parameters were set for a size of functional neighborhood (SNF) of 0.95, threshold of flow ratios between phenotypes (ratioThreshold) of 1, and flow threshold (corThreshold) of 0.1.

Pathway enrichment analysis

Web-based gene set analysis toolkit (WebGestalt; www.webgestalt.org/) with Gene Ontology (GO) biological process functional databases was used for pathway enrichment analysis [41]. Target gene symbol lists were input and analyzed for

overrepresentation against the *Homo sapiens* genome protein-coding reference set.

Data availability

Bulk RNA sequencing data are available under accession number GSE134431 on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) [6]. All data presented in the current study will be made available by the investigative team upon reasonable request.

Results

Differential gene expression analysis detected significant differences between diabetic foot ulcer (DFU) and uninvolved diabetic foot skin (DFS) groups. Principal component analysis demonstrated that DFU and DFS samples could be segregated by their gene expression profiles (Fig. S1). Moreover, hierarchical clustering of gene expression could divide the DFU and DFS patients into two distinct groups (Fig. 1a, b).

Next, gene set enrichment analysis was performed to compare pathways in DFU vs. DFS. None of the hallmark, curated, or ontology gene sets were significantly (p-adj <0.05) upregulated in DFU vs. DFS, suggesting that DFU and DFS shared enrichment in common pathways. In the hallmark (H) gene set, 36 gene sets were upregulated in DFU, with greatest enrichment in genes upregulated by reactive oxygen species (normalized enrichment score (NES) $=1.58$, false discovery rate (FDR) $q=0.84$), genes upregulated in response to *TGF-β1* (NES $=1.57$, FDR $q=0.45$), and genes regulated by *NF-κB* in response to *TNF-α* (NES $=1.53$, FDR $q=0.42$) (Fig. 1c, d). Notably, upregulation of reactive oxygen species is associated with cellular senescence [42], *TGF-β1* is a canonical senescence marker [43], and *TNF-α* is both an inducer of senescence and SASP protein [44, 45]. Other senescence-related gene sets with NES greater than 1 in DFU included genes mediating apoptosis (NES $=1.26$, FDR $q=0.57$) and genes involved in p53 pathways and networks (NES $=1.23$, FDR $q=0.57$). After establishing that DFU and DFS samples had distinctive patterns of gene expression, the roles of specific cellular senescence markers in each group were investigated.

Upregulation of cellular senescence profile in diabetic foot ulcers

Senescence marker expression was compared between the two groups: DFU vs. DFS. Cellular proliferation markers, including *IGFBP2* (Benjamini–Hochberg adjusted $p=0.0019$) and *TGFα* ($p=0.0057$), were significantly upregulated in the DFU group (Fig. 2a). Cell cycle arrest genes differed in expression between the DFS and DFU groups (Fig. 2b). *CDKN1A* ($p=1.6\times10^{-8}$), an inhibitor of G1 cyclin-dependent kinases, was significantly upregulated in the DFU group. In contrast, *TP53* ($p=0.033$), a tumor suppressor gene with a role in DNA damage foci formation and different stress responses, was significantly downregulated [46]. Furthermore, inflammatory and wound healing markers were significantly upregulated in the DFU group, including *IL-1α* ($p=2.3\times10^{-5}$), *CXCL8* ($p=0.0020$), *SERPINE1* ($p=0.018$), and *MMP10* ($p=0.042$) (Fig. 2c). Specific gene expression levels suggest that DFU and DFS groups have distinct phenotypes with respect to cellular senescence and wound healing.

Similarly, DFU and DFS gene expression was compared for the validated senescence gene set, SenMayo (Fig. S2a) [35]. Of the 125 genes in SenMayo, 14 were significantly upregulated in DFU, including *IL-1α*, *CXCL8*, *SERPINE1*, and *MMP10* evaluated above, as well as *EREG* (Benjamini–Hochberg corrected $p=2.3\times10^{-8}$), *AREG* ($p=3.6\times10^{-4}$), *IL1B* ($p=1.4\times10^{-3}$), *PGF* ($p=1.8\times10^{-3}$), *IGFBP2* ($p=1.9\times10^{-3}$), *JUN* ($p=4.2\times10^{-3}$), *IGFBP6* ($p=0.013$), *PLAUR* ($p=0.014$), *BMP2* ($p=0.029$), and *VEGFA* ($p=0.043$). Two of the SenMayo genes were downregulated: *C3* ($p=3.4\times10^{-4}$), a complement protein, and *CXCL12* ($p=3.4\times10^{-3}$), a homeostatic chemokine. Among the top 20 genes found in SenMayo-enriched human cells, 5 were significantly upregulated in DFU, and none were downregulated (Fig. S2b). The upregulated genes were *CSTA* (Benjamini–Hochberg corrected $p=1.0\times10^{-10}$), *CXCL8* ($p=2.0\times10^{-3}$), *S100A11* ($p=6.4\times10^{-3}$), *S100A12* ($p=0.012$), and *S100A8* ($p=0.046$). Three of these upregulated genes are in the S100 family, which consists of calcium-binding proteins involved in inflammation, cancer, and epidermal differentiation [47]. Next, gene set enrichment analysis of the SenMayo gene set was performed. Like the other gene sets analyzed, the SenMayo gene set was not significantly

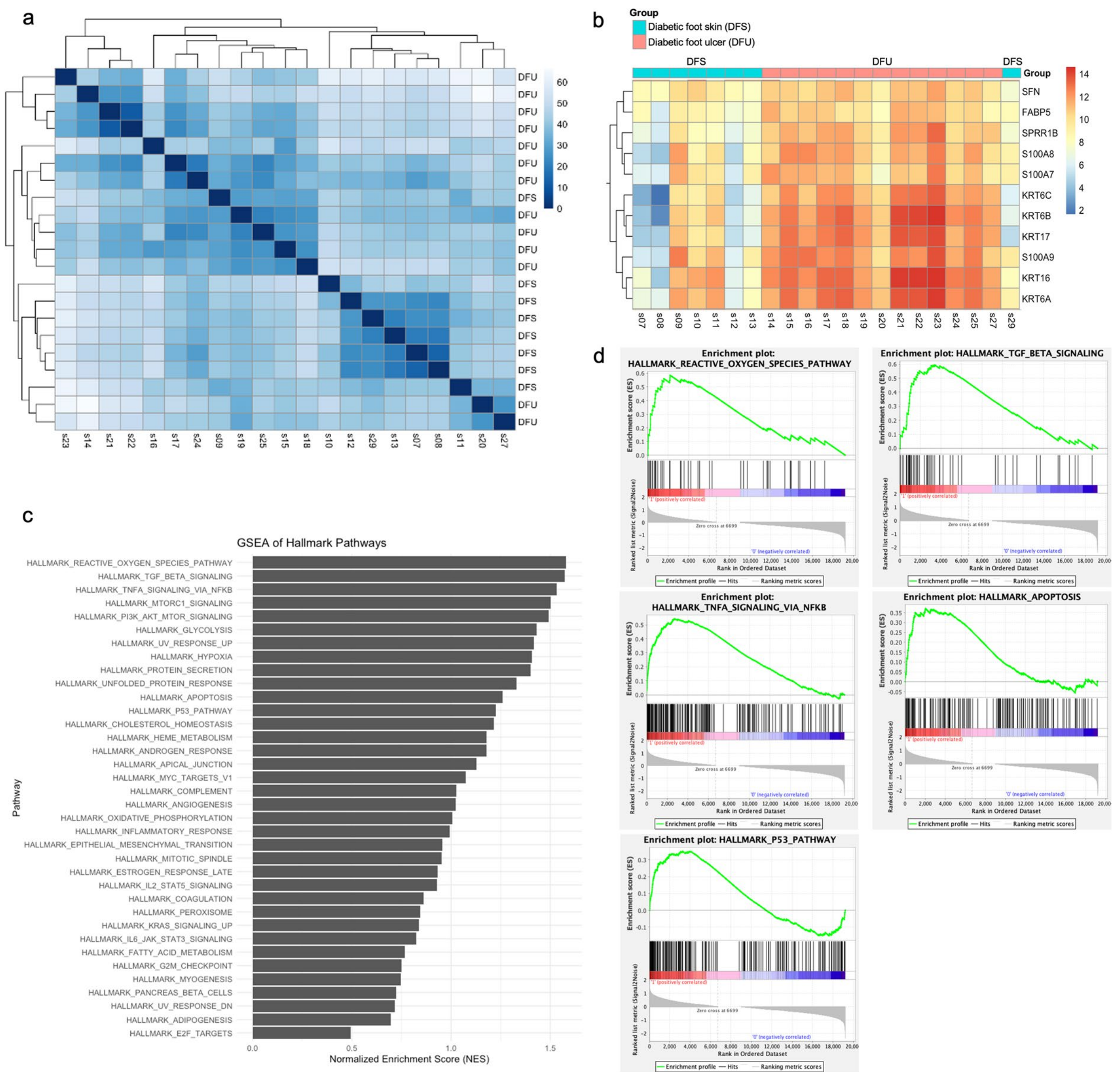


Fig. 1 Hierarchical clustering of RNA sequencing of samples from the diabetic foot ulcer (DFU, $n=13$) and uninvolved diabetic foot skin (DFS, $n=8$) groups. **a** Heat map of sample-to-sample distances. **b** Heat map of count matrix. **c** Gene set enrichment analysis of hallmark gene set, with pathways enriched in DFU compared to DFS. **d** Gene set enrichment analysis of top 3 upregulated pathways in DFU (reactive oxygen species, *TGF-β* signaling, and *TNF-α* signaling via *NF-κB*), as well as apoptosis and p53 pathways

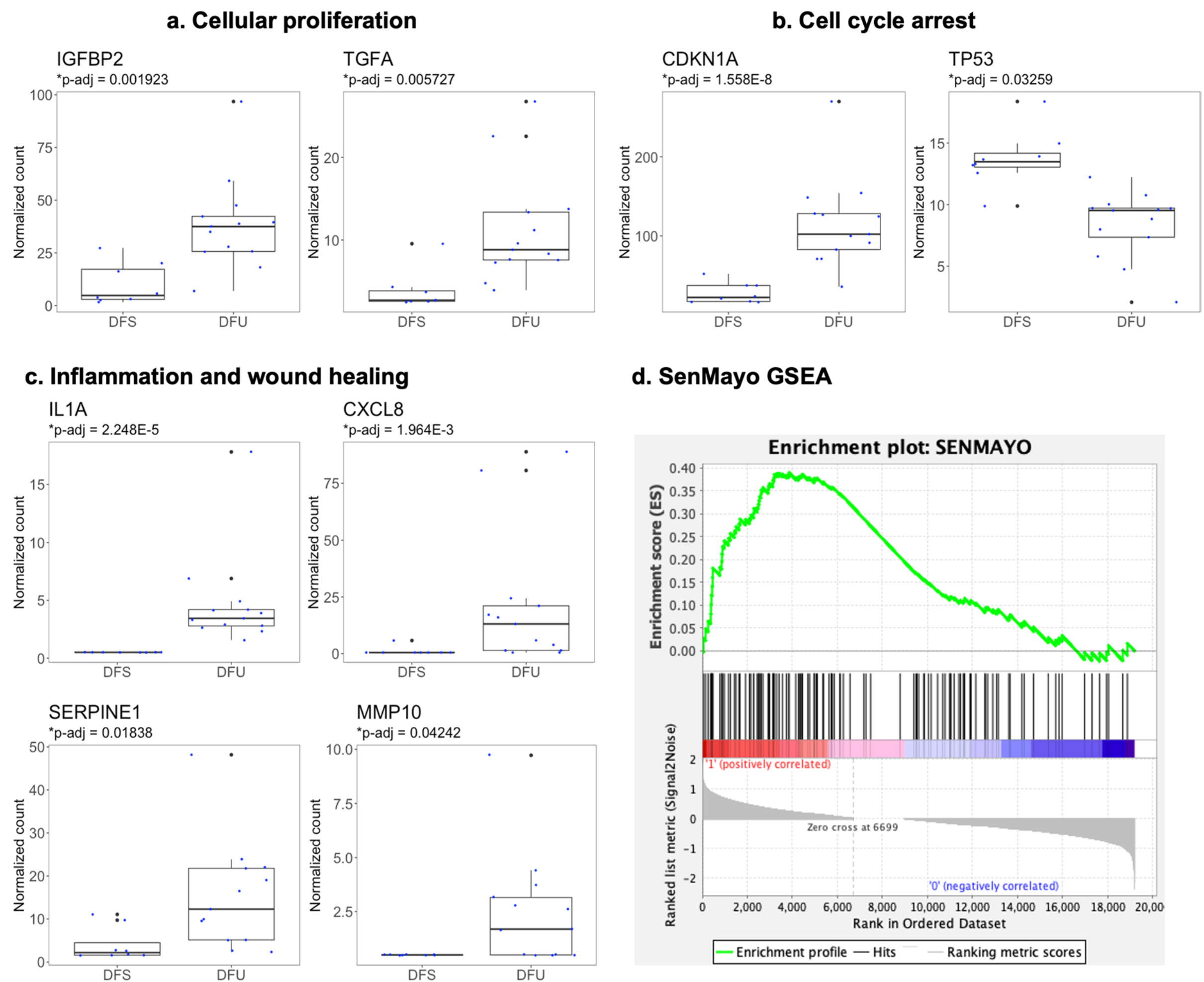


Fig. 2 Differences in cellular senescence profiles, comparing samples from the diabetic foot ulcer (DFU, $n=13$) and uninvolved diabetic foot skin (DFS, $n=8$) groups. Boxplots include 25th (Q_1) and 75th (Q_2) percentiles, interquartile range (IQR), median, and potential outliers ($Q_1-1.5$IQR or $Q_3+1.5$IQR). Wald tests with Benjamini–Hochberg correction were performed to compare DFS and DFU for each gene. Only markers with significantly different expression levels between the two groups (p-adj < 0.05) are presented

upregulated, but it showed enrichment in DFU (NES = 1.04, FDR $q=0.45$) (Fig. 2d).

Cellular senescence profile in diabetic foot ulcer protein–protein interaction networks

To gain more insight into protein–protein interactions, gene network analysis was performed. Prioritized DFU and edge-centered DFS protein–protein interaction networks were derived, and overall networks were significantly different between the DFU and DFS groups (Fig. 3). Paths obtained for the DFS edge-centered network were mostly isolated from each other and included eight senescence-associated markers (*MITF*, *IL-1β*, *MMP2*, *VCAM1*, *CDKN1A*, *MMP9*, *CCL2*, and *TP53*), which were all sources of their genetic paths (Fig. 3a). DFS networks had a mixture of activating and inhibiting protein–protein interactions, with half of the senescence-associated genes activating (*MMP2, VCAM1, CDKN1A,* and *MMP9*), three inhibiting (*MITF*, *IL-1β*, *CCL2*), and *TP53* activating five and inhibiting six downstream genes. In stark contrast, DFU-prioritized and edge-centered networks had paths that were interconnected, and most genes had higher degrees, or more protein–protein interactions from each gene (Fig. 3b,

S3). As senescence-associated genes were used as sources, greater interactions and paths suggest that senescence had greater impact and more downstream consequences in DFU. Eleven senescence-associated markers (*IGFBP4*, *SERPINE1*, *CCL2*, *CDKN1A*, *MMP2*, *TP53*, *CXCL1*, *VCAM1*, *CXCL8*, *MMP9*, and *SPP1*) were included in the prioritized network as sources (Fig. 3b). All protein–protein interactions were activating except for the one from *CDKN1A*. Notably, while all senescence-associated markers in the DFS network were in the DFU network except *MITF* and *IL-1β*, these markers interacted with different genes in the DFU network, leading to different genetic paths in DFS and DFU states.

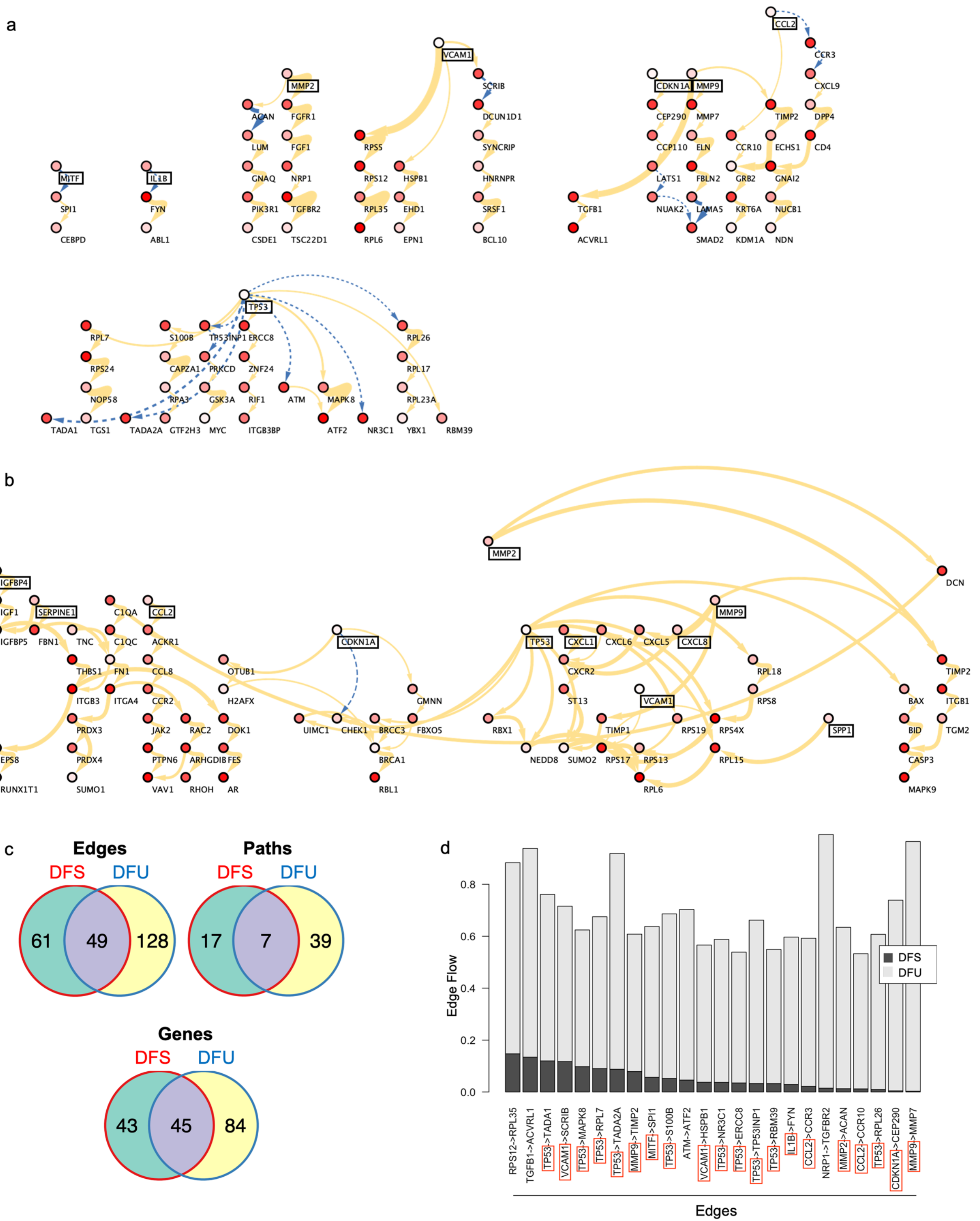
There were more highly expressed genes (129 DFU > 88 DFS), protein–protein interactions, or edges (177 DFU > 110 DFS), and genetic paths (46 DFU > 24 DFS) in diabetic wounding with little overlap (49 common edges, 45 common genes, and 7 common paths) between the DFU and DFS groups (Fig. 3c). Moreover, DFU samples had higher edge flows between genes, suggesting greater protein–protein interaction activity (Fig. 3d). Because cellular senescence markers were used as pathway sources, this indicates greater activity and contribution of cellular senescence in DFU compared to DFS. Key edges, which are protein–protein interactions with greatest flow differences, differed between the DFU and DFS groups. Notably, many key edges involved senescence-associated markers as sources, including *TP53*, *VCAM1*, *MMP9*, *MITF*, *IL-1β*, *CCL2*, *MMP2*, and *CDKN1A*. In fact, all senescence-associated genes were sources of protein–protein interactions or signaling pathways, in the DFS and DFU protein–protein interaction networks. In fact, senescence-associated genes comprised a large proportion of sources in both networks. Their positions indicated that senescence-associated genes were key regulators in the DFS and DFU protein–protein interaction networks.

Although senescence-associated genes were key components of both DFU and DFS protein–protein interaction networks, they differed in expression and interaction partners. In the DFU network, senescence-associated genes had greater expression and more flow to downstream genes, suggesting that these signaling pathways are critical for disease phenotype. Half of the senescence-associated genes in the DFS network had inhibitory interactions, but *CDKN1A* was the only gene that had an inhibitory interaction in the DFU network. Furthermore, all senescence-associated genes that were present in both DFU and DFS networks interacted with different genes in either network. In addition to having more senescence-associated genes in the DFU network, the same senescence-associated genes shifted from inhibiting a set of target genes in DFS to activating a completely different set of genes in DFU. Overrepresentation analysis of the target genes revealed different biological processes associated with DFU and DFS networks (Fig. S4a-c). While the DFS network was enriched in RNA catabolism and apoptosis, the DFU networks were enriched in inflammatory and immune responses, extracellular matrix organization, and protein metabolism.

Network analysis was also performed on protein–protein interaction networks with the top 20 genes in SenMayo-enriched human cells as the gene source list. Similarly, the networks reflected a larger impact of SenMayo genes on DFU than DFS networks (Fig. S5). Again, the SenMayo genes differed in type of interaction, i.e., activating or inhibitory, and interaction partners in DFU and DFS edge-centered subnetworks. Overrepresentation analysis was also performed on targets of the top 20 SenMayo genes in the prioritized DFU protein–protein interaction network (Fig. S4d). Like the DFU-prioritized network with senescence markers as the source list, the targets were enriched in immune responses and inflammation, specifically, upregulating migration and chemotaxis, especially of leukocytes, and cytokine signaling.

Discussion

To prevent infections and amputations, DFUs require frequent and aggressive management, including wound debridement and dressings, offloading pressure, and infection control [4, 10]. Even with current treatments, patients with DFUs are at risk for significant morbidity and mortality; they are associated with a 2.5 times higher risk of death at 5 years compared to those without DFUs [10]. Understanding the gene pathways in DFUs could reveal new and more effective targets for DFU management. In this work, we elucidated the role of senescence-associated genes in DFU compared to DFS. By leveraging a computational system biology approach, we further described senescence-associated gene activity by contrasting their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◂**Fig. 3** Arrow color represents activation (solid yellow) or inhibition (dashed blue) of target protein. Arrow thicknesses represents weight of interactions. Node colors represent node flows. Senescence-associated markers are boxed in black. **a** Uninvolved diabetic foot skin (DFS) edge-centered protein–protein interaction network. **b** Diabetic foot ulcer (DFU) prioritized protein–protein interaction network. **c** Significant edges, paths, and genes found in the prioritized protein–protein interaction networks of diabetic foot ulcers (DFU, $n=13$), uninvolved diabetic foot skin (DFS, $n=8$), or both. **d** Key edges, or protein–protein interactions, in the diabetic foot ulcer (DFU) network compared to the uninvolved diabetic foot skin (DFS) network. Senescence-associated markers are boxed in red

interactions in DFS and DFU protein–protein interaction networks. To our knowledge, this is the first report characterizing cellular senescence pathways in human diabetic foot ulcers.

Cellular senescence has been postulated to be a key driver of diabetic foot ulcer pathogenesis [12, 26, 48]. Consistent with previous studies, we found that DFUs had elevated expression of senescence-associated genes and pathways compared to DFS [12, 26]. Importantly, in our studies of human chronic wounds, we found that elevated expression of *CDKN1A*, as obtained from QRT-PCR, predicted wound chronicity (data not shown). Moreover, we compared $p16^{INK4a}+$cells in diabetic and non-diabetic chronic wounds, as $p16^{INK4a}$ is a canonical marker of senescence, and demonstrated that diabetic wounds had increased senescence burden. However, some senescence-related genes that were upregulated in other wound models, such as *CDKN2A*, *CYR61*, *PDGFα*, and *CXCR2*, were not significantly upregulated in DFU that we studied [2, 26, 31]. These discrepancies could be due to differences in depth of skin sampled or wound types, chronicity, and models.

Our analysis is consistent with previous reports showing that *TP53* is a crucial part of the senescence response in wound healing [31, 49, 50]. We observed *TP53* downregulation as opposed to upregulation found in a previous study, which could be explained by the chronicity of diabetic foot ulcers, likely resulting in a late senescence phenotype [31]. However, other studies have shown that absence of p53, the protein encoded by *TP53*, was associated with a markedly increased SASP, which was consistent with our findings [51]. Moreover, *TP53* was shown to have different gene interactions in the context of different diseases [40]. Other analyses of senescence gene networks also describe *TP53* as having some of the highest degree scores [52] and being a crucial regulator of cellular senescence [53].

The sole gene with an inhibitory interaction in the prioritized DFU network was *CDKN1A*, which, notably, had an activating interaction in the DFS network. p21, the protein encoded by *CDKN1A*, is known to protect against genotoxic stress but also mediate cellular senescence [54, 55]. According to our DFS network, these contrasting roles of *CDKN1A* could be mediated by interactions with different downstream partners. In fact, the inhibitory interaction of p21 in the DFU network could be consistent with actions of p21 to inhibit cell cycle and DNA repair pathways, particularly in cellular senescence [54, 55]. In addition, p21 was shown to mediate senescent phenotypes in p53-dependent and -independent pathways [55, 56]. In the DFU network, *CDKN1A* and *TP53* had separate pathways as well as a common pathway that converged on *BRCA1*, supporting the idea of having both *TP53*-dependent and -independent gene activity.

This work shows that senescence-associated genes interact with different genes in DFS vs. DFU and that these interactions are integral to each phenotype, implicating senescence in DFU pathophysiology. Analysis of single-cell transcriptomic or proteomic data could uncover the signaling and cellular mechanisms underlying the pathogenesis of DFU and roles of senescent cells in the dynamic wound healing process. Understanding cellular mechanisms could also lead to more precise targeting of cell types or signals in the development of therapeutics for wound care management.

Our findings also support further study of the use of senolytics, agents that selectively eliminate senescent cells, and senomorphics, agents that inhibit SASP factors, to treat or prevent DFUs. The identification of key senescence-associated genes and gene interactions could aid in the design and choice of senolytics and senomorphics for DFU therapeutics and potential monitoring of the progression or improvement of DFUs.

In summary, many senescence-associated genes were found to be differentially expressed and interacted with different genes in full-thickness skin biopsies of DFU wound edges compared to uninvolved DFS. The difference in the roles of senescence-associated genes in DFS and DFU protein–protein interaction networks likely suggests that they contribute to DFU formation or progression.

Limitations of the study

Because of the lack of a rigorous, robust transcriptomic definition of cellular senescence profile in skin, some senescence markers could have been excluded from this analysis. Multiple senescence gene sets have been proposed, and some differ from the gene set used in this work [35, 57, 58]. However, multiple sources were reviewed to obtain a list of pertinent senescence-associated genes, and the targeted differential gene analysis was balanced with the use of unbiased network analysis.

The use of bulk RNA sequencing data prevented the study of distinct cellular gene expression and cell types. However, senescent cells are rare in vivo, and bulk RNA sequencing provides more depth than most single-cell transcriptomic techniques [59]. Moreover, by studying protein–protein interaction networks in addition to differential gene expression, we obtained substantially more insight into how transcriptomic activity differed in DFS vs. DFU. Future studies could validate these findings by quantifying target or downstream proteins of the senescence-associated genes in animal models or patient samples.

Furthermore, although we did not obtain the topology of the entire protein–protein interaction network, using NetDecoder, we captured the key information flows that differentiated protein–protein interaction networks in the DFS vs. DFU phenotypes [40]. This enabled us to study context-dependent roles of senescence-associated genes in the etiology of DFU. Future studies could aim to evaluate DFU transcriptomics, proteomics, or epigenomics and study signaling at a single-cell resolution. They could also contribute to unveiling sex differences in wound pathology [60].

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