Altered regulation of mesenchymal cell senescence in adipose tissue promotes pathological changes associated with diabetic wound healing

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Pathologic diabetic wound healing is caused by sequential and progressive deterioration of hemostasis, inflammation, proliferation, and resolution/remodeling. Cellular senescence promotes wound healing; however, diabetic wounds exhibit low levels of senescent factors and accumulate senescent cells, which impair the healing process. Here we show that the number of p16INK4B+PDGFRα+ senescent mesenchymal cells in adipose tissue increases transiently during early phases of wound healing in both non-diabetic mice and humans. Transplantation of adipose tissue from diabetic mice into non-diabetic mice results in impaired wound healing and an altered cellular senescence-associated secretory phenotype (SASP), suggesting that insufficient induction of adipose tissue senescence after injury is a pathological mechanism of diabetic wound healing. These results provide insight into how regulation of senescence in adipose tissue contributes to wound healing and could constitute a basis for developing therapeutic treatment for wound healing impairment in diabetes.
Cellular senescence is a process in which cells cease dividing, and it is activated in response to numerous stressors, including exposure to genotoxic agents, nutrient deprivation, hypoxia, mitochondrial dysfunction, and oncogene activation. One of the features of senescent cells is that they undergo distinctive phenotypic alterations called senescence-associated secretory phenotype (SASP), wherein they secrete cytokines, chemokines, growth factors, and matrix metalloproteases (MMPs). Senescent cells exert beneficial or detrimental effects on both tissue remodeling and dysfunction through cell-to-cell communication and SASP expression. Beneficial effects of senescence include tissue remodeling and regeneration. Increased senescent cells are observed in mouse skin during normal wound healing, heart regeneration in zebrafish and neonatal mice, and tissue regeneration in salamanders and zebrafish. By contrast, chronically accumulated senescent cells exhibit an anti-apoptotic phenotype and a SASP, which induce detrimental effects including lung fibrosis, kidney and heart dysfunction, impairment of the central neuronal system, and muscle weakness.

During normal wound healing, the numbers of senescent fibroblasts and endothelial cells increase transiently in the skin, and wound closure is accelerated by the secretion of platelet-derived growth factor-AA (PDGF-AA), which induces myofibroblast differentiation. Senescent cells are also induced in fibroblasts during skin wound healing by either cellular communication network factor 1 (CCN1) or CCN2, which both inhibit excess fibrosis. Transiently induced to allow matrix formation and then regresses. By contrast, chronic diabetic wounds show chronically larger numbers of senescent fibroblasts and macrophages that produce high levels of matrix-degrading proteases and inflammatory cytokines. These enzymes cause uncontrolled inflammation and inhibit wound healing. When more than 15% of cells isolated from venous ulcers are senescent fibroblasts, wound healing is delayed. However, the regulatory mechanisms that govern these properties of cell senescence, specifically concerning their contribution to modifying tissue homeostasis and pathological conditions by producing various cytokines and growth factors, remain unknown.

Adipose tissue is attracting attention for promoting wound healing. Within subcutaneous adipose tissue, stromal vascular cells and their subsets release growth factors and cytokines critical for neovascularization and wound repair. Subcutaneous tissue has garnered attention particularly in obesity and type-2 diabetes because increased inflammation can alter the outcome of wound repair through the production of large numbers of hormones and cytokines, such as tumor necrosis factor α (TNF-α), Interleukin 6 (IL-6), and plasminogen activator inhibitor 1 (PAI-1); these proteins are the major components of the SASP. Senescent cell accumulation in the adipose tissue of patients with diabetes and obesity is associated with insulin resistance and systemic inflammation. Furthermore, subcutaneous adipose tissue in obese subjects is characterized by an excessive amount of interstitial fibrosis and phenotypic changes in human pre-adipocytes, which may contribute to tissue deterioration.

In this study, we investigated cellular senescence in skin and subcutaneous adipose tissue during the wound healing process using a Lepr<sub>db/db</sub> type-2 diabetic mouse model. We found that growth of subcutaneous adipose tissue reached the wound site in control mice; however, in the diabetic Lepr<sub>db/db</sub> mice, the adipose tissue remained below the granulating tissue, and thereby impaired wound healing. In addition, transplantation of Lepr<sub>db/db</sub>-derived adipose tissue into control mice impaired wound healing. In the adipose tissue of control mice, senescent cells increased at 2 days post-wound (DPW) and decreased at 8 DPW, whereas senescent cells in Lepr<sub>db/db</sub> mice remained low at 2 DPW and increased slightly at 8 DPW. The composition of SASP factors differs between the control and Lepr<sub>db/db</sub> mice, resulting in the inhibition of fibroblast migration. Our results demonstrate that diabetic adipose tissue impairs transient senescence during pathological healing, which causes deteriorated wound healing, suggesting that cellular senescence of adipose tissue could be a therapeutic target for diabetic ulcers.

**Results**

**Type-2 diabetic mice models have impaired wound healing.** C57BLKS/J lar +/+ Lepr<sub>db/+</sub>/Lepr<sub>db/+</sub> (Lepr<sub>db/+</sub>) and C57BLKS/J lar -/+ +Lepr<sub>db</sub> (Lepr<sub>db/+</sub>) mice were used in this study as type-2 diabetic and control mouse models, respectively. Blood glucose level ≤200 mg/dL (Lepr<sub>db/+</sub>) or > 300 mg/dL (type-2 diabetic, Lepr<sub>db/db</sub>) was used to confirm the absence or presence of diabetes, respectively (Fig. 1a). Full-thickness excisional skin wounds were created on the backs of Lepr<sub>db/db</sub> and Lepr<sub>db/+</sub> mice, and the wound closure rate was evaluated at 2, 5, and 8 DPW. At each time-point, the wound closure rates were impaired to a greater extent in Lepr<sub>db/db</sub> mice than in Lepr<sub>db/+</sub> mice (Fig. 1b–c). Histological analysis involving hematoxylin and eosin (H&E) staining and Masson trichrome staining, indicated the presence of adipose tissues immediately under the wound eschar region in Lepr<sub>db/+</sub> mice at 2 DPW albeit not in Lepr<sub>db/db</sub> mice (Fig. 1d). At 8 DPW in Lepr<sub>db/+</sub> mice, there was less adipose tissue under the wound eschar as the wound closed. Conversely, in Lepr<sub>db/db</sub> mice, we observed increased cell infiltration beneath the wound eschar region, and thus more adipose tissue and collagen deposition at the wound closing region (Fig. 1d). Our quantitative data indicates significantly more cell infiltration and collagen deposition in Lepr<sub>db/db</sub> mice along with significantly less area covered by adipose tissue (Fig. 1e–f).

Next, we used antibodies specific for platelet-derived growth factor receptor α (PDGF-Rα) and a smooth muscle actin (α-SMA) to identify mesenchymal stromal cells that produce ECM and play important roles in wound healing of subcutaneous tissue of the skin. PDGF-Rα is a cell surface receptor tyrosine kinase expressed in mesenchymal stromal cells in a variety of tissues and is also used as a marker for adipose progenitor cells. Differentiation of mesenchymal stromal cells into α-SMA + myofibroblasts is necessary for acute wounds to heal but increased PDGF-Rα activation causes progressive fibrosis in many organs, including the skin. Immunohistochemical analysis indicated a greater percentage of α-SMA + cells in interstitial connective tissue in Lepr<sub>db/+</sub> mice (Fig. 1h–i, and Supplementary Fig. 1). However, Lepr<sub>db/db</sub> mice showed a higher percentage of PDGF-Rα + cells but a lower percentage of α-SMA + cells in interstitial connective tissue compared with Lepr<sub>db/+</sub> mice (Fig. 1h–i). In addition, Lepr<sub>db/db</sub> mice showed an increased population of cells positive for both PDGF-Rα and α-SMA in interstitial connective tissue (Fig. 1h–i).

The panniculus carnosus is a thin striated muscular layer with interstitial connective tissue under it. The panniculus carnosus is a thin striated muscular layer with interstitial connective tissue under it. The panniculus carnosus is a thin striated muscular layer with interstitial connective tissue under it.
The skin wound region of Lepr<sup>db/db</sup> mice. The excisional skin wound was sutured closed after transplantation to stabilize the transplanted tissue (Fig. 2a). We evaluated the histological wound healing score at 8 DPW using H&E staining and Masson trichrome staining<sup>36</sup>. Three investigators each assigned a histological score in a blinded fashion, and the corresponding intraclass correlation coefficient (ICC) was 0.79. The histological wound healing scores for mice transplanted with Lepr<sup>db/db</sup> adipose tissue (Lepr<sup>db/db</sup> ATT mice) were significantly lower than those for mice transplanted with Lepr<sup>db/+</sup> adipose tissue (Lepr<sup>db/+</sup> ATT mice) and for sham control mice (Fig. 2b–c). Immunohistochemical analysis indicated a higher percentage of PDGFRα+ cells and a lower percentage of α-SMA+ cells in the interstitial connective tissue of Lepr<sup>db/db</sup> ATT mice compared with Lepr<sup>db/+</sup> ATT mice (Fig. 2d–e). Moreover, cells...
positive for both PDGFRα and α-SMA were more prevalent in the interstitial connective tissue of Leprdb/db ATT mice (Fig. 2d–e), which was also observed for impaired wound healing in Leprdb/db mice. We also detected greater expression of Pdgfra but not Acta2 in skin cells of Leprdb/db ATT mice using quantitative reverse transcription PCR (qRT-PCR) (Fig. 2f). These results suggest that transplantation of adipose tissue derived from Leprdb/db mice into Leprdb/+ mice impairs wound healing.

To ascertain the role of cell movement in transplanted adipose tissue, we investigated the localization of CM-Dil–labeled adipose tissue–derived cells in wound regions. We found CM-Dil+/ adipose cells in layers below the interstitial connective tissue at 2 DPW (Fig. 2g,h). Quantitative data are shown as means ± SE in either dot plots or box-and-whisker plots with IQRs and 1.5 times the IQR. p-values were determined using a two-tailed Student’s t-test or the Tukey method for one-way ANOVA (*p < 0.05 and **p < 0.001).

Fig. 2 Transplantation of adipose tissue derived from Leprdb/db mice into Leprdb/+ mice impairs wound healing. a Schematic diagram of the procedure for the adipose tissue transplantation (ATT) experiments. b Representative image of H&E staining and MT staining of a wound at 8 DPW and (c) the quantitative analysis of the wound healing histological score (n = 8 in the ATT group and n = 4 in the sham group). d, e Representative images of PDGFRα and α-SMA immunostaining of a wound edge at 8 DPW and quantitative data on the percentage of PDGFRα and α-SMA + cells in interstitial connective tissue (n = 4 for each group). f Relative levels of Acta2 and Pdgfra mRNA in Leprdb/+ and Leprdb/db mouse skin at 8 DPW. g, h Localization of CM-Dil–labeled adipose tissue–derived cells within wound regions and quantitative data (n = 3 for each group).
Senescence-related gene expression levels were transiently increased in subcutaneous adipose tissue during wound healing. Senescent cells play beneficial roles in wound healing by expressing SASP factors including PDGF-AA, CCN1, VEGF, and Serpine1, which promote the production of ECM and prevent excessive fibrosis. By contrast, accumulation of senescent cells is observed in diabetic ulcers. We speculated that different types of cellular senescence occur between normal and diabetic wound healing in response to wounds. Hence, we investigated the time-dependent change in the expression of senescence-related factors during wound healing in Leprdb/db and Leprdb/db + mice. We harvested skin and subcutaneous adipose tissue at pre-wounding, 2 DPW, and 8 DPW. Mouse skin and adipose tissue are divided by interstitial connective tissue. In skin tissue, damage induced the expression of cellular senescence markers. Cdkn1a mRNA transcription levels increased at 8 DPW relative to those at pre-wound and 2 DPW in both Leprdb/db + and Leprdb/db + mice (Fig. 3a). Trp53 transcript level decreased at 2 DPW relative to that of the pre-wound level in Leprdb/db + mice, but the level in Leprdb/db + mice was not significantly different (Fig. 3a). Cdkn2b is a marker of cell senescence. The transcription level of Cdkn2b mRNA increased at 2 DPW relative to that of the pre-wound level in Leprdb/db + mice and decreased at 8 DPW relative to that of the level at 2 DPW in Leprdb/db + mice (Fig. 3a). The mRNA transcription level of the SASP-related factor Serpine1 was essentially the same in both mouse models, but the Serpine2 transcription level increased at 8 DPW in Leprdb/db + mice (Fig. 3a).

Next, we measured mRNA levels in subcutaneous adipose tissue during wound healing. The levels of Cdkn2b and Trp53 mRNA in Leprdb/db + mice were higher at 2 DPW than at pre-wounding while the level of Cdkn2b transcript was lower at 8 DPW (Fig. 3a). In Leprdb/db + mice, Cdkn2b and Trp53 transcription levels did not significantly change during wound repair, and increased levels of Cdkn1a, Serpine2, and Tgfb1 transcription, relative to 2 DPW, were observed at 8 DPW (Fig. 3a). The mRNA transcription levels of Serpine1 and Iil6 did not change in Leprdb/db + mice during wound healing, but Leprdb/db + mice exhibited a time-dependent decrease in Serpine1 and a transient increase in Iil6 levels (Fig. 3a). To clarify these differences in gene expression patterns related to senescence during the wound healing process between Leprdb/db + and Leprdb/db + mice, principal component analysis (PCA) was performed (Fig. 3b–c). In skin tissue, PCA plots indicate that both Leprdb/db + mice and Leprdb/db + mice had similar patterns related to senescence during the wound healing process (Fig. 3b). Conversely, PCA plots in adipose tissue indicate that Leprdb/db + mice experienced large changes in senescence-related gene transcription at 2 DPW, whereas at 8 DPW, the transcription of these genes returned to similar levels to what was observed pre-wounding (Fig. 3c). By contrast, Leprdb/db + mice showed minimal changes in senescence-related gene transcription during wound healing (Fig. 3c).

To identify the cell type that exhibits senescence in subcutaneous adipose tissue, we performed immunohistochemistry for p15INK4B and PDGFRα. Encoded by CDKN2B, p15INK4B is an INK4 class of cell-cycle inhibitors. We verified that the percentage of PDGFRα + cells was higher at 8 DPW in Leprdb/db + adipose tissue and that PDGFRα mRNA expression was higher in 8 DPW but significantly lower in Leprdb/db + adipose tissue (Fig. 4a–c). In Leprdb/db + adipose tissue, the percentage of p15INK4B + in PDGFRα + cells increased at both 2 and 8 DPW, and the fluorescence intensity of p15INK4B in PDGFRα + cells transiently increased at 2 DPW (Fig. 4a, d–e). However, in Leprdb/db + adipose tissue, an increase in the percentage of p15INK4B + in PDGFRα + cells was delayed at 8 DPW relative to that observed for Leprdb/db + mice (Fig. 4a, d–e). In addition, p15INK4B fluorescence intensity in PDGFRα + cells in Leprdb/db + mice was higher at 2 DPW and was similarly high at 8 DPW (Fig. 4a, d–e). To determine if p15INK4B is a senescence marker, we performed co-immunostaining with anti-p15INK4B, p16INK4A, and p21CIP1/WAF1 antibodies. We observed that 75% of p15INK4B + cells expressed either p16INK4A, p21CIP1/WAF1, or both (Supplementary Fig. 2a–b).

We also determined the expression of SPIDER-β-Gal, a fluorescent probe for detecting SA-β-Gal, in PDGFRα + cells. In Leprdb/db + adipose tissue, the percentage of SPIDER-β-Gal + in PDGFRα + cells was higher at 2 DPW and lower at 8 DPW (Supplementary Fig. 3a–b). However, in Leprdb/db + adipose tissue, the percentage of SPIDER-β-Gal + PDGFRα + cells was higher at 8 DPW than at pre-wounding and 2 DPW (Supplementary Fig. 3a–b).

These results suggest that cellular senescence rapidly occurs in Leprdb/db + adipose tissue after wounding, and cell senescence is transient. However, in Leprdb/db + adipose tissue, cell senescence is delayed after wounding, and this delay may allow for accumulation of senescent cells in diabetic wound healing.

Senescent PDGFRα + cells accumulate in subcutaneous adipose tissue during wound healing in diabetic patients. To determine whether the number of senescent cells increases in subcutaneous adipose tissue during wound healing in diabetic and non-diabetic patients, we performed immunohistochemical analysis of PDGFRα + cells and looked for expression of p15INK4B and γH2A.X, which is a DNA damage–induced cell senescence marker. Patient demographics are presented in Table 1. In non-diabetic patients, a negative correlation was observed between the percentage of p15INK4B + cells in PDGFRα + cells and the time post-wounding (Fig. 4a, c). By contrast, in diabetic patients, we observed a high positive correlation between the percentage of PDGFRα + cells that were either p15INK4B or γH2A.X + and the time post-wounding (Fig. 4b, c, d). To determine if p15INK4B is a marker of senescence in human adipose tissue, we performed co-immunostaining with antibodies against p15INK4B, p16INK4A, and p21CIP1/WAF1. We found that 80% of cells that express p15INK4B also express either p16INK4A, p21CIP1/WAF1, or both (Supplementary Fig. 4a–c). These results indicate that cell senescence gradually accumulates in diabetic adipose tissue during wound healing, but in non-diabetic patients, cell senescence occurs robustly in adipose tissue during the early stages of wound healing.

SASP factors derived from Leprdb/db mice adipose tissue impair wound healing. Overall, our results suggest that the difference in cell senescence between diabetic and non-diabetic adipose tissue influences wound healing. Because adipose-derived cells stay under the interstitial connective tissue during wound healing, SASP factors may contribute to wound healing. Therefore, we determined the differences in the expression levels of SASP factors in Leprdb/db + and Leprdb/db + adipose tissue. Adipose tissue was collected from Leprdb/db + and Leprdb/db + mice at pre-wound, 2 DPW, and 8 DPW, and organ culture was performed to harvest SASP-containing culture media. A proteome profiler antibody array was used to investigate whether SASP factors were present in the culture media. At pre-wound, adipose tissue in Leprdb/db + mice had higher expression levels of IGFBP3 and IGFBP5, but Leprdb/db + mice have higher expression levels of CCL6 and CCL11. At 2 DPW, both Leprdb/db + and Leprdb/db +
adipose tissue had increased expression levels of various secretory factors; however, the composition was different (Fig. 5a, b, and Supplementary Fig. 5). The Lepr<sup>db/+</sup> adipose tissue exhibited higher expression levels of Adiponectin, Ang2, CCL2, CRP, CXCL3, IL1Ra, MMP9, VEGF, and CCN4 (Fig. 5a, b, and Supplementary Fig. 5). By contrast, Lepr<sup>db/db</sup> adipose tissue had higher expression levels of IL11, CCL11, and MMP3 (Fig. 5a, b, and Supplementary Fig. 5). At 8 DPW, the levels of VEGF, IGFBP family members, and Serpin F1, which are important factors for wound healing<sup>3,41</sup>, increased in Lepr<sup>db/+</sup> adipose tissue-derived conditioned media, but Lepr<sup>db/db</sup> conditioned media continued to have increased expression levels of CCL6, CCL11, and CXCL2.

**Fig. 3 Cellular senescence in subcutaneous adipose tissue during wound healing in Lepr<sup>db/+</sup> and Lepr<sup>db/db</sup> mice.**

- **a** Relative mRNA expression of senescence-related genes at pre-wound, 2 DPW, and 8 DPW in Lepr<sup>db/+</sup> and Lepr<sup>db/db</sup> mice (n = 7 for each group).
- **b, c** Principal component analysis (PCA) of the levels of senescence based on senescence-related gene expression at pre-wound, 2 DPW, and 8 DPW for Lepr<sup>db/+</sup> and Lepr<sup>db/db</sup> mice in skin and adipose tissue. Quantitative data are presented as means and medians with IQRs and 1.5 times the IQR and are shown as box-and-whisker plots. p-values were determined using the Tukey method for one-way ANOVA (*p < 0.05).
Finally, to determine whether the conditioned media derived from Leprdb/+ or Leprdb/db adipose tissue promotes or inhibits wound healing, we performed wound healing assays that involved treating wounds with conditioned media collected from 2DPW adipose tissue. We found that the conditioned medium derived from Leprdb/+ adipose tissue promotes wound closure relative to the control medium at 1 and 2 d after wounding, while the conditioned medium derived from Leprdb/db adipose tissue delays wound closure relative to Leprdb/+ adipose tissue at 3 and 4 d after wounding (Fig. 6c, d).
Fig. 4 p15INK4B expression in PDGFRα+ cells in subcutaneous adipose tissue during wound healing in Leprdb/+ and Leprdb/db mice. a Representative images of adipose tissue following immunostaining for PDGFαR and p15INK4B. Samples were collected at pre-wounding, 2 DPW, and 8 DPW. b–e Percentage of PDGFαR+ cells (Leprdb/+ pre-wounding: n = 3; Leprdb/+ 2 DPW: n = 4; Leprdb/+ 8 DPW; n = 3; Leprdb/db pre-wounding: n = 3; Leprdb/db 2 DPW: n = 3; Leprdb/db 8 DPW; n = 3) and PDGfra mRNA levels in adipose tissue at pre-wounding, 2 DPW, and 8 DPW (Leprdb/+ pre-wounding: n = 6; Leprdb/+ 2 DPW: n = 7; Leprdb/+ 8 DPW: n = 7; Leprdb/db pre-wounding: n = 7; Leprdb/db 2 DPW: n = 7; Leprdb/db 8 DPW; n = 6). f–i Percentage of PDGFRα+ and p15INK4B+ cells and fluorescence intensity of p15INK4B in PDGFRα+ cells (Leprdb/+ pre-wounding: n = 3; Leprdb/db 2 DPW: n = 4; Leprdb/db 8 DPW; n = 3; Leprdb/db pre-wounding: n = 3; Leprdb/db 2 DPW: n = 3; Leprdb/db 8 DPW; n = 3). Quantitative data are presented as box-and-whisker plots with IQRs and 1.5 times the IQR. p-values were determined using the Tukey method for one-way ANOVA (*p < 0.05 and **p < 0.001).

Discussion
Cellular senescence contributes to wound healing in both the normal and pathological healing processes, thereby reducing the role that cellular senescence plays in wound healing impairment, and modulating this senescence presents a therapeutic approach to reduce this impairment. In normal wound healing, the number of senescent cells in mice skin increases 3–6 DPW and decreases 9 DPW, and genetic depletion of p16INK4A-expressing senescent cells delays wound healing. Senescent fibroblasts and endothelial cells in the dermis accelerate wound closure by secreting platelet-derived growth factor (PDGF) AA, which binds to its tyrosine kinase receptor PDGFα, thereby inducing myofibroblast differentiation.

Given that the expression pattern of senescence-related genes, including Cdkn2b, by PCA did not differ between Leprdb/db and Leprdb/+ in skin cells post-wounding, skin senescence in diabetic wounds should be similar to that during normal wound healing. In contrast to expectations, we observed a rapid but transient increase in senescent cells in subcutaneous adipose tissue for the control; however, the number of senescent cells in diabetic Leprdb/db adipose tissue gradually increased post-wounding. Transplantation of subcutaneous adipose tissue of diabetic Leprdb/+ into normal dermal tissue inhibits wound healing and leads to more PDGFRα+ cells in Leprdb/db interstitial connective tissue layer, while a-SMA + myofibroblasts of interstitial connective tissue infiltrated the wound site with adipose tissue in control. These data indicate that the senescence function of subcutaneous adipose tissue may induce PDGFRα+ cells to differentiate into myofibroblasts in the control interstitial connective tissue layer, but the delay in cellular senescence and the difference in SASP components in diabetic subcutaneous adipose tissue may limit the differentiation of PDGFRα+ cells into myofibroblasts, resulting in impaired wound healing.

We found that SASP factor expression levels differ between Leprdb/db+ and diabetic Leprdb/db-derived adipose tissue, which affects wound healing in vitro. In diabetic wounds, VEGF and CCL2 enhance wound healing by promoting angiogenesis and regulating macrophages, respectively. VEGF secretion increased at 2 DPW in Leprdb/+ mouse adipose tissue but not in Leprdb/db adipose tissue. CCL2 secretion also increased at 2 DPW in adipose tissue in Leprdb/+ mice. The levels of CCN4, a senescence regulator that enhances wound healing by regulating dermal fibroblast cell migration, proliferation, and ECM expression, increased at 2 DPW in Leprdb/+ mouse adipose tissue. By contrast, diabetic Leprdb/db adipose tissue exhibited higher secretion of IL11, CCL11, and MMP3 at 2 DPW. IL11 is a member of the IL6 family of cytokines and binds to IL11 receptor subunit alpha (IL11RA), which is expressed on stromal cells and promotes tissue fibrosis. In atopic dermatitis, the number of IL11-expressing cells is elevated in skin biopsy specimens relative to controls, and a significant correlation exists between IL11 and type I collagen deposition.

MMPs, known SASP factors, are important regulators of ECM degradation and deposition, and the timing and level of MMP activation are vital for determining whether successful wound healing or chronic non-healing is observed. In normal wound healing, MMP9 expression increases concurrent with fibroblast migration into the wound area, and MMP3 levels increase at re-epithelialization. In this study, the levels of MMP9 secretion increased in Leprdb/+ derived adipose tissue at 2 DPW, and MMP3 levels increased at 8 DPW. However, in Leprdb/db-derived adipose tissue, MMP3 increased at both 2 DPW and 8 DPW, suggesting that dysregulation of MMPs in adipose tissue results in wound healing impairment in Leprdb/db mice. Furthermore, the secretion levels of IGFBP3 and Serpin F1 in Leprdb/+ adipose tissue increased at 8 DPW. IGFBP3 is one of 6 structurally related IFGPs that bind to IGF peptides with high affinity, and the IGFBP3:IGF1 complex binds to fibrin clots and concentrates at wound sites to facilitate wound healing. Serpin F1 is a pigment epithelium-derived factor (PEDF), and PEDFs contribute to the resolution of wound healing by causing regression of immature blood vessels and stimulating maturation of the vascular microenvironment, which promotes a return to tissue homeostasis after injury. Our findings suggest that Leprdb/+ adipose tissue–secreted SASP factors promote cell migration. By contrast, Leprdb/db adipose tissue–secreted SASP factors may potentially induce wound healing impairment.

In this study, we focused on acute wounding in our mouse model. We have previously demonstrated the importance of transient senescence in the healing of acute muscle injury. Moreover, we found that this transient senescence does not occur during chronic muscle inflammation, which can be ameliorated by inducing senescence among mesenchymal stroma cells. Therefore, we suspect that diabetes may cause changes to the characteristics of acute senescence post wounds on the skin. Following acute wounding of diabetic mice, senescence is delayed in subcutaneous adipose tissue; however, senescent cells are present in subcutaneous adipose tissue of diabetic patients over 60 d post-wounding, which suggests a chronic wound. Previous studies have demonstrated the importance of transiently induced senescence for acute wound healing whereas chronic senescence induces pathological conditions. For example, transient senescence promotes collagen deposition after acute damage and limits fibrosis. By contrast, SA-β-Gal+ senescent cells are present in chronic wounds, and the presence of more than 15% senescence in populations of cells isolated from venous ulcers may indicate a “difficult to heal” ulcer.

In this study, we could not determine how delayed senescence contributes to the accumulation of senescent cells in diabetic chronic wounds. While senescent cells generated by acute inflammation are removed by immune cells through the action of SASP, accumulated senescent cells acquire immune evasion and anti-apoptotic properties in the chronic inflammatory environment. In the future, it will be necessary to determine how the senescence induction after cell damage is related to the accumulation of senescent cells found in diabetic wounds. Regarding the accumulation of senescent cells in subcutaneous adipose tissue, Rouault et al. recently reported that SA-β-Gal activity is seven times higher in subcutaneous than in omental adipose tissue when following a prospective cohort of...
### Table 1: Information of patients with diabetes and controls.

| Donor/Group | Sex | Age | DM Diagnosis | HbA1c (NGSP) [%] | Diagnosis | Wound (Source of the adipose tissue) | Time post-wounding | Comorbidity | Past Medical History (PMH) | Medication |
|-------------|-----|-----|--------------|-----------------|-----------|-------------------------------------|-------------------|-------------|---------------------------|------------|
| Non-DM1     | Male | 65  | -            | -               | Electric injury, Burn | Abdomen | 19 | No | Postoperative hepatoma Myocardial infarction, cerebral infarction, postoperative colorectal cancer | Celecoxib, rebamipide |
| Non-DM2     | Male | 82  | -            | -               | Burn | Thigh | 39 | Chronic renal impairment, dementia | Esomeprazole magnesium hydrate, acetaminophen, aspirin, carvedilol, amiodarone hydrochloride, silodosin |
| Non-DM3     | Male | 65  | -            | -               | Burn | Lower leg | 39 | Hypertension, hypercholesterolemia, noncerebral stroke | - |
| Non-DM4     | Male | 45  | -            | -               | Burn | Hip | 39 | Smoke inhalation | Traumatic intracranial hemorrhage Breast cancer |
| Non-DM5     | Female | 70  | -            | -               | Pressure ulcer | Chest | 70 | Chronic obstructive pulmonary disease (COPD) | Flucloxacillin furoate, umeclidinium bromide, tiotropium bromide, cilnidipine, allopurinol, sodium risedronate, rabeprazole, domperidone, syakuyaku-kangaz (kampo medicine) |
| Non-DM6     | Male | 21  | -            | -               | Pressure ulcer | Ischial region | 84 | Spinal cord injury (Paraplegia) | - |
| DM1         | Female | 76  | +            | 7.1             | Chronic traumatic ulcer | Elbow | 11 | Thoracic compression fracture | Denosumab, prednisolone (15 mg/day), fenofibrate, pioglitazone hydrochloride hydrate, vonaprazan fumarate, cilostazol, salmeterol, sodium ferrous citrate, duloxetine |
| DM2         | Male | 64  | +            | 7.3             | Burn | Thigh | 13 | Hypertension | unknown |
| DM3         | Male | 60  | +            | 5.2             | Burn | Back | 17 | Multi-hepatoma, liver cirrhosis, esophageal varices, hypertension | Rikkunshito (kampo medicine), esomeprazole |
| DM4*        | Male | 60  | +            | 5.2             | Burn | Abdomen | 31 | Multi-hepatoma, liver cirrhosis, esophageal varices, hypertension | Rikkunshito (kampo medicine), esomeprazole, rikkunshito (kampo medicine), insulin human (genetical recombination), mefenamic acid, famotidine, cilostazol, linezolid, lactulose |

*DM4 refers to a different patient with a similar medical history and treatment compared to DM3.*
227 individuals with severe obesity. Metabolic complications, including type 2 diabetes and dyslipidemia, are more prevalent in patients with high SA-β-Gal levels. Moreover, eliminating accumulated senescent cells in adipose tissue by targeting them to the apoptotic pathway improved glucose tolerance, enhanced insulin sensitivity, lowered circulating inflammatory mediators, and promoted adipogenesis in obese mice. Therefore, targeting the cellular senescence of subcutaneous adipose tissue may be effective for treating diabetic wounds.

We identified a transient increase in p15INK4B + senescent PDGFR-α + cells in adipose tissue during the normal wound healing process; however, in diabetic Lepdb/db mice, p15INK4B + senescent PDGFR-α + cells steadily accumulated over the course of wound healing. Accumulation of p15INK4B + senescent PDGFR-α + cells is also observed in diabetic patients, and non-diabetic patients have a decreased number of senescent cells during wound healing. PDGFR-α + mesenchymal stromal cells in skeletal muscle exhibit transient senescence, and this promotes muscle regeneration after acute muscle injury. PDGFRα + vimentin+ cells in neonatal hearts exhibit transient senescence to promote regeneration. Using qRT-PCR and immunohistochemical analysis, we found that the levels of p15INK4B encoded by the Cdkn2b gene, significantly increased in Lepdb/db adipose tissue at 2 DPW. CDKN2B expression levels are highest in subcutaneous adipose tissue relative to other tissues such as bone marrow, the central nervous system, skeletal muscle, and the internal organs. Furthermore, CDKN2B expression levels in subcutaneous adipose tissue are correlated with BMI.

Although the detailed function of p15INK4B is not as well understood as those for p16INK4A and other senescence-related factors, the function and protein structure of p15INK4B are predicted to be similar to p16INK4A, and p15INK4B is upregulated in TGF-β–related cell senescence. p15INK4B binds to CDK4 and CDK6, preventing their binding to cyclins and thereby inhibiting cell cycle progression. The antiproliferative action of TGF-β is also mediated through the inhibition of c-Myc expression. c-Myc inhibits the expression of p15INK4B and p21CIP1/WAF1 in proliferating cells, and suppression of c-Myc by TGF-β limits c-Myc availability and suppresses the activity of p15INK4B and p21CIP1/WAF1. TGF-β expression and activation are rapidly induced in response to injury, and TGF-β controls wound healing by acting as a potent chemoattractant for monocytes and fibroblasts. Although the role of TGF-β in the control of diabetic wound healing remains to be fully explained, increased levels of TGF-β1 are linked with type-2 diabetes, and TGF-β1 contributes to diabetic wound healing. The regulation of TGF-β–related cell senescence may be a therapeutic target for diabetic ulcers.

Our results suggest that the transient increase in the number of senescent PDGFRα + cells in adipose tissue is important for wound healing, and that there is a decrease in cell senescence in diabetic wounds during the acute wound-healing phase. Hence, impairment of wound healing is concurrent with the accumulation of senescent cells. However, our study has several limitations. We analyzed subcutaneous adipose tissue; however, intradermal adipose tissue contributes to wound healing through regulation of adipocyte precursor proliferation and mature intradermal adipocyte repopulation in the skin after wounding. The anatomical location of intradermal adipose tissue and subcutaneous adipose tissue is separated by the panniculus carnosus in mice, but human skin does not have a detectable panniculus carnosus. It is also difficult to confirm the existence of intradermal adipose tissue in humans because of the obvious inability to conduct lineage-tracing studies. Even though intradermal and subcutaneous adipose tissue are not physically demarcated in humans, increasing evidence suggests that there is a functional distinction

Table 1 (continued)

| Donor/Group | No. | Sex | Age | DM Diagnosis | HbA1c (%) | Post Wound Healing(PMH) | Diagnosis | Source of the adipose tissue |
|-------------|-----|-----|-----|-------------|----------|-------------------------|-----------|-----------------------------|
| DMS1        | +   | Male | 70  | +           | +        | Postoperative gangrene   | Fournier  | Hip                          |
| DMS2        | +   | Female | 82  | -           | +        | Gallstone, diabetic retinopathy | +         | Lower leg                    |

*Same patient as DM3*
Fig. 5 Cellular senescence in subcutaneous adipose tissue during wound healing in diabetic patients. a, b Representative images of adipose tissue immunostained for PDGFRα, p15INK4B, and γH2AX, during wound healing in diabetic and non-diabetic patients. c, d Correlation between the percentage of p15INK4B+ or γH2AX+ cells in PDGFRα+ cells and time post-wounding (n = 6 for each group). Correlations were examined statistically using Pearson’s correlation coefficient, and 95% confidence intervals are shown with translucent fill corresponding to each marker color.
between these tissues. Hence, careful interpretation of our results is needed, but at a minimum, we have demonstrated that cellular senescence in subcutaneous adipose tissue contributes to both normal and diabetic wound healing. However, the interstitial connective tissue under the panniculus carnosus of mouse differs anatomically from that of humans. Mouse interstitial connective tissue separates the panniculus carnosus and subcutaneous adipose tissue. The muscular layer of the panniculus carnosus is located above interstitial connective tissue. Although the muscular layer is mostly absent in humans, several muscles are considered as vestigial rudiments of the panniculus carnosus. Recent studies demonstrated that human subcutaneous adipose tissue consists of fibrous connective tissue layer that demarcates two layers in subcutaneous adipose tissue: superficial and deep. It is unclear which layer human stromal cells correspond to, but even in humans, myofibroblasts that may promote wound healing are regulated by subcutaneous adipose tissue.

Next, our study focused on PDGFRα+ mesenchymal cell senescence in adipose tissue. Macrophages are among the senescent cell populations in diabetic wounds, and their abundance is correlated with senescent cell burden in adipose tissue. Although PDGFRα+ cells play important roles in adipose tissue homeostasis, further study is needed regarding the role that macrophage senescence plays in adipose tissue during wound healing. In this study, we used the Leprdb/db mouse diabetes model, which is widely used as a typical delayed healing model, and our findings are similar to what is observed in type-2 diabetic patients. To better understand adipose tissue–related mechanisms in diabetic wound healing, further study is needed using a type-1 diabetes model, which exhibits adipose tissue atrophy and fibrosis. Finally, we only used the male Leprdb/db mouse diabetes model in this study, because several studies have shown that the prevalence of diabetes ulcers is higher among males than females and that the risk of amputation in...
diabetic ulcer is higher in males than in females\textsuperscript{78}. However, sex is acting as a modiﬁer of wound healing mechanism\textsuperscript{79}. Further studies are needed to elucidate common and sex-speciﬁc mechanisms of wound healing and cellular senescence. In summary, we demonstrate that transient mesenchymal cell senescence occurs in adipose tissue in physiological wound healing; however, accumulation of cell senescence occurs concurrently with expression of different components of SASP in diabetic wound healing. Our data suggest that impairment of senescence in adipose tissue contributes to intractable wound healing in diabetes (Fig. 7).

Methods

Human wound samples. Six wound tissue samples from five diabetic patients (age: 68.7 ± 9.0 years old; HbA1c(NGSP): 7.02 ± 2.15%) and six wound tissue samples from six non-diabetic patients (age: 58.0 ± 21.7 years old) were used in the study. The tissues used in the study were debrided and disposed during surgery for treatment, and no additional excisions were made for the study. The adipose tissues used in this study were included among the debrided tissue and was located in the peripheral wound site. Samples were recruited after agreement was obtained which were used for histological wound-healing scoring. For scoring purposes\textsuperscript{36}, a n percentage of wound closure was calculated as follows: day n area/day 0 area × 100 (%).

Adipose tissue transplantation. To perform adipose tissue transplantation, 100–120 mg of subcutaneous adipose tissue was collected from the back of Lepr\textsuperscript{db/db} and Lepr\textsuperscript{+/+} mice and labeled with Cell Tracker Vybrant CM-Dil Cell-Labeling Solution (V22888; Thermo Fisher Scientiﬁc, MA, USA). Labeling of adipose tissue was performed according to the protocol. Briefly, tissue was incubated with CM-Dil at 37 °C for 5 min and at 4 °C for 15 min. The labeled adipose tissue was transplanted onto the back of Lepr\textsuperscript{db/db} mice, and the wound was sutured with nylon thread. In Lepr\textsuperscript{+/+} mice, sham surgery was performed without adipose tissue transfer.

Histological analysis and wound healing scoring. Wound tissue and adipose tissue were harvested with marginal skin and fixed in 4% paraformaldehyde at 4 °C overnight. The following day, the tissue was cut into 5 mm sections and was parafﬁn-embedded. Each slide was stained with H&E and Masson trichrome, which were used for histological wound-healing scoring. For scoring purposes\textsuperscript{38}, an examiner assessor re-epithelization, epithelial thickness index, keratinization, granulation tissue thickness, scar elevation index, and remodeling. Histology sections were randomized, blinded, and evaluated by three investigators. Each parameter was objectively quantiﬁed and scored using ImageJ software. In this validated scoring system, the lowest score, 0, is associated with an open unhealed wound as is evident immediately and within the ﬁrst day post-injury, whereas the highest score, 12, is associated with a completely closed and healed wound without excessive scarring. All images were captured using a BZ-X700 ﬂuorescence microscope (KEYENCE, Osaka, Japan).

Immunoﬂuorescence staining. Parafﬁn-embedded sections were deparafﬁnized and rehydrated for immunostaining. Antigen retrieval was performed in a microwave oven (95–98 °C for 10 min) using citrate buffer (10 mM sodium citrate, pH 6.0). After cooling, the slides were washed twice with deionized water and once with 1X Tris-buffered saline with Tween-20 (TBST) for 5 min each. The sections were blocked with 1% bovine serum albumin (BSA) in TBST for 15 min at room temperature (RT) and were then incubated with primary antibodies overnight at
For multiplex staining, stripping was performed in a microwave oven (95 °C for 10 min) with TSA Plus Working Solution (Fluorescein, Cyanine 3, and Cyanine 5; Vector Laboratories, Burlingame, CA, USA) for 30 min at RT in the dark. The sections were then washed in TBST three times for 5 min each and treated with TSA Plus Working Solution (Fluorescein, Cyanine 3, and Cyanine 5; Vector Laboratories, Burlingame, CA, USA) for 10 min at RT in the dark. For multiplex staining, stripping was performed in a microwave oven (95 °C for 10 min) using citrate buffer. After cooling, staining with different tyramide fluorescent labels was performed according to the above procedure. Nuclei were labeled with Cellstain DAPI solution (1:1000, 4′,6-diamidino-2-phenylindole; Dianojio, Kumamoto, Japan), and after further washes, the sections were mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). The following primary antibodies were used: rabbit anti-p15INK4B (150; ab50303; Abcam, Cambridge, UK), rabbit anti-p16INK4A (1:200; clone 5F22; Sigma-Aldrich), rabbit anti-p21 (1:1000; clone 5G7; Sigma-Aldrich), rabbit anti–PDGF receptor receptor (1:1000 (mouse) and 1:500 (human); D1E1E; XP; Cell Signaling Technology), rabbit anti–α-smooth muscle actin (1:500; D4KSN; XP; Cell Signaling Technology), and rabbit anti–phospho-histone H2A.X(Ser139) (1:180; Ser139, 20E3; Cell Signaling Technology). These primary antibodies were used after dilution with SignalStain Antibody Diluent (Cell Signaling Technology). All images were captured using a BZ-X700 fluorescence microscope (KEYENCE).

**SPIDER-β-Gal staining.** Gelatin-embedded frozen tissue was used. Ten micrometer-thick frozen sections were washed three times with TBST and were incubated with SPIDER-β-Gal staining solution (1:50 in McIlvaine Buffer Solution, pH 6.0; Dianojio, Kumamoto, Japan) for 30 min at 37 °C. Nuclei were labeled with Cellstain DAPI solution (1:1000; Dianojio) and stained sections were mounted in VECTASHIELD (Vector Laboratories) after further washing.

**RNA extraction and quantitative reverse transcription PCR (qR-PCR).** Total RNA was isolated from skin wound tissues and subcutaneous adipose tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and was reverse transcribed into cDNA using the Script cDNA Synthesis Kit (1708891; Bio-Rad). QoSUniversal Advanced SYBR Green MicroBio (Bio-Rad) and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used for qR-PCR with the following conditions: 95 °C for 30 s and 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. Expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or beta-actin (ACTB) levels. The primer sequences used for the PCR analysis are shown in Supplementary Table 1.

**Organ culture and SASP characterization.** Subcutaneous adipose tissue under the wound for five Leprdb/db or Leprdb–/– mice in each group (0 DPW (before wound), 2 DPW, and 8 DPW) was collected and put into phosphate-buffered saline (PBS) supplemented with 2% penicillin/streptomycin. The tissue was then washed and transferred to Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The volume of the culture medium was 1 mL for every 60 mg of adipose tissue, the medium was changed once at 24 h post-extraction, and the culture medium was collected at 48 h post-extraction for use in the following assay. The culture medium from adipose tissue culture was characterized using the Proteome Profiler Mouse XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA). Developed films were imaged and integrated density analysis was performed using Image J, version 1.5 (National Institutes of Health).

**Cell preparation and in vitro wound-healing assays.** Skin fibroblasts were collected from male C57BL/6 mice. After euthanasia, skin was harvested and digested in Liberase TL (5401020001, Merck) for 120 min at 37 °C. The digested skin slurry was filtered through a 100 μm cell strainer (EASYstrainer Cell; Greiner Bio-One, Kremsmuenster, Austria) and through a 70 μm cell strainer (Greiner Bio-One). Cells were suspended in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and were cultured in a 75 cm² tissue culture flask. Cells reached 80–90% confluence after incubation for 1–2 weeks, and the cells were passaged. Cells from passage 2 were used for the in vitro studies. In vitro wound healing was studied using 2-well Culture-Inserts (Bdidi, Bavaria, Germany). Mouse skin-derived fibroblasts were cultured in 2-well Culture-Inserts with adipose tissue–cultured media collected from 2DPW adipose tissues. Phase contrast images were obtained every 24 h and immediately after removing the 2-well Culture-Insert using Promovert and Axioview208 microscopes (Carl Zeiss, Jena, Germany).

**Statistics and reproducibility.** Mice were only excluded from the study if they had visible wounds from fighting. The established scientific standard of n ≥ 3 was applied throughout this study. All experimental procedures were independently repeated using at least three mice per experimental group. We did not use any specific method of randomization to determine how animals were allocated to experimental groups. The investigators were not blinded to allocations during experiments or outcome assessments, although they were blinded during the histological assessment. Statistical analyses were performed using R (The R Foundation for Statistical Computing, Vienna, Austria). Statistical significance between two groups was determined using an unpaired t-test. A one-way or two-way analysis of variance (ANOVA) was conducted to assess differences among three or more groups. Pairwise comparisons were made only when the ANOVA test identified a statistical significance. p-values for multiple comparisons were adjusted using the Tukey method. Statistical analyses were performed using EZR, which is a graphical user interface for R. Two-sided p-values < 0.05 were considered statistically significant. Quantitative data are presented as either the mean ± standard error of the mean (SEM) or median with interquartile range (IQR) and 1.5 × IQR. Box-and-whisker plots and bar plots were generated using ggplot2, a plotting system for R based on The Grammar of Graphics (The R Foundation for Statistical Computing, Vienna, Austria). The R packages FactoMineR and factoextra were used to generate heat maps, Ward’s hierarchical agglomerative clustering, and principal component analyses.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All data generated or analysed during this study are included in this published article and its supplementary information files. Source data can be found in Supplementary Data 1.
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Author contributions
A.K., Y.S., and T.S.C. designed the studies. A.K., Y.S., N.M., M.M., S.Y., T.S., and T.S.C. performed the experiments and analyzed the data. A.K., Y.S., Y.T., M.F., and T.S.C. contributed to the drafting and reviewing of the paper.

Competing interests
The authors declare no competing interests.

Additional information
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