Local and transient inhibition of p21 expression ameliorates age-related delayed wound healing

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Running Title: p21 inhibition accelerates wound healing

Keywords: p21, CDKN1A, wound healing, aging, fibroblasts

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/wrr.12763
Abstract

Non-healing chronic wounds in the constantly growing elderly population represent a major public health problem with high socio-economic burden. Yet the underlying mechanism of age-related impairment of wound healing remains elusive. Here we show that the number of dermal cells expressing cyclin dependent kinase inhibitor p21 was elevated upon skin injury, particularly in aged population, in both man and mouse. The nuclear expression of p21 in activated wound fibroblasts delayed the onset of the proliferation phase of wound healing in a p53-independent manner. Further, the local and transient inhibition of p21 expression by in vivo delivered p21-targeting siRNA ameliorated the delayed wound healing in aged mice. Our results suggest that the increased number of p21+ wound fibroblasts enforces the age-related compromised healing, and targeting p21 creates potential clinical avenues to promote wound healing in aged population.

Introduction

The aged population is subjected to a higher risk of developing non-healing chronic wounds (1). A recent prospective study showed that advanced age of >65 years, is associated with a significant delay of wound healing (2). In animals, the healing of burn wounds (3) and excisional wounds (4) takes longer in aged mice than in young adult mice. Despite a strong causal link, the mechanism of age-related delayed wound healing remains elusive. Previous studies suggest that the expression of cell cycle checkpoint genes such as CDKN1A (p21) and CDKN2A (p16), which are robust in vivo markers for cellular senescence (5), are markedly increased in age-associated chronic wounds (6, 7).

p21\(^{Waf1/Cip1}\), also known as cyclin-dependent kinase inhibitor 1 (CDKN1A), is a potent CDK inhibitor that mediates cell cycle G1/S arrest and induces cellular senescence (8). p21 also constitutes a critical regulator of cellular proliferation, differentiation and senescence in mesenchymal stem cells (9), which are believed to be crucial progenitor cells for tissue repair.
p21 is required for hair follicle development in skin by oscillating its expression during hair follicle cycle progression (11). More interestingly, the African spiny mouse (12) and MRL (Murphy Roths Large) mouse (13) are endowed with the capacity to fully regenerate lost tissues. Of note, MRL mice express reduced p21 levels and are capable of regenerating ear punches with full regeneration of cartilage, dermal tissue, hair follicles and sweat glands without scarring (14). Subsequently, the researchers found that genetically modified p21−/− mice phenocopy this regenerative phenotype (14). Therefore, p21 may qualify as a molecular target to enhance wound healing and skin regeneration in elderly adults.

In this study, we showed that p21 expression is transiently upregulated upon skin wounding, and persists at high level in wounds of aged human individuals and mice. Transient and localized inhibition of p21 can promote wound healing in elderly individuals that often suffer from difficult-to-treat wounds with extended scar formation.

**Materials and Methods**

**Mice and human samples**

Aged (2-year-old) C57BL6/JRj mice were purchased from Janvier Labs (France). Young C57BL6/J mice at an age of 8-10 weeks were purchased from Charles River (Germany). p21-deficient (p21−/) mice (B6.129S6(Cg)-Cdkn1a<sup>tm1Led</sup>/J, JAX 016565) were purchased from Jackson Laboratory. Mice were constantly maintained under specific pathogen-free conditions. All experiments were carried out in compliance with the German Law for Welfare of Laboratory Animals. The animal experiments were approved by the local ethical committee with project number 1204.

Sections (5 μm) of formalin-fixed paraffin-embedded healthy skin and wound tissue from young and elderly patients were obtained from the Department of Dermatology and Allergic Diseases, University Hospital of Ulm with informed consent, and were approval by the ethical committee at University of Ulm. The wound tissues were the re-excision biopsies shortly after
the initial excision of skin tumors for histological examination. The selected re-excision biopsies were free of tumor cells.

**CDKN1A (p21) knockdown by siRNA**

Mouse *CDKN1A* (p21) siRNAs (Qiagen, #GS12575) and Allstars negative control siRNA (Qiagen, #1027281) were purchased from Qiagen. 3T3 cells (mouse embryonic fibroblast cell line) were seeded at 1x10⁵ cells per well in 6-well plate in serum-free medium for 3 h before the treatment with etoposide at final concentrations of 1 µM, 5 µM or 25 µM for 1 h in serum-free medium to induce p21 expression, and the solvent DMSO was used as control. Thereafter, the etoposide containing medium was removed and replaced with the complete medium containing 10% FBS. The basic medium was DMEM, supplemented with 1x GlutaMAX, 1x Penicillin/streptomycin, and 1x MEM non-essential amino acids, purchased from Thermo Fisher Scientific. Cells were cultured in 37°C incubator with a humidified atmosphere containing 5% CO₂.

Four siRNA molecules with different sequences targeting mouse *CDKN1A* (accession no. NM_001111099 and NM_007669) were tested in this study. 3T3 cells were transfected with mouse *CDKN1A*-siRNAs or control siRNA at a final concentration of 30 nM using Lipofectamine RNAi MAX (Life Technologies) diluted in Opti-MEM reduced serum medium (Life Technologies). Transfection medium was replaced by culture medium 6 h after transfection. 24 h after transfection, cells were pulsed with 25µM etoposide for 1 h in serum-free medium. Thereafter, the etoposide containing medium was replaced with culture medium, and cells were cultured for another 24 h. Cells were harvested 2 days after transfection for *CDKN1A* mRNA and p21 protein quantification. The siRNA Mm_Cdkn1a_3 (Qiagen, #SI02652510) with sense strand 5’-CUGUGUGUCUUAAUUAUUATT-3’ and antisense strand 5’-UAAUAUUAAAGACACACACAGAG-3’ showed the best silencing effect of p21 on etoposide treated 3T3 cells, and was selected for the subsequent *in vivo* experiments.
For *in vivo* delivery, CDKN1A-siRNA or control siRNA was prepared with BCC Injectin *in vivo* siRNA delivery kit (BioCellChallenge #I5000, France) according to the manufacturer’s instruction. Briefly, for each injection, 5 µg siRNA diluted in 7 µl RNase-free water was mixed with 3 µl of glucose buffer, and then mixed with 5 µl Injectin reagent. The resulted 15 µl of mixture was incubated at room temperature for 15 min before injection. The preparation of siRNA-Injectin mixture was scaled up according to the number of injections.

**Wound healing model**

Wound-healing studies were performed using mice of both genders. Prior to injury, C57BL6/J mice or p21−/− mice were anaesthetized by intraperitoneal injection of a ketamine (10 g/l)/xylazine (8 g/l) solution (10 µl/g body weight). After shaving, the exposed dorsal skin was disinfected with 70% ethanol. Thereafter, four full-thickness excisional wounds were created at two sites in the middle of the dorsum using 6 mm biopsy round knives (Stiefel, Germany).

One day after wounding, CDKN1A-siRNA or control siRNA was prepared in BCC Injectin for intradermal injections around the wounds as described above. Each wound received three injections of 15 µL of siRNA-Injectin mixture, which lead to 15 µg siRNA per wound (60 µg per mouse). Mice received second dose of siRNA on day 5 post-wounding. Each wound region was digitally photographed at day 0, 3, 5, 7 and 10 post-wounding, and wound areas were measured using Photoshop software (Adobe Systems). Wound sizes at any given time point after wounding were expressed as percentage of initial (day 0) wound area. Photography and wound area analyses were done in a blinded fashion.

**Real time reverse transcription PCR (qPCR)**

From fresh mouse wound tissue, total RNA was extracted with RNeasy fibrous tissue kit (Qiagen, #74704), and cDNA was reverse transcribed with the first strand cDNA synthesis kit.
using Oligo(dT)$_{18}$ Primer (Thermo Fisher Scientific, #K1612) according to the manufacturer’s procedures. RNA concentrations were measured by Nanodrop (Thermo Fisher Scientific).

Real time PCR was performed in a volume of 10 µl containing QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 µM forward and reverse primer, 5 ng cDNA using a LightCycler 480 II (Roche). Reactions were performed at 95°C for 15 min and followed by 40 cycles at 94°C for 15s, 60°C for 20s and 72°C for 20s. The relative expression of mouse CDKN1A (p21) was normalized to mouse 18S rRNA. The primer sequences of mouse CDKN1A (accession number NM_007669) are forward 5’-TCCACAGCGATACCAGACA-3’, reverse 5’-GGACATCACCAGGATTGGAC -3’, and the primer sequences of mouse 18S (accession number NM_011296) are forward 5’-GATCCCAGACTGGTTCCTGA-3’, reverse 5’-GTCTAGACCGTTGGCCAGAA-3’.

For formalin-fixed paraffin-embedded human wound samples, total RNA was extracted from 8 sections of 5 µm paraffin sections for each sample, by using RNeasy FFPE kit (Qiagen) according to the manufacturer’s instruction. cDNA was reverse transcribed with the first strand cDNA synthesis kit (Thermo Fisher Scientific). qPCR was performed in a LightCycler 480 II (Roche). The relative expression of human CDKN1A (p21) was normalized to human ribosomal protein L13a (RPL13A). The primer sequences of human CDKN1A (accession number NM_000389) are forward 5’-GGGTCGAAAACGGCGGCAGACCAGC-3’, reverse 5’-GGCCTTTGAGGCCCTCGCGCT -3’; and the primer sequences of human RPL13A (accession number NM_012423) are forward 5’- CCGACCGTGCGAGGTAT -3’, reverse 5’-CACCATCCGCTTTTTCTTGTCC-3’.

**Western blotting**

Wound tissues or cultured cells were harvested at the indicated time points and homogenized in RIPA buffer (Sigma-Aldrich) supplemented with 2 mM Na$_3$VO$_4$, 10 mM NaF (Sigma-Aldrich) and protease inhibitors (Roche) with FastPrep-24 homogenizer in Lysing Matrix D.
tubes (MP Biomedicals, Germany). The homogenates were then centrifuged at 12,000x g for 15 min at 4°C. The supernatant was collected, aliquoted and stored in -80°C. The protein concentration was determined by Bradford assay (Bio-Rad, Germany). Fifty µg protein from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Whatman, UK). Membranes were blocked with 5% BSA in TBS supplemented with 0.1% Tween-20 and incubated with primary antibody against mouse p21 (Clone F-5, Santa Cruz, #sc-6246), or mouse p53 (Abcam, #ab31333), or mouse β-actin (Santa Cruz, #sc-1615) at 4°C overnight. Thereafter, membranes were incubated with secondary anti-mouse IgG antibody conjugated to HRP (Dianova, Germany) for 1 hour at room temperature. Immunoreactions were detected by chemiluminescence using Vilber Fusion Fx7 system (Vilber, Germany). Densitometry analyses were performed with Bio-1D quantification software (Vilber, Germany). The expression of p21 or p53 was normalized to the loading control β-actin in the same lane.

Immunofluorescence staining

For formalin-fixed paraffin-embedded human skin and wound samples, 5 µm paraffin sections were rehydrated through 100%, 96% 80% and 70% ethanol and finally distilled water. Antigen retrieval was carried out by heating the sections in 1x target retrieval solution (DAKO, Germany) in steam for 15 min. Slides were incubated with 5% BSA in PBS to block non-specific antibody binding, and then incubated with primary anti-human p21 antibody (Clone EA10, Millipore, #OP64, 1:100 dilution) at 4°C overnight. Mouse IgG served as isotype control. Alexa Fluor 647 conjugated goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific, 1:200 dilution) were used as secondary antibody. For costaining with human fibroblast-specific protein 1 (FSP1, S100A4), anti-FSP1 antibody (Abcam, #ab41532 1:100 dilution) and Alexa Fluor 488 conjugated goat anti-rabbit IgG (Invitrogen, Thermo Fisher Scientific, 1:200 dilution) were used.
For cryopreserved mouse wound tissues, 5 µm cryosections were fixed with cold acetone (-20°C) for 5 min and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, followed by 30 min incubation with 5% BSA (Sigma-Aldrich) in PBS to block non-specific antibody binding. Sections were incubated with rabbit anti-mouse p21 antibody (Clone EPR18021, Abcam, #ab188224, 1:100 dilution) or rat anti-mouse p21 antibody (Clone HUGO291, Abcam, #ab107099, 1:100 dilution) at 4°C overnight. Respective IgG served as the isotype controls. After washing with PBS for three times, the slides were incubated with Alexa Fluor 488 conjugated secondary antibodies (Invitrogen, Thermo Fisher Scientific, 1:200 dilution) for 1 h at room temperature. The sections were subsequently labeled with anti-PDGFRα (R&D Systems, #AF1062, 1:100 dilution), or anti-α-SMA (Clone ASM-1, Progen, #61001, 1:100 dilution), or anti-CD45 (Clone IBL-3/16, Abcam, #ab23910, 1:100 dilution); or anti-cytokeratin 14 (CK14) (Clone LL002, Abcam, #ab7800, 1:100 dilution), and Alexa Fluor 647 conjugated respective secondary antibodies (Invitrogen, Thermo Fisher Scientific, 1:200 dilution). Nuclei were counterstained with DAPI (Sigma-Aldrich). Coverslips were mounted with fluorescent mounting medium (DAKO).

For cell proliferation, Ki67 antibody (Clone SP6, Thermo Fisher Scientific, #RM-9106) was used as the primary antibody. For cell death, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the fluorescein labelled in situ cell death detection kit (Roche, #11684795910), according to the manufacturer’s instruction.

Microphotographs were documented by using a Zeiss Axiophot microscope with an AxioCam digital color camera and AxioVision software v4.7 (Zeiss, Germany). The numbers of fluorescence labelled cells were analyzed using ImageJ on single channel binary images.

**Statistical analysis**

Quantitative data are presented as mean ± SD. Numbers of biological replicates (independent samples or mice) are stated in the figure legends. Statistical analyses were performed with
GraphPad Prism software (GraphPad version 5.0). As indicated in the corresponding figure legends, two-tailed unpaired \( t \)-test with Welch’s correction, or nonparametric Mann-Whitney test was used to determine statistical significance between the two groups; and one-way ANOVA with Tukey’s multiple comparison test or two-way RM ANOVA with Bonferroni post-tests was used to determine the statistical significance in multiple comparison. The alpha error was set at 0.05. Where stated in figure legends, we performed log transformation for mRNA and protein expression data, and logit transformation for percentage data, before statistical testing to approximate normal distribution with equal variances.

Results

Number of p21\(^+\) cells is elevated in wounds of elderly patients

We first checked the abundance of p21\(^+\) cells in normal skin from young (31-38 years old, \( n = 5 \)) and elderly (61-78 years old, \( n = 6 \)) donors (Table 1). Immunofluorescence staining of skin sections showed that the p21\(^+\) cells were present in the dermis, and the percentage of p21\(^+\) cells in total dermal cells was similarly low in both young and aged skin at homeostasis (Figure 1A, Table 1).

We then looked into wound tissue from elderly and young patients by examining re-excision biopsies from patients that underwent initial excision of tumors. Immunofluorescence staining of p21 was performed on paraffin sections of re-excision samples from young (17-37 years old, \( n = 5 \)) and elderly (70-86 years old, \( n = 6 \)) patients, where average duration between the two excisions was 46 days (Table 2). In wound sections from a 17-year-old young patient, 11.10% of total dermal cells were positive for p21, while p21\(^+\) cells comprised 23.85% of total dermal cells in wound sections from a 78-year-old elderly patient (Figure 1B, Table 2). Quantitative analysis of total dermal p21\(^+\) cells from all skin and wound samples revealed that wounding significantly increases p21\(^+\) cell numbers in wound margins compared to healthy dermis (Figure 1C). More importantly, the number of p21\(^+\) cells was significantly higher in
wound margins of elderly compared to young patients, despite different anatomic locations and wound ages (Figure 1C). In line with the immunofluorescence data, qPCR of human CDKN1A (p21) from total RNA purified from the formalin-fixed paraffin-embedded wound sections showed that the expression of CDKN1A was significantly higher in wounds from elderly compared to young patients (Figure 1D). These data suggest that p21+ cell numbers in dermis increase dramatically upon wounding, with higher extent in elderly people.

To further explore the location and identity of dermal p21+ cells, we performed histological and immunostaining on abdominal skin sections from a 56 years old donor. H&E staining clearly showed epidermal, papillary dermal, and reticular dermal layers (Figure 1E). The immunofluorescence staining of p21 showed that the p21+ cells were present in both papillary and reticular dermis without anatomic preference (Figure 1F). The p21+ cells in human dermis are fibroblast-specific protein 1 (FSP1)+ fibroblasts (Figure 1G).

**p21 expression increases during wound healing, and persists in aged mice**

To determine whether p21 expression is regulated during wound healing, we performed 6 mm full-thickness excisional wounds on back-skin of C57BL/6 mice. Uninjured skin and wound tissues were harvested at various time points, and p21 expression was quantitatively assessed by qPCR (Figure 2A) and western blot analysis (Figure 2B, C). p21 expression was significantly upregulated in wounds 3 days post-wounding compared to unwounded skin and declined to basal levels thereafter (Figure 2A-C). This data suggests that under injury conditions, p21 is markedly upregulated in order to arrest cell cycle.

We then performed similar wounding experiments in aged (2 years old) C57BL6/JRj mice, and compared wound expression of p21 between aged and young (8-10 weeks old) C57BL6/J mice. Aged skin had a ~ 2-fold higher basal expression of p21 at both RNA and protein levels. The expression of p21 in wounds from aged mice peaked at day 3 post-wounding just as occurs in wounds from young mice, but elevated to a higher extent (5.1-fold of mRNA and...
3.1-fold of protein of day 0) (**Figure 2D-F**). More importantly, the expression of p21 remained substantially high in wounds of aged mice at day 10 post-wounding (3.2-fold of mRNA and 1.8-fold of protein day 0), while in wounds of young mice p21 expression declined to baseline (**Figure 2D-F**).

The expression of p21 was further verified by immunofluorescence staining of p21 on cryosections of uninjured skin or wounds from young and aged mice. The wound tissue was harvested on day 3 post-wounding, when p21 expression level was highest (**Figure 2D-F**). In uninjured skin, the percentage of p21+ cells was low in young mice (1.15%), and slightly but statistically significant higher in aged mice (5.21%). Wounding induced substantial increase of p21+ cells in wound margin in both young and aged mice. More importantly, the percentage of p21+ cells was markedly higher in aged wounds (21.03%) compared to young wounds (8.12%) (**Figure 2G, H**), which recapitulates the difference of the percentages of p21+ cells in human wounds from aged and young donors (**Figure 1A-C**). The anti-mouse p21 antibody showed minimum non-specific labeling on uninjured skin or wound from p21-/- mice (**Figure S1**), indicating the high specificity of this antibody for immunostaining.

These data indicate that p21 expression is elevated early upon injury, but then bifurcates. The p21 expression declines to normal after wound closure in young mice but persist at high level in aged mice.

**Local and transient inhibition of p21 accelerates wound healing in aged mice**

To investigate the physiological function of p21 on wound healing in aged mice, we planned to transiently reduce p21 expression in the wounded area by employing in vivo delivery of siRNA targeting murine CDKN1A (p21). We first selected the most effective CDKN1A-siRNA in vitro using murine embryonic fibroblast 3T3 cells. Treatment of 3T3 cells with etoposide, a chemical agent causing DNA double-strand breaks, for 1 h induced upregulation of CDKN1A in a dose-dependent manner 24 h after treatment (**Figure S2A**). Knockdown of
CDKN1A expression by four different siRNA sequence targets was evaluated in 25 µM etoposide treated 3T3 cells. The most effective CDKN1A-siRNA showed 86.2% ± 1.8% suppression of CDKN1A mRNA (Figure S2B), and was subsequently used for in vivo wounding experiments.

We further evaluated the knockdown efficiency of in vivo delivered CDKN1A-siRNA by immunoblotting of p21 protein in wounds that had been intradermally injected with 15 µg CDKN1A-siRNA or control-siRNA (Figure 3A). Compared to control siRNA, a single dose of injected CDKN1A-siRNA resulted in 52.4%, 38.3% and 27.6% reduction of p21 protein in wounds at days 3, 5 and 7 post-wounding, respectively (Figure 3A, B).

To evaluate the effect of p21 inhibition on wound healing, 6 mm full-thickness excisional wounds were produced on the back-skin of aged (> 2-year-old) and young wild type (WT) mice. The selected CDKN1A-siRNA was intradermally injected around the wounds at day 1 and day 5 post-wounding. Aged (> 1.5-year-old) and young p21−/− mice were used as the genetic control for transient silencing of CDKN1A. As expected, wound healing in aged WT mice was substantially delayed compared to young counterparts (Figure 3C, D). More interestingly, in aged WT mice, injection of CDKN1A-siRNA significantly accelerated healing at all analyzed time points in comparison to control siRNA (Figure 3C, D). In young adult WT mice CDKN1A-siRNA showed beneficial effect only at early stage of wound healing, but with no significant impact on the overall healing process (Figure 3C, D). Notably, in aged mice, p21−/− wounds healed similarly to CDKN1A-siRNA treated WT wounds, at all analyzed time points. (Figure 3C, D). Wounds on aged and young p21−/− mice showed the same healing kinetics (Figure 3C, D), suggesting that in the absence of p21, aging has no impact on wound healing. These findings indicate that the inhibition of p21 at the early stage of skin injury accelerates wound healing in aged mice, and that this effect is p21-dependant. Local and transient inhibition of p21 is sufficient to obtain a profoundly faster healing effect,
and in consequence avoids any unpredicted adverse effect of systemic prolonged p21 silencing due to p21’s multi-functional roles.

**Wounding-induced p21 inhibits fibroblast proliferation**

To identify which cell type in wounds express p21, we performed co-immunostaining of p21 and cell type markers on cryosections of wounds at day 3 post-wounding. p21-expression was primarily restricted to platelet-derived growth factor receptor alpha (PDGFRα)+ and alpha-smooth muscle actin (α-SMA)+ fibroblasts in the wound margins (**Figure 4A, B**), and absent from wound infiltrated and resident CD45+ leukocytes (**Figure 4C**), and CK14+ keratinocytes (**Figure 4D**). Recent findings suggest the function of p21 is dependent on its subcellular location (15-17). p21 in wound fibroblasts was localized to the nucleus (**Figure 4A’, B’**), suggesting that it functions in wound fibroblasts as a cell cycle inhibitor. Similarly, in uninjured skin, p21 expression was found in PDGFRα+ fibroblasts (**Figure S3A**), but not in leukocytes or keratinocytes (**Figure S3B, C**), although the number of p21+ cells was low.

To investigate whether the regulation of p21 expression during wound healing is p53 dependent or independent, we assessed the expression of p21 and p53 in the same wounds at the different time points by western blot analysis. We found distinct expression patterns of p21 and p53 during wound healing. p53 expression was decreased at day 3 post-wounding and restored at later stages (**Figure 4E, F**), consistent with earlier reports of p53 expression kinetics in swine wounds (18). By contrast, p21 expression was transiently upregulated at day 3 post-wounding (**Figure 4E, F**). This data suggests that the wounding induced p21 upregulation is not downstream of p53, and independent of p53 signaling.

To investigate the function of p21 upregulation in wound fibroblasts, we compared cell proliferation and cell death between wounds from p21−/− and WT mice. Cell proliferation was analyzed by Ki67 staining of wound margins of p21−/− wounds and showed significantly
higher levels at day 6, but significantly lower at day 10 post-wounding compared to WT wounds (Figure 4G, H). The proliferating cells were not p21+ cells (Figure S4). Cell death as indicated by TUNEL staining, did not differ under both conditions (Figure 4G, I). These findings indicate that the inhibition of p21 expression in activated wound fibroblasts bypasses cell cycle arrest and promotes an earlier onset of the proliferation phase, that leads to accelerated healing.

Discussion
In this study, we have identified that the elevated nuclear expression of p21 in activated wound fibroblasts is an early response after skin injury, particularly in elderly population. p53-independent p21 signaling in wound fibroblasts delays the onset of the proliferation phase, which is believed to be one of the mechanisms underlying age-related impairments in wound healing. The local and transient inhibition of p21 expression is sufficient to accelerate the compromised wound healing in aged mice.

The inhibition of p21 bypasses a cell cycle arrest thereby enhances cell proliferation. p21 plays protective effects on stem cell (eg. mesenchymal stem cells (9)) exhaustion, which may underline long-term homeostasis throughout entire life span. During tissue injury, however, p21-mediated cell cycle arrest is unfavorable for repair. Our study indicates that p21 induced cell cycle arrest is dispensable for tissue repair. Low level of damage can be tolerated in order to accelerate the repair process.

The classic p21 signaling upon DNA damage is known to be p53-dependent (19). Yet p21 plays regulatory roles in normal tissue development and cell differentiation in a p53-independent manner (20). Our data of the distinct kinetics of p21 and p53 expression during wound healing points to a p53-independent transient upregulation of p21 upon skin injury. This conclusion is supported by the previous observation that loss of p53 in MRL mice did not show reduced tissue and appendage regeneration during ear hole healing (14). In addition,
p21 has been shown to regulate hair follicle cycle in a p53-independent fashion (11). It would be interesting in the future study to identify the upstream regulator of p21 upon wounding.

In this study, we have shown that p21\(^+\) cells in human wounds are dermal FSP1\(^+\) fibroblasts in both papillary and reticular dermal layers, and in mouse wounds are confined to PDGFR\(\alpha^+\), \(\alpha\)-SMA\(^+\) fibroblasts. On the other hand, timely regulation of p21 in keratinocytes is important for skin barrier function after injury. It has been shown that p21 expression is elevated in the epidermis of K14-Dicer-ablated mice, and p21 suppression accelerates reepithelialization in these mice (21). Therefore, the contribution of keratinocytes to the accelerated healing upon p21 inhibition cannot be ruled out.

High expression of p21 has been implicated in tissue fibrosis in heart (22), kidney (23, 24), lung (25, 26), and liver (27). During skin wounding, upregulation of p21 delays proliferation phase, and may induce prolonged inflammation and promote scarring. However, on the contrary, under conditions of optimal wound care, healed wounds in elderly patients reveal a shorter maturation time and a better scar quality (28). Recently, it was reported that aging promotes skin regeneration by suppressing skin-derived SDF1 (29). Therefore, in future experiments it would be highly interesting to investigate which role p21 plays in skin fibrosis and scarring after wound healing.

Inhibition of p21 promotes liver regeneration (30-33), but also facilitates hepatocarcinogenesis (30, 32), suggesting that tumor suppression is achieved at the expense of delayed tissue repair and loss of regeneration ability. Cartilage and skin regeneration has been demonstrated by complete regeneration of full-thickness ear holes in MLR mice and p21\(^-\) mice on a mixed B6/129Sv background (14). Our major and clinically relevant finding in this study is that local and transient p21 inhibition in wounds is sufficient to promote wound repair without compromise of its tumor suppressive function. Of note, two variables – age of the donor and age of the wound, are present in our small cohort human samples. Since p21 expression increases shortly after wounding and returns back to the normal level as we...
have seen in young mice, more patient data would be beneficial and necessary for future clinical translational study. p21 inhibition will certainly not be used in acute wound repair but would be an option in compromised wound healing of the elderly with long-term treatment. As a consequence, the hypothetic risk of tumor formation needs to be cautiously monitored.

This proof of concept study paves the way for developing transient and localized inhibition of p21 as a new strategy to improve wound healing after surgery, or even treat non-healing wounds in elderly patients. Nevertheless, the efficiency of CDKN1A knockdown by in vivo delivery of CDKN1A-siRNA was relatively low, and has limitations for therapeutic purpose. Therefore, it is a medical need to develop a synthetic potent p21 inhibitor, in order to explore its potential of reducing scarring and promoting skin regeneration in elderly patients in the future studies.

Acknowledgements

We thank Ishani Banik and Nouman Khalid for their technical assistance. This study was supported by research grants from the German Research Foundation (SFB1149) to K.S.-K., the Fritz-Thyssen-Stiftung (2016-01277) to Y.R., and the Baustein Program from the Medical Faculty, University of Ulm (LSBN.0100) to D.J.

Conflict of Interest

The authors declare no competing financial interests.
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Table 1. Percentage of p21^+ cells in total dermal cells in human healthy skin

| Donor No. | Age | Age group | % of p21^+ cells |
|-----------|-----|-----------|------------------|
| K14-03    | 31  | young     | 2.32%            |
| K17-03    | 35  |           | 3.11%            |
| K91-03    | 38  | young     | 1.82%            |
| K108-03   | 33  |           | 1.62%            |
| K186-04   | 32  |           | 4.07%            |
| K46-04    | 73  |           | 3.50%            |
| K87-03    | 61  |           | 5.70%            |
| K92-03    | 67  | aged      | 1.63%            |
| K166-04   | 76  |           | 0.76%            |
| K268-04   | 78  |           | 3.28%            |
| K522-03   | 74  |           | 0.63%            |

Table 2. Percentage of p21^+ cells in total dermal cells in human skin wounds

| Patient No. | Gender | Age | Anatomic location | Wound age (days) | Age group | % of p21^+ cells |
|-------------|--------|-----|-------------------|------------------|-----------|------------------|
| 1851-16     | M      | 35  | back              | 34               |           | 10.36            |
| 1668-16     | M      | 37  | lower arm         | 97               |           | 10.82            |
| 1931-15     | M      | 28  | pre-auricular     | 36               | young     | 11.13            |
| 1657-16     | F      | 35  | upper arm         | 119              |           | 4.06             |
| 1624-16     | M      | 17  | left foot         | 49               |           | 11.10            |
| 1791-16     | M      | 78  | scalp             | 5                |           | 23.85            |
| 1715-16     | M      | 73  | flank             | 53               |           | 13.94            |
| 1811-16     | M      | 86  | parasternal       | 21               | aged      | 12.26            |
| 2344-16     | M      | 76  | temple            | 19               |           | 19.15            |
| 1600-16     | M      | 85  | scalp             | 14               |           | 14.26            |
| 1429-16     | M      | 70  | back              | 64               |           | 13.26            |
Figure Legends

Figure 1. Higher percentage of p21+ dermal cells in skin wounds from elderly patients. (A-B), representative microphotographs depicting immunostaining for human p21 (red) on sections from healthy skin of representative young and elderly donors (A) or on wound sections from representative young and elderly patients (B). Nuclei were counterstained with DAPI (blue), and mouse IgG (mIgG) served as isotype control. The dashed lines in (A) indicate the epidermal-dermal junction, and the white arrow heads in (A) indicate p21 positive cells. e, epidermis; d, dermis; wm, wound margin. (C), percentage of p21+ cells in total dermal cells of healthy skin from 5 young and 6 elderly donors, and of wounds from 5 young and 6 elderly patients. **, p<0.01; ***, p<0.001; n.s., not significant, by two-way RM ANOVA with Bonferroni post-tests after logit transformation. (D), mRNA expression of human CDKN1A relative to RPL13A in wounds from 5 young and 6 elderly patients. *, p<0.05 by two-tailed unpaired Welch’s t-test after log transformation; AU, arbitrary unit. (E-F), H&E staining (E) and immunofluorescence staining of p21 (F) on abdominal skin sections from a 56 years old donor. The dashed lines delimited epidermal, papillary dermal, and reticular dermal layers. e, epidermis; pd, papillary dermis; rd, reticular dermis. (G), representative immunofluorescence images of p21 (red), FSP1 (green) and DAPI (blue) of the area indicated by white box showed in (F). Scale bars: A-B = 50 µm, E-G = 100 µm.

Figure 2. Kinetics of p21 expression during wound healing in young and aged mice. (A-C), p21 is transiently upregulated during wound healing in mice. Total RNA and protein lysate were prepared from wound tissues harvested at days 0, 3, 6, and 9 post-wounding. (A) mRNA expression of murine CDKN1A relative to 18S in wounds, mean ± SD, n = 4. (B) western blot analysis of p21 protein expression in 4 independent wounds at each time point. β-actin served as loading control. (C) semi-quantitative densitometry analysis of the blot showed in (B), mean ± SD, n = 4. **, p<0.01; ***, p<0.001 by one-way ANOVA with
Tukey’s multiple comparison test after log transformation. (D-F), the extent of p21 upregulation during wound healing is higher in aged mice. Total RNA and protein lysate were prepared from wound tissues from 8-week old young mice or 2-year old aged mice, at days 0, 3 and 10 post-wounding. (D) mRNA expression of murine CDKN1A relative to 18S, mean ± SD, n = 4. (E) representative immunoblotting of p21 in wounds from young and aged mice at the indicated time points. Y, young; A, aged. (F) semi-quantitative densitometry analysis of two independent blots showed in (E), mean ± SD, n = 4. Statistical significance was assessed by using two-way RM ANOVA with Bonferroni post-tests after log transformation, **, p<0.01 and ***, p<0.001 young vs. aged at indicated time point. *, p<0.05 and ###, p<0.001 day 3 and 10 vs. day 0 in wounds from aged mice. (G), representative immunofluorescence images of p21 (green) and DAPI (blue) on cryosections from uninjured skin and day 3 wounds from young and aged mice. e epidermis; d, dermis; f, hair follicle; es, eschar; wm, wound margin. Scale bar: 100 µm. (H), quantification of percentages of p21+ cells showed in (G). Data are mean ± SD, n = 4. *, p<0.05; ***, p<0.001 by one-way ANOVA with Tukey’s multiple comparison test after logit transformation.

Figure 3. Local and transient inhibition of p21 expression accelerates wound healing in aged mice. (A-B), CDKN1A-siRNA or control siRNA were intradermally injected around wounds at 15 µg/wound 1 day after wounding. (A) representative immunoblotting of p21 protein in wound tissue at days 3, 5, and 7 post-wounding. β-actin served as loading control. (B) semi-quantitative densitometry analysis of two independent blots showed in (A), showing the p21 protein expression in CDKN1A-siRNA treated wounds relative to control-siRNA treated wounds (1 AU, indicated by the dashed line). Data are mean ± SD, n = 4. **, p<0.01, ***, p<0.001 by two-tailed unpaired Welch’s t-test after log transformation. AU, arbitrary unit. (C-D), 6-mm full-thickness excisional wounds were produced on young (8-week old) and aged (122-week old) C57BL/6J (WT) mice, or young (10-12-week old) and aged (78-80-week old) mice.
week old) p21\(^{-/}\) mice. Control-siRNA or CDKN1A-siRNA was intradermally injected to WT wounds at 15 µg/wound at days 1 and 5 post-wounding. Wounds on p21\(^{-/}\) mice were left untreated. Each wound region was digitally photographed and analyzed at the indicated time points. (C) representative clinical pictures of wounds of each group at the indicated time points. (D) quantitative analysis of all wound areas per group at the indicated time points, expressed as percentage of initial wound size at day 0. The dashed line at 100% indicates the initial wound area at day 0. *, p<0.05; **, p<0.01; ***, p<0.001 by Mann-Whitney test.

**Figure 4. p21 is expressed in wound fibroblasts independently of p53 and suppresses proliferation.** (A-D), representative immunofluorescence images of p21 (green) and PDGFR\(\alpha\) (A), \(\alpha\)-SMA (B), CD45 (C), and CK14 (D). Nuclei were counterstained with DAPI (blue). (A'-B'), high magnification images of white boxes indicated in A and B. (E), western blot analysis of p21 and p53 protein expression in 2 independent wounds at indicated time points. \(\beta\)-actin served as loading control. (F), semi-quantitative densitometry analysis of the blot showed in E, mean ± SEM. AU, arbitrary unit. (G), representative images of immunofluorescence staining of Ki67 (red) and TUNEL staining (green) on cryosections of uninjured skin (day 0) and wounds from WT and p21\(^{-/}\) mice at days 3, 6, and 10 post-wounding. Nuclei were stained with DAPI (blue). (H-I), percentages of Ki67\(^+\) (H) and TUNEL\(^+\) (I) cells in total cells at each time point. Mean ± SD, n = 4. *, p<0.05 by two-tailed unpaired t-test. Scale bars: A-D, A'-B' = 50 µm; G = 100 µm.

**Supplemental Figure 1. Specificity control of anti-mouse p21 antibody.** Cryosections of uninjured skin or day 3 wound from p21\(^{-/}\) mice were labeled with anti-mouse p21 antibody to check the antibody specificity. Rabbit IgG served as the isotype control. p21 showed in green and DAPI in blue. e, epidermis; d, dermis; f, hair follicle; wb, wound bed. Scale bars: 100 µm.
Supplemental Figure 2. Silencing p21 by siRNA in 3T3 fibroblasts with induced p21 expression. (A), mRNA expression of murine CDKN1A relative to 18S in 3T3 fibroblasts 24 h after the treatment with etoposide at concentrations of 1 µM, 5 µM, and 25 µM for 1 h. CDKN1A expression in DMSO-treated control 3T3 cells (0 µM) served as 1 AU. (B), relative mRNA expression of CDKN1A in CDKN1A-siRNA or control-siRNA transfected, 25 µM etoposide treated 3T3 cells. Non-transfected etoposide treated 3T3 cells and untreated 3T3 cells served as controls. Murine 18S served as reference gene for normalization. CDKN1A expression in untreated 3T3 cells served as 1 AU. Mean ± SD, n = 3. **, p<0.01; ***, p<0.001 by one-way ANOVA with Tukey’s multiple comparison test after log transformation. AU, arbitrary unit.

Supplemental Figure 3. p21 is expressed in skin fibroblasts. Representative images of uninjured skin from wildtype mice co-immunolabeled with p21 (green) and PDGFRα (red) (A), or CD45 (red) (B), or CK14 (red) (C). Nuclei were counterstained with DAPI (blue). Scale bars: 50 µm.

Supplemental Figure 4. Proliferating cells are not p21+ cells. Representative images of day 3 wounds from aged mice co-immunolabeled with p21 (green) and Ki67 (red). Nuclei were counterstained with DAPI (blue). Scale bars: 50 µm.
Fig. 1

A 31-year-old 73-year-old 73-year-old

B 17-year-old 78-year-old 78-year-old

C

D

E

F

G

Accepted Article

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