Influencing the homing and differentiation of MNCs in hereditary hemorrhagic telangiectasia

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DPP4 inhibition enhances wound healing in endoglin heterozygous mice through modulation of macrophage signaling and differentiation

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Manuscript in preparation

Introduction

In non-pathological wound healing, inflammation and subsequent repair is tightly regulated through complex processes and signaling pathways[1-3]. After infiltrating immune cells (e.g. neutrophils, M1 macrophages, T cells) clear the cell debris and foreign bodies, they are gradually replaced by a population of cells with regenerative capacities (e.g. M2 macrophages, epithelial progenitor cells, fibroblasts)[4,5]. During wound inflammation and repair, the transmembrane protein endoglin (*ENG,* CD105) is highly expressed in endothelial cells and expression coincides with the infiltration of the immune cells[6].

Endoglin is a transforming growth factor beta (TGFβ) co-receptor, facilitating the TGFβ-receptor (TGFβR) complex binding several TGFβ family growth and differentiation factors including for example TGFβ isoforms, activins, NODAL, and bone morphogenetic proteins (BMPs) and many others[7,8]. TGFβ transduces its signal via a canonical and a non-canonical pathway. The canonical signaling pathway of TGFβ starts with binding of TGFβ/BMP cytokines to a heterotetrameric complex consisting of two type I and two type II receptors. While binding of TGFβ to a complex containing ALK5 induces Smad2 and/or Smad3 phosphorylation, BMP binds to a complex harboring ALK1/2/3/6, phosphorylating Smad1/5/8[8-10]. Phosphorylation of the SMADs propagate the signal intracellularly, and co-SMAD4 binding together with the phosphorylated SMADs causes the formation of a SMAD complex. The SMAD complex translocates to the nucleus where transcription of multiple genes is affected. The TGFβ/BMP pathway has been studied most extensively in endothelial cells (ECs). In ECs, TGFβ signaling via the ALK1-SMAD1/5/8 pathway results in proliferation and migratory signals, whereas signaling via ALK5-SMAD2/3 results in a decrease in proliferation and migration.

Endoglin has an important function in initiation and regulation of angiogenesis by stimulating ECs[10-12]. In ECs, endoglin mainly stimulates the ALK1-SMAD1/5/8 pathway resulting in angiogenesis and limits activation of the ALK5-SMAD2/3 pathway, which promotes a quiescent vascular state[10]. However endoglin can still promote/activate the SMAD2/3 signaling via the formation of a heteromeric complex of ALK1 combined with ALK5, underlining the complexity of endoglin-TGFβ signaling[9,13].

The prominent role of endoglin in vessel formation is most apparent in the vascular disorder hereditary hemorrhagic telangiectasia type 1 (HHT1), where endoglin haploinsufficiency causes endothelial hyperplasia and impaired angiogenesis[14-17]. The mouse model for HHT1, the endoglin heterozygous (*Eng*$^{+/-}$) mouse displayed impaired mononuclear cell (MNC) homing towards sites of ischemic damage[18]. Furthermore, MNCs expressed increased levels of dipeptidyl peptidase 4 (DPP4)[19]. Homing of MNCs is primarily regulated by the stromal cell-derived factor 1(SDF1)- CXC chemokine receptor type 4 (CXCR4) axis. MNCs express the CXCR4 receptor, specific for the chemokine SDF1[20]. SDF1 is upregulated and released into the bloodstream upon cell damage and stress, creating a chemokine gradient leading the MNCs expressing CXCR4 towards the site of tissue damage[21]. The SDF1-CXCR4 axis is negatively regulated by enzymatic activity of DPP4. DPP4 cleaves the di-amino-terminal peptides of SDF1, thereby inactivating the homing signal[22]. DPP4 is membrane-bound, but its extra-cellular domain can be cleaved resulting in an soluble form with its enzymatic function still intact[23]. The generation of a soluble form of DPP4 occurs under hypoxic conditions and is mediated by matrix metalloproteases[24]. In HHT1, both CXCR4 and DPP4 were found in elevated surface expression levels on MNCs[19] suggesting that the SDF1-CXCR4 axis is dysregulated in these patients.

Besides impaired homing of MNCs towards SDF1, HHT1 patients and endoglin heterozygous mice harbor an impaired immune response[25-28], suggesting that endoglin not only affects angiogenesis, but also plays an important role in the inflammation phase during the wound healing process[6,18,29,30]. In *Eng*$^{+/-}$ mice, wound healing was reported to be delayed due to reduced keratinocyte proliferation and nitric oxide (NO) avaliability[30]. Furthermore, while fibroblast activation is a necessary action for wound repair, *Eng*$^{+/-}$ wound fibroblasts were found to be hyperactive and aggravated fibrosis through increased AKT signaling[31].

We previously reported that in *Eng*$^{+/-}$ animals the inflammatory (M1-like) and regenerative (M2-like) macrophage content are imbalanced in the infarcted heart. Others also found wound healing in *Eng*$^{+/-}$ to be disrupted[30]. MNCs are essential during the inflammatory response and wound healing[4,32,33] and in previous research we were able to restore immune cell homing and increase cardiac repair in *Eng*$^{+/-}$ mice by the use of DPP4 inhibition[19,34]. Furthermore, DPP4 inhibitor treatment increased the number of M2-like macrophages[34]. We hypothesized that macrophage function and/or differentiation in the *Eng*$^{+/-}$ mice in dermal wound healing is impaired as well, and could potentially be restored by DPP4 inhibition. Therefore in this study, we aimed to use DPP4 inhibition to improve *Eng*$^{+/-}$ MNC function in dermal wound repair.

Material and methods

Mice and dorsal skin wounding experiments
Experiments and analyses were conducted on male endoglin wild type (*Eng*$^{+/+}$, referred to as WT) and heterozygous (*Eng*$^{+/-}$) transgenic mice. All mouse strains were kept on a C57BL/6Jico background (Charles River).

Wild type and *Eng*$^{+/-}$ mice were anesthesized with subdermal Ketamin (100 mg/kg) and Xylazin (10 mg/kg) injection. The dorsal area was depilated using razor followed by application of depilating crème. After sterilization of the skin with 70% ethanol, 4 x 6 mm punches were made through the dorsal skin fold as described previously[35]. The wound area was measured horizontally and vertically with digital caliper, and photographed.

DPP4 inhibitor Sitagliptin (Merck) was dissolved in demineralized water to 1mg/ml, of which 10 ul was topically applied to the wound areas, in total 10 ug Sitagliptin per applied dosis. Demineralized water was used as control treatment. Wound size was measured at day 0, 2, 4, 6, 8 and 10 post-wounding. During measurements, the mice were anesthesized with 2% isoflurane. Mice were divided in a 5-day and 10-day follow-up period post-wounding. Mice were carbon dioxide-euthanized after which skin biopsies were dissected. Mice were randomally allocated into groups of n=6-7 for each treatment. Animal health and behavior were monitored on a daily basis by the research and/or animal care staff, all trained in animal care and handling. All mouse experiments were approved by the regulatory authorities of Leiden University (the Netherlands) and were in compliance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Immunofluorescent analysis
Skin was dissected from carbon dioxide-euthanized mice and placed into a container with PBS. The tissue was fixated overnight (O/N) at 4°C in 4% paraformaldehyde in PBS, and then washed with PBS, 50% EtOH and 70% EtOH for 1 h each, followed by embedding in paraffin wax. Paraffin tissue sections of 6 µm thickness were mounted onto coated glass slides (VWR SuperFrost Plus microscope slides). The tissue were stained as previously described using antigen retrieval (Duim 2015). Primary antibodies were incubated O/N at 4°C and directed against: rat anti-mouse MAC3 (CD107b, dilution 1:200, BD Biosciences), rabbit anti-mouse Mannose Receptor (CD206, dilution 1:300, NFκB-p-p65 (1:1000 #11260, Signaling Antibodies) and goat anti-mouse p-IKKα/β (1:1000, #SC21661, Santa Cruz). Appropiate fluorescent-labelled secondary antibodies (ThermoFisher Scientific) were incubated for 1.5 h, at 1:250 dilutions. The slides were mounted with Prolong Gold-DAPI Antifade (# P36931, ThermoFisher Scientific) reagent. Photos were taken with fluorescent digital slide scanner (Pannoramic scanner, Sysmex) and analyzed with CaseViewer software (3DHistech).

Macrophage differentiation and cell culture
Femur and tibia were isolated from WT and *Eng*$^{+/-}$ mice. Bone marrow was flushed using syringes filled with PBS. Cells were seeded onto culture dishes in RPMI 1640 culture media (#11875093, Gibco, ThermoFisher Scientific), supplemented with 10% FBS (#10270, Fetal Bovine Serum, Gibco, ThermoFisher Scientific), 0.2% Penicillin-Streptomycin and 1 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF, #315-03, Peprotech). Media was refreshed at day 3, with added DPP4 inhibition, either 100µM Sitagliptin (Merck) or 100µM Diprotin A (Sigma-Aldrich) to the culture medium. On day 6 of culture, cells were placed onto serum-free RPMI media, and LPS was added for 24 hrs to stimulate an inflammatory response. Cells were stimulated with TGFβ 5ng/ml and/or freshly added DPP4 inhibitor for 60 minutes before washing with cold PBS, and lysis of the cells for western blotting, see description below.

Western blotting
Cultured macrophages were lysed on ice with cold radio immunoprecipitation assay (RIPA) lysis buffer (in house) supplemented with phosphatase inhibitors((1M NaF Sigma Aldrich #S7920, 10% NaPi Avantor #3850-01, 0.1M NaVan Sigma Aldrich # S6508), protease inhibitors (Complete protease inhibitor cocktail tablets, Roche Diagnostics, #11697498001) and protein concentration was measured using BCA protein assay (Pierce BCA Protein Assay Kit, #23225, ThermoFisher Scientific). Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane (# IPVH00010, PVDF membrane, Millipore). The blots were blocked for 60 minutes using 10% milk in Tris-Buffered Saline and 0.1% Tween-20 solution and incubated O/N at 4°C with mouse anti-mouse anti-β-Actin (1:10.000 dilution, A5441, Sigma-Aldrich), rabbit anti-mouse phosphorylated Smad2 (Cell signaling, #3101), total Smad2/3 (BD Biosciences, BD610842), mouse anti-mouse phosphorylated ERK1/2 (Sigma-Aldrich, #M8159), rabbit anti-mouse total ERK1/2 (a.k.a. p44/42 MAPK, Cell Signaling, #4695, clone 137F5), rabbit anti-mouse phosphorylated p38 (Cell Signaling Technology, #9211), rabbit anti-mouse phosphorylated AKT (Cell Signaling Technology, #9271), rabbit anti-mouse total-AKT (pan) (Cell Signaling Technology, #4691), Vinculin (Sigma #V9131). and rabbit anti-mouse total p38 (Santa Cruz Biotechnology Inc. #535). Blots were incubated for 1 h with horse radish

peroxidase anti-goat (ThermoFisher Scientific, #32230), anti-rabbit (ECL rabbit IgG, HRP-linked whole Ab, GE Healthcare, #NA934V) or anti-mouse (ECL mouse IgG, HRP-linked whole Ab, GE Healthcare, #NA931V). Blots were developed in a Kodak X-omat 1000 processor with Thermo Scientific SuperSignal West Dura (Extended Duration Substrate) or SuperSignal West Pico and exposed to Fuji SuperRX medical X-ray film. Analysis was performed using Image J (National Institute of Mental Health, Bethesda, Maryland, USA).

ELISA assay

MNC media samples were used for ELISA detection of MCP-1 (MCP-1 ELISA construction kit, Antigenix America Inc. #RRF423CKC) and IL-6 (IL-6 ELISA construction kit, Antigenix America Inc. #RRF600CKC) according to the manufacturer's protocol. Plates were read at 450nm using a microplate reader.

HEK293T cell culture and luciferase reporter assay

HEK293T cells were seeded at density of $1.0x10^5$ cells/ml in 48-well plates in DMEM media (Gibco, ThermoFisher Scientific) supplemented with 10% FBS (Fetal Bovine Serum, Gibco, ThermoFisher Scientific, #10270), and transfected with a NFκB subunit p65 reporter plasmid using PEI (Fermentas) transfection reagent, as described previously[36]. Twenty-four hours after transfection, cells were serum-starved for 8 h followed by O/N treatment with Sitagliptin (Merck) at the indicated concentrations. Next, cells were stimulated with TNF-α (50 ng/ml) for 8 h and cell lysates were prepared for measuring luciferase activity with a Perkin Elmer luminometer. The internal control, β-Galactosidase Reporter plasmid, was used in the same samples to normalize transfection efficiency.

Statistics

All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated using one-way ANOVA for difference between multiple groups and statistical significance was accepted at $p<0.05$. In case the p value was significantly different between groups, T-testing was performed. Adjustment for multiple comparisons was made with either Tukey's or Dunnett's testing and unpaired students T-testing for testing between two groups. Survival curves were tested with a log-rank Mantel-Cox test. Analysis was performed using Graphpad Prism v6 software.

Results

DPP4 inhibition using Sitagliptin enhances wound closure in *Eng*$^{+/-}$ mice

Wild type (WT) and *Eng*$^{+/-}$ mice were first assessed in their capacity for wound healing over time. We confirmed that *Eng*$^{+/-}$ indeed display reduced wound healing compared to wild type litter mates (Fig. 1A and B). Next we analyzed the effect of treatment with the DPP4 inhibitor Sitagliptin on dermal wound closure. Interestingly, applying Sitagliptin to the wound of wild type mice only delayed closure of the epidermis (Fig. 1A and C). In the *Eng*$^{+/-}$ mice treatment with the DPP4 inhibitor Sitagliptin normalized wound repair to wild type levels (Fig. 1A and D).

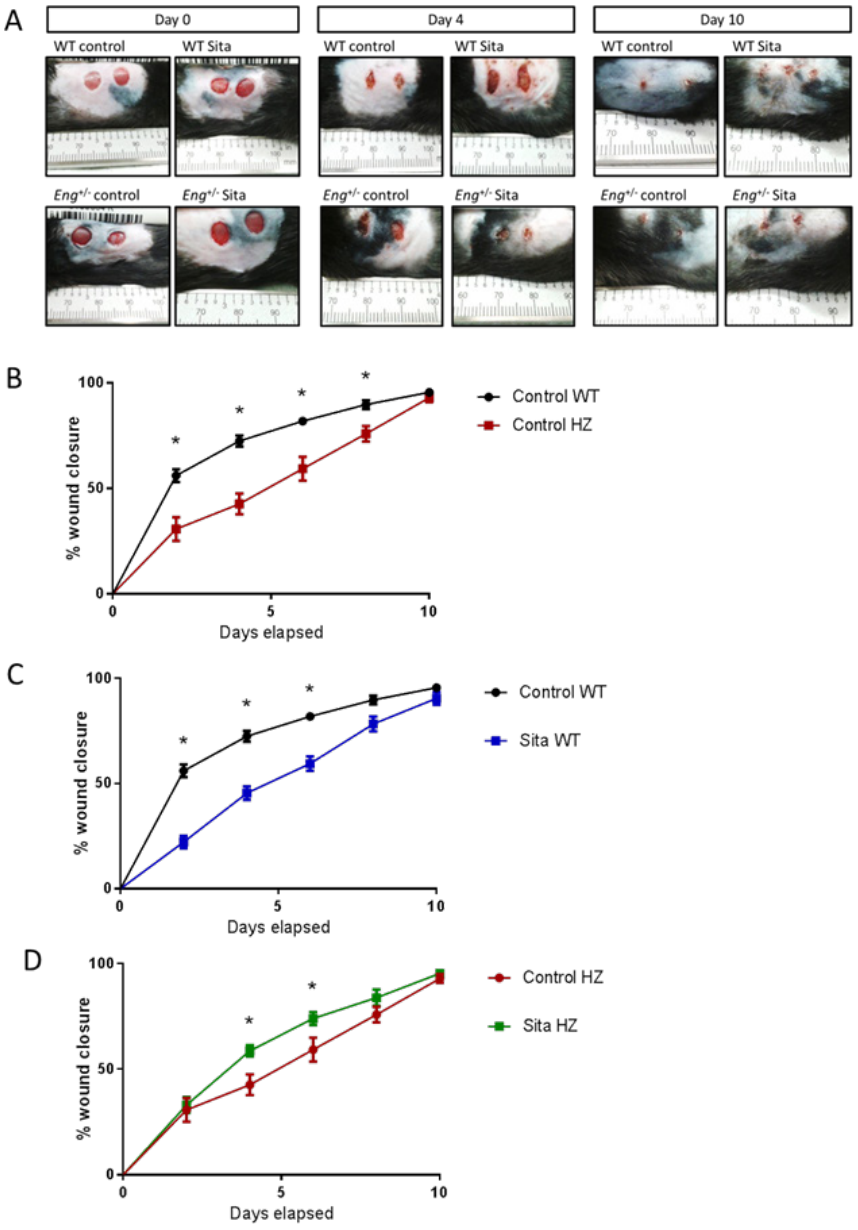

Fig. 1 Sitagliptin enhances tissue repair in Eng+/- mice. A) WT and Eng+/- wound closure over time, day 0, 4 and 10. Wounds from WT and Eng+/- mice treated with DPP4 inhibitor Sitagliptin (Sita) or placebo control. Wound closure in Eng+/- mice is delayed compared to wild type mice. Sitagliptin enhances tissue repair in Eng+/- mice. B) Quantification of average wound closure over time. Treatment with DPP4 inhibitor Sitagliptin. WT control vs. Eng+/- (in graph: HZ-heterozygous) control treated animals. C) WT control vs. WT Sitagliptin treated animals. Sitagliptin delays tissue repair in wild type mice. D) Eng+/- control vs. Eng+/- Sitagliptin treated animals. Sitagliptin enhances tissue repair in Eng+/- mice.

DPP4 inhibition decreases fibrosis in the *Eng*$^{+/-}$ wound area

Since Sitagliptin had a different effect on wound closure in WT versus *Eng*$^{+/-}$ mice, we performed further tissue analyses to gain more insight in this observation. Although DPP4 inhibitors are mainly used in T2DM to prevent cleaving of incretins – DPP4 has a lot more known and unknown targets. One of the events in wound healing is the activation of fibroblasts, cells that are necessary to create new extracellular matrix ECM and collagen for wound contraction and scar formation[37]. Since DPP4 inhibition is reported to have an anti-fibrotic effect[38-41], we analyzed the effect of Sitagliptin on the expression of αSMA, a marker for myofibroblasts formation. αSMA was quantified in sections 5 days post-wounding. In control treated *Eng*$^{+/-}$ mice, αSMA content was significantly increased in the wound area compared to WT animals (Fig. 2). Upon Sitagliptin treatment the αSMA levels in the wounding area of WT mice were not affected. However, the αSMA levels in the wounding area of *Eng*$^{+/-}$ mice were decreased significantly, suggesting myofibroblast presence and/or activity was inhibited by DPP4 inhibition.

DPP4 inhibition increases the number of M2 macrophages in the wound area

We previously reported that in *Eng*$^{+/-}$ animals the inflammatory (M1-like) macrophages were increased compare to reparative (M2-like) macrophage numbers and these mice show a disrupted cardiac repair. Therefore we next investigated whether or not macrophage function and/or differentiation in the *Eng*$^{+/-}$ mice in dermal wound healing is impaired as well. To further determine the impact of DPP4 inhibition, we analyzed the presence of the MAC3^{+}/CD206^{+} M2-like (referred to as 'M2') macrophages near the wound area (Fig. 3A). Topical Sitagliptin treatment on dermal wounds significantly increased the numbers of M2 in both WT and *Eng*$^{+/-}$ mice (Fig. 3B).

DPP4 inhibition blunts macrophage stress responses to LPS stimulation

Because of the change in macrophage population (towards the reparative type) observed when we applied topical Sitagliptin treatment, we next questioned whether or not there is an effect of DPP4 inhibition on the intracellular signaling of both the WT and *Eng*$^{+/-}$ macrophages. And moreover, whether there is a change response to signals present in the tissue and/or disrupted signaling in the *Eng*$^{+/-}$ macrophages. To investigate whether DPP4 inhibitor specific effects would vary we also added a second competitive DPP4 inhibitor– Diprotin A- in addition to Sitagliptin. The main difference between the two inhibitors is that Diprotin A has reversible DPP4 inhibition, whereas Sitagliptin inhibits DPP4 irreversibly. We then investigated activation of TGFβ and stress related intracellular signaling pathways. Murine monocytes were isolated from bone marrow aspirates, and stimulated with GM-CSF for 7 days to induce differentiation into macrophages. DPP4 inhibition was added to the culture at day 3 to pre-sensitize the cells and ensure optimal DPP4 inhibition.

Activation of the canonical TGFβ pathway via ALK5 - SMAD2/3 phosphorylation was determined via pSMAD2 (pSMAD1/5/8 was not detectable in our cells). In both the WT and *Eng*$^{+/-}$ cells pSMAD2 activation was increased when TGFβ was added to the culture (similar to findings by Letarte et al. 2005 in HHT1-HUVECs[42]) (Fig. 4A and B). Surprisingly, upon addition of Sitagliptin pSMAD2 decreased in *Eng*$^{+/-}$ and WT, and also the total SMAD levels went down, even when stimulated with TGFβ.

To understand the effect of Sitagliptin on inflammation, we stimulated the macrophages with

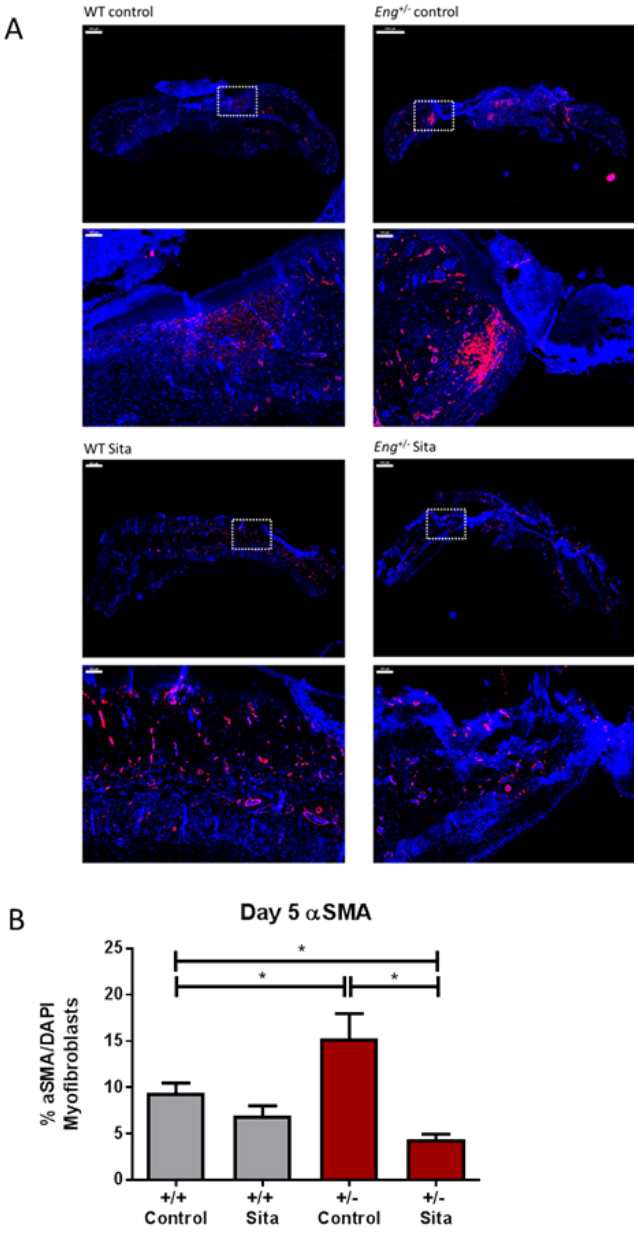

Fig. 2 Sitagliptin treatment decreases activated fibroblast presence in the skin surrounding wounded area. A) Histological analysis of activated myofibroblast presence during Eng+/- wound healing. Immunofluorescence staining of αSMA on PFA-fixed Paraffin-embedded wounds from WT and Eng+/- mice at day 5 post-wounding. Upper panels: skin section overview. Lower panels: magnification of inset depicted in upper panels. Scale bars WT control, WT sita and Eng+/- = 500µm, Eng+/- control=1000µm. All scale bars of the insets/magnifications=100µm B) Quantification of activated fibroblasts of immunofluorescence staining of aSMA on PFA-fixed Paraffin-embedded wounds from WT and Eng+/- mice at day 5 post-wounding.

LPS, 24hrs before harvesting. Induction of pSMAD2 increased markedly, and this effect was even more pronounced in the *Eng*$^{+/-}$ cells. When TGFβ was added, pSMAD2 increased in both WT and *Eng*$^{+/-}$ cells. With Sitagliptin inhibition a decrease was observed in the *Eng*$^{+/-}$ macrophages, whereas we did not observe a difference in the WT cells. PSMAD2 is strongly induced in the LPS-treated cells, and co-treatment with TGFβ induces pSMAD2 to an even greater extend. LPS with Sitagliptin added does not show any pSMAD2 difference compared to the LPS only signal (Fig. 4). Co-treatment of both TGFβ and Sitagliptin does not show any additional increase. Sitagliptin's inhibitory effect was not detectable in the

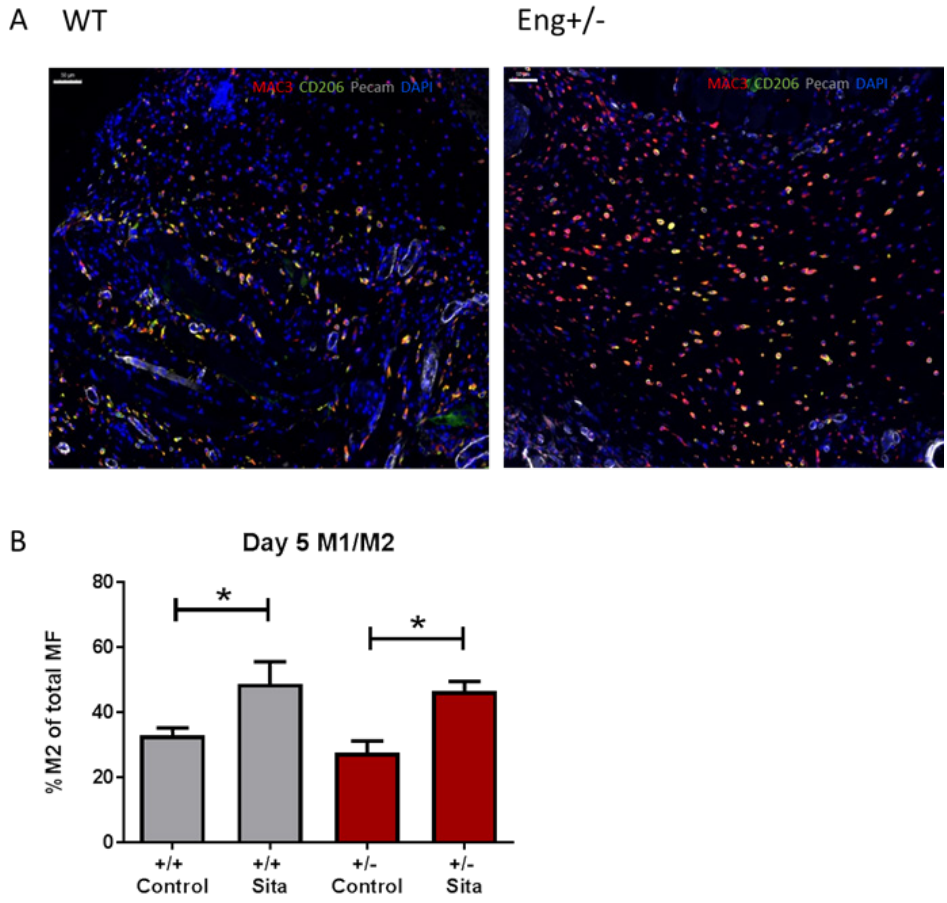

Fig. 3 DPP4 inhibition results in an increased reparative macrophage presence. A) Histological analysis of macrophage presence during Eng+/- wound healing. Representative immunofluorescence staining image of MAC3 and CD206 on PFA-fixed paraffin-embedded wounds from WT and Eng+/- mice at day 5 post-wounding. MAC3=red, CD206=green, Pecam=white, DAPI=blue. Scale bar = 50µm B) Sitagliptin treatment increases M2 presence in the skin surrounding wounded area. Percentage CD206+ cells (M2 macrophages) of total MAC3+ macrophages present in the wound border zone.

LPS treated macrophages. Overall, while pSMAD2 signaling is affected by DPP4 inhibition, it does not vary significantly between WT and *Eng*$^{+/-}$ (Fig. 4B). These results suggest the macrophage signaling affected by DPP4 inhibition are probably not SMAD–mediated and involve other TGFβ-related pathways. Therefore, we next investigated the effects of DPP4 inhibition on non-SMAD TGFβ signaling. Activation of the non-canonical/SMAD TGFβ pathway and AKT is part of a survival pathway, and also involved in cell proliferation and M2 differentiation. pAKT was found increased in *Eng*$^{+/-}$ at endogenous levels. In both WT and *Eng*$^{+/-}$ cells pAKT decreases upon Sitagliptin addition, though in WT macrophages the inhibition of pAKT is more pronounced. This could indicate that the response via AKT signaling is blunted in *Eng*$^{+/-}$ macrophages.

The combination of TGFβ and Sitagliptin inhibits AKT phosphorylation (Fig. 4A). The total amounts of AKT were also decreased by Sitagliptin addition. LPS stimulation of the macrophages caused an increase of pAKT; this induction was even more pronounced in the *Eng*$^{+/-}$ (Fig. 4A).The increase in pAKT to additional TGFβ stimulation was similar in both WT and *Eng*$^{+/-}$ macrophages. With the addition of Sitagliptin we observed either no effect or a slight increase in the WT macrophages, whereas the signal steadily decreased in the *Eng*$^{+/-}$ cells. The combination of TGFβ with Sitagliptin gave varying results in the WT cells, but

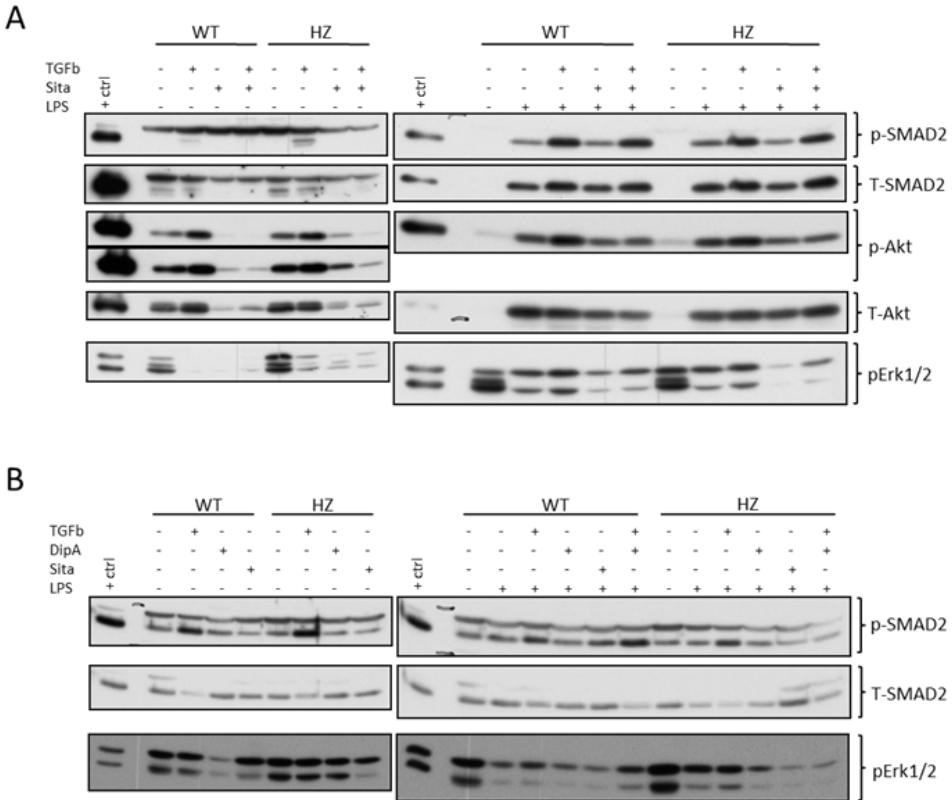

Fig. 4 DPP4 inhibition decreases intracellular signaling in cultured macrophages in response to LPS. Western blot results of bone-marrow derived macrophages stimulated with Sitagliptin, Diprotin A, TGFβ or LPS in varying combinations. N=3, representative blot results for both phosphorylated and total SMAD2, AKT and ERK1/2.

showed a reproducible decrease in the *Eng*$^{+/-}$ macrophages, returning the amount of pAKT to Sitagliptin-only levels. We conclude that both in physiological/non-inflammatory and in inflammatory conditions TGFβ-induced pAKT is negated by DPP4 inhibition.

ERK signaling is known to be involved in cell proliferation[43], survival[44], and pro-inflammatory cell responses[45,46]. Therefore we analyzed the effect of Sitagliptin on pERK1/2 in both WT and *Eng*$^{+/-}$ macrophages. Interestingly, the endogenous level of pERK1/2 was already increased in the *Eng*$^{+/-}$ macrophages. Treatment with either Sitagliptin or DipA led in both WT and *Eng*$^{+/-}$ cells to a decrease in phosphorylation, although more pronounced in the *Eng*$^{+/-}$ macrophages. TGFβ did not change the pERK1/2 levels in either WT or *Eng*$^{+/-}$ cells. Sitagliptin also inhibited the TGFβ-induced ERK1/2 phosphorylation. Co-treatment with TGFβ and Sitagliptin decreased pERK1/2 in equal amount for both wild type and *Eng*$^{+/-}$ cells. Total levels of ERK1/2 were not affected.

LPS stimulation increased pERK1/2 in both WT and *Eng*$^{+/-}$ macrophages. Treatment with Sitagliptin or co-treatment with TGFβ to the LPS pre-treated cultures consistently decreased the pERK1/2 response in both WT and *Eng*$^{+/-}$ macrophages. This indicates that LPS and/or TGFβ -induced pERK1/2 is repressed by DPP4 inhibition, indicating an anti-inflammatory response by the DPP4 inhibitor. We confirmed the anti-inflammatory effect of Sitagliptin via a luciferase reporter assay for NFκB expression in HEK293T cells. Addition of the DPP4 inhibitor resulted in a significant decrease in NFκB expression (Fig. 5).

We conclude that Sitagliptin is able to reduce macrophage stress signaling responses after LPS stimulation, most likely influencing the polarization of these cells towards a more reparative phenotype, the M2 cells. As we now prove that DPP4 inhibition is able to influence TGFβ-related signaling and cell stress responses, this might explain the difference in wound repair in the wild type and *Eng*$^{+/-}$ mice, and we speculate that endoglin heterozygosity most likely impairs the correct functioning of these macrophages.

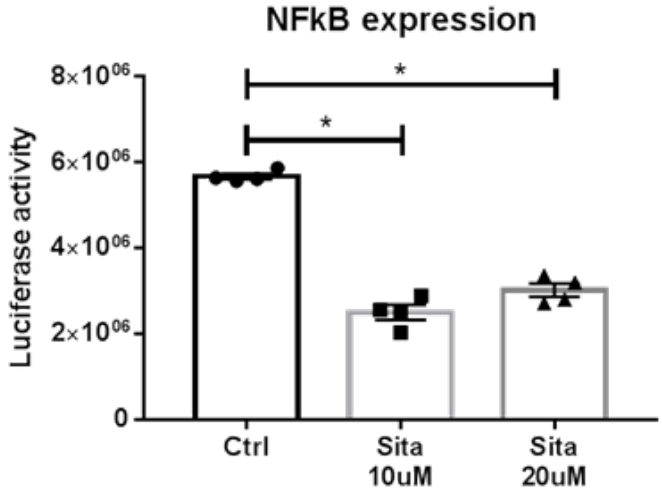

Figure 5. NFκB expression decreases with DPP4 inhibition. Luciferase reporter assay on lysates of HEK293T cells. Sitagliptin was added to the culture at 10µM and 20µM. *=p<0.05

The inflammatory response of *Eng*$^{+/-}$ macrophages is modulated by DPP4 inhibition

The major increase of M2 macrophages present in the wound area raised the question how DPP4 inhibition is able to alter the inflammatory response. We therefore determined the *in vitro* expression of one of the key inflammatory cytokines involved in the macrophage chemotaxic response, Monocyte Chemoattractant Protein-1 (MCP-1, CCL2)[47]. ELISA analysis of the supernatants showed that MCP-1 levels are higher in the *Eng*$^{+/-}$ macrophages compared to the wild type cells (Fig. 6A), indicating a possible hyper-inflammatory status in *Eng*$^{+/-}$ animals.

When Sitagliptin was added to the culture, no changes were detectable in extracellular MCP-1 levels from WT cells (Fig. 6C), while in the *Eng*$^{+/-}$ macrophages the MCP-1 level decreased significantly (Fig. 6D), restoring MCP-1 expression to WT levels (Fig. 6B). When pretreating the macrophages with LPS, we observed a similar trend, MCP-1 level was higher in the *Eng*$^{+/-}$ macrophages. However there was no significant difference in MCP-1 expression level after Sitagliptin addition (Fig. 7 A and B). LPS treatment may therefore abolish the effects of Sitagliptin we observed without LPS addition to the culture.

Another important cytokine in dermal wound healing is interleukin-6 (IL-6). IL-6 was found to be involved in keratinocyte proliferation[48,49] and macrophage differentiation[50,51]. When the macrophages were stimulated with LPS to induce IL-6 production, the IL-6 level was decreased in the *Eng*$^{+/-}$ cells (Fig. 7C) (As previously reported by Scharpfenecker et al.[52]). Sitagliptin treatment however did not affect IL-6 cytokine levels (Fig. 7D). These results

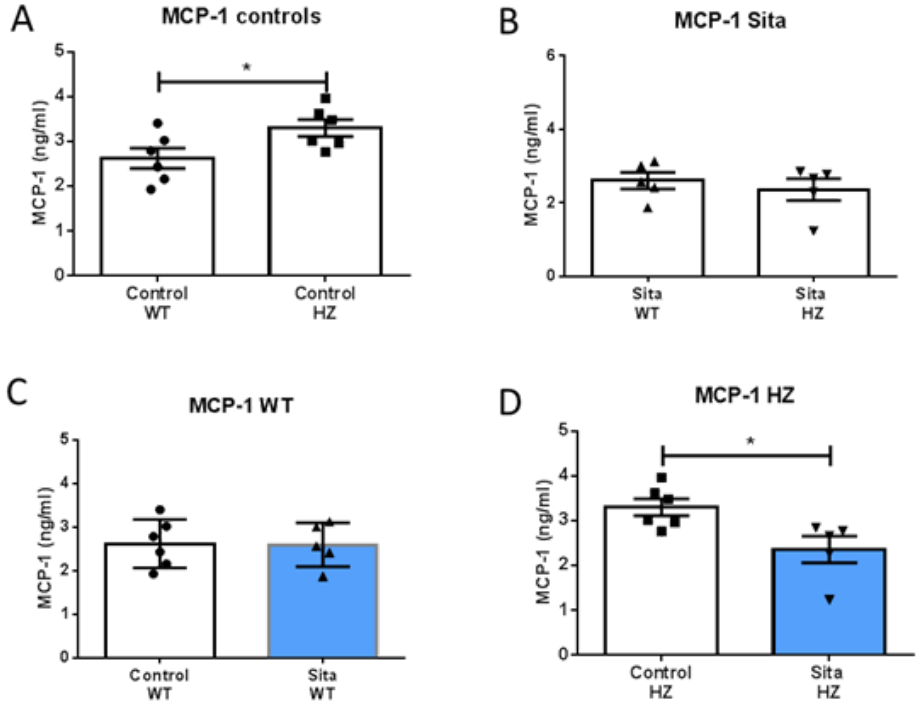

Figure 6. MCP-1 levels and the effect of DPP4 inhibition in cultured murine macrophages. Macrophage culture media samples were used for ELISA detection of MCP-1. Measurements were performed with media samples from n=5-6 mice per group. SEM, *=p<0.05

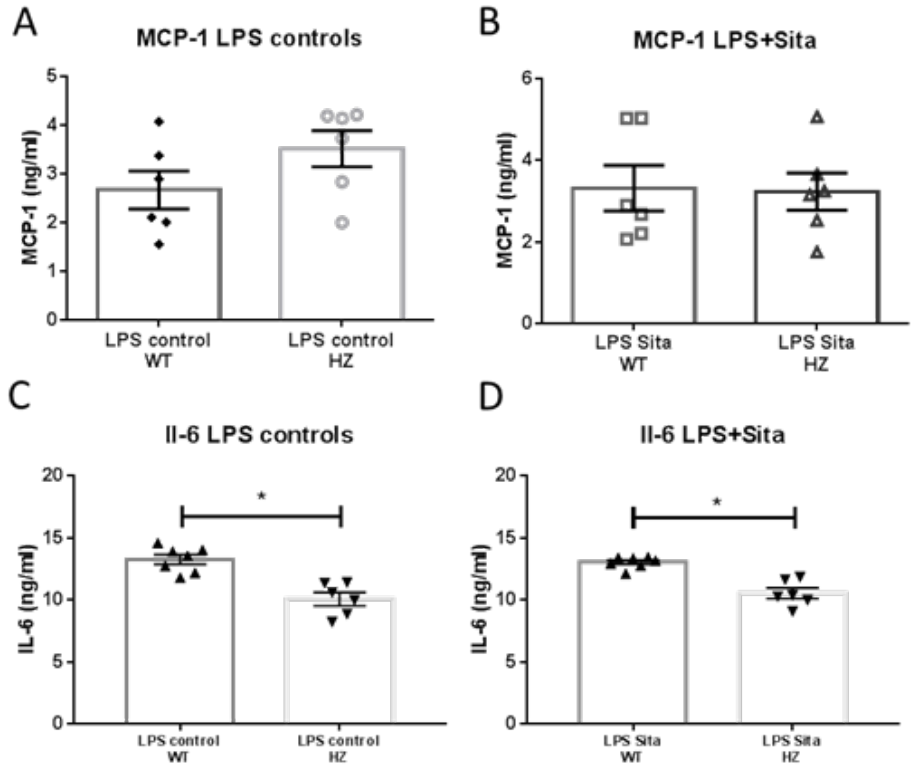

Figure 7. Effect of DPP4 inhibition on expression of inflammatory-related cytokines IL-6 and MCP-1 in macrophage culture after LPS stimulation. Macrophage culture media samples were used for ELISA detection of MCP-1 and IL-6. LPS was added for 24 hrs. A) MCP-1 control treated macrophages B) MCP-1 Sitagliptin treated macrophages C) IL-6 control treated macrophages D) IL-6 Sitagliptin treated macrophages. Measurements were performed with media samples from n=5-6 mice per group. SEM, *=p<0.05

suggest that low IL-6 levels at baseline might reduce keratinocyte proliferation, and could affect macrophage function as well.

To further unravel the mechanism explaining the localized wound tissue responses, we next examined tissue samples from the *in vivo* wounding study. We analyzed the expression of inflammatory markers localized directly next to the wounding site. Tissue sections were stained for inflammatory markers involved in the NFκB complex and pathway: p-p65 (NFκB complex subunit) and pIKKα/β (protein necessary for NFκB complex phosphorylation[53]) (example of tissue staining can be found in Supplementary Fig. 2), were quantified. Phosphorylated IKKα/β levels were increased in the $Eng^{+/-}$ wound sections (Fig. 8A). Sitagliptin treatment decreased pIKKα/β levels in $Eng^{+/-}$ wounds to comparable levels as WT wounds (Fig. 8A). Although a trend towards increased pIKKα/β is visible in the WT wounds treated with Sitagliptin, no significant effect on the pIKKα/β levels were observed (which could indicate a reverse response of IKK to DPP4 inhibition). This could indicate an anti-inflammatory effect of Sitagliptin in the $Eng^{+/-}$ wounds, the slight increase in pIKKα/β in WT animals signifies increased inflammation and could therefore explain the delayed wound healing observed in these animals.

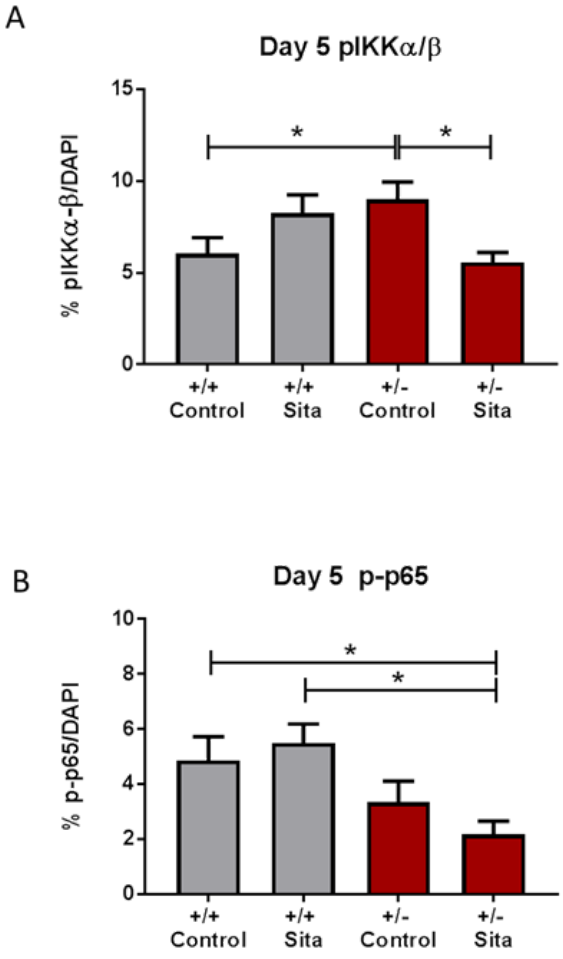

Figure 8. Sitagliptin treatment decreases the inflammatory cytokine response in Eng+/- wounds. A) Quantification of pIKKα/β levels in the wound area, 5 days post-wounding. B) Quantification of p-p65 levels in the wound area, 5 days post-wounding. Single or duplet measurements were made per individual mouse, SEM, n=4 mice per group,*=p<0.05

Phosphorylated p65 was not significantly different between WT and *Eng*$^{+/-}$ wounds (Fig. 8B). Interestingly, while no effect was observed on the p-p65 levels in the WT wounds treated with Sitagliptin, Sitagliptin treatment decreased the levels of phosphorylated p65 in the *Eng*$^{+/-}$ wounds compared to WT p-p65 levels (Fig. 8B). In summary, DPP4 inhibition decreases NFκB-mediated inflammatory response in the wound area of *Eng*$^{+/-}$ mice.

Discussion

Eng$^{+/-}$ mice are affected in their immune response[25-28], characterized by a skewed M1/M2 balance towards the pro-inflammatory M1 macrophages[34] and a prolonged and delayed wound healing[30,34]. Previously we showed that DPP4 inhibition could direct *Eng*$^{+/-}$ macrophage homing and/or differentiation towards the more reparative M2 macrophages. We aimed to restore dermal wound repair by using topical DPP4 inhibition.

The present study demonstrates that topical treatment with DPP4 inhibitor Sitagliptin results in an improved dermal wound repair in *Eng*$^{+/-}$ mice. Furthermore, we now show that in the *Eng*$^{+/-}$ macrophages, Sitagliptin is able to exert anti-inflammatory effects, reversing the *Eng*$^{+/-}$ macrophages to a less inflammatory phenotype. At the wounding site, *Eng*$^{+/-}$ mice had increased levels of myofibroblasts present. Whereas we observed only a slight decrease in wild type mice, treatment with Sitagliptin significantly decreased the myofibroblast content in the wounds of *Eng*$^{+/-}$ animals. These results imply an anti-fibrotic effect of DPP4 inhibition, consistent with findings in other disease studies like kidney or liver fibrosis[38-41]. Conversely, in diabetic Ob/Ob mice, myofibroblasts were found increased when treated with DPP4 inhibitor Linagliptin[54], of course the diabetic Ob/Ob–specific model/setting could be the reason of the pro-fibrotic outcome of DPP4 inhibition. However, this study also demonstrated that DPP4 inhibition did prove to have an anti-inflammatory effect, via the lowering of pro-inflammatory markers like cyclooxygenase-2 and MIP-2 (macrophage inflammatory protein-2)[54]. In our current study, we provide a mechanism for the anti-inflammatory effects in the *Eng*$^{+/-}$ mice, We show that in the *Eng*$^{+/-}$ mice Sitagliptin treatment decreased p-p65, pIKKα/β *ex vivo* and MCP-1 *in vitro.* Furthermore DPP4 inhibition decreased phosphorylation of other stress-related signaling like ERK1/2 and AKT in cultured macrophages, possibly accounting for the accelerated wound healing observed in these mice.

Because macrophages have major immune-regulatory functions in tissue repair[55], we focused only on this cell type. Determining the local effects on macrophage population, we showed that Sitagliptin increased the number of M2 macrophages present at the skin wounding site. However, the increase in M2 in the WT mice did not result in increased tissue repair, indicating that M2 macrophages are not sufficient or always beneficial in tissue repair[56,57].

Investigating bone marrow-derived macrophage responses to TGFβ and LPS stimulation, the intrinsic differences in stress signaling varied between wild type and *Eng*$^{+/-}$ macrophages. Already at baseline conditions, pERK1/2 was increased in *Eng*$^{+/-}$ macrophages. Ras-ERK1/2 pathway activation is associated with being pro-cell survival[44], moreover, with pro-inflammatory effects[58], suggesting this could be the reason of prolonged inflammation in the *Eng*$^{+/-}$ mice.

Activation of the SMAD/AKT pathway is associated with polarization towards M2 macrophages[59,60]. However, in our macrophage culture addition of Sitagliptin in both non- and inflammatory conditions reduced total AKT and p-AKT expression dramatically, in both the WT and *Eng*$^{+/-}$ cells. Therefore the M2 polarization in our skin model is most likely AKT-independent. The PI3K-AKT pathway has also been reported to be involved in proliferation and importantly, inducing macrophage activation[61]. This provides a correlation of macrophage activation with DPP4 inhibition; decreasing AKT signaling which leads to a decrease in macrophage activation. AKT therefore may in part be responsible for the anti-inflammatory responses we observed *in vivo* and *in vitro*.

Besides a disturbed inflammatory response, we also report that *Eng*$^{+/-}$ mice displayed

increased fibroblast presence, and also co-localized with increased AKT phosphorylation (as reported by Pericacho et al.[31]). DPP4 inhibition decreased pAKT, thereby increasing wound repair via fibroblast activity; thus possibly providing the mechanism of accelerated wound healing in our study. Overall, we conclude that the inflammatory response of macrophages is increased in endoglin heterozygous conditions, and the intracellular macrophage signaling response to DPP4 inhibition seems to be increased in *Eng*$^{+/-}$ macrophages. DPP4 inhibition is able to decrease inflammatory markers in vivo. We conclude that DPP4 inhibitor treatment is most likely only beneficial for dermal wound healing in *Eng*$^{+/-}$ conditions.

The inflammation and stress-related signaling aberrations found in *Eng*$^{+/-}$ macrophages highlight the complexity of the immunological defects present in HHT1. Whereas in this study we focused on macrophages, the effects of DPP4 inhibition on other immune cells and tissue cells is still largely unknown. For example, DPP4 inhibition was shown to increase re-epithelization in a diabetic model using Ob/Ob mice[54]. Therefore in future research it is important to keep in mind the varying effects between DPP4 inhibitors[62] and also the complexity of the signaling pathways and reactions. Furthermore the other cell types affected by endoglin heterozygosity or DPP4 inhibition, and involved in tissue repair are also to be considered, such as endothelial[63] and epidermal progenitor cells[64], keratinocytes and fibroblasts[30,38,65].

In conclusion, the results presented in this paper suggest an inhibitory effect of DPP4 inhibition on inflammation and a decrease of local cell stress/proliferation responses in endoglin haploinsufficiency context. These findings suggest that the mechanisms by which DPP4 inhibition is able to positively direct wound healing in *Eng*$^{+/-}$ mice is by decreasing the pro-inflammatory signaling in the macrophages and injured tissue. Further research should focus on finding a direct correlation between canonical/non-canonical TGFβ signaling and DPP4 inhibition, and could lead to revealing new mechanisms in the pathogenesis of HHT1.

Acknowledgements
This work was financially supported by the Netherlands Institute for Regenerative Medicine (NIRM, FES0908), the Dutch Heart Foundation (NHS2009B063), and by the Netherlands Cardiovascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences (CVON-PHAEDRA consortium).

Conflict of interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author Contributions
M.G, I.E.H, W.B and C.D conceptualized the study set up and design. C.D, K.L, and K.K performed experiments. C.D wrote the manuscript. All authors reviewed the manuscript.

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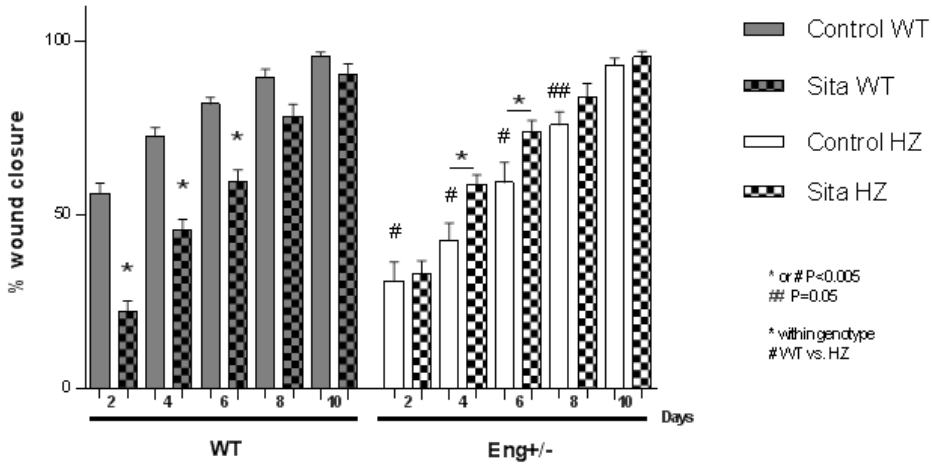
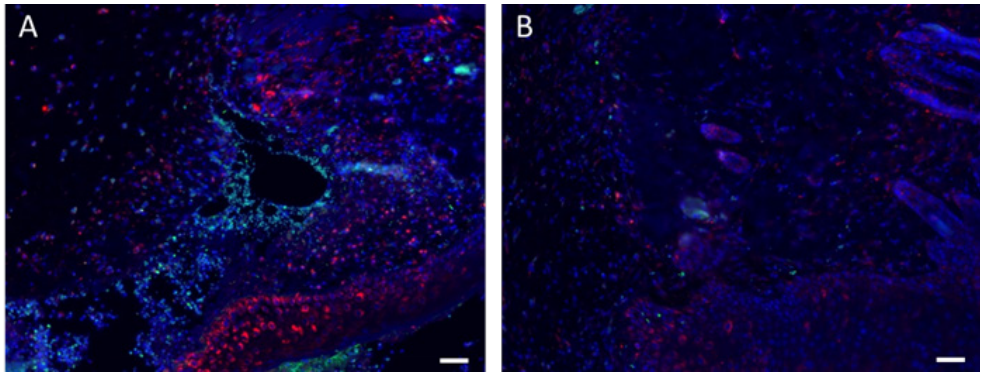
Supplementary figures



Supplementary Figure 1. Sitagliptin enhances tissue repair in Eng+/- mice. A) Quantification of wound closure over time. Sitagliptin delays tissue repair in wild type mice. Sitagliptin enhances tissue repair in Eng+/- mice. WT = wild type mice, HZ = Endoglin heterozygous mice.



Supplementary Figure 2. Example of p-p65 (NFκB complex subunit) and pIKKα/β (regulators of NFκB activation) staining.
A. Day 5 post-wounding, Eng+/- skin sample control treated. P-p65 in green, pIKKα/β in red, DAPI in blue. Scale bar = 50µm. B. Day 5 post-wounding , Eng+/- skin sample Sitagliptin treated. P-p65 in green, pIKKα/β in red, DAPI in blue. Scale bar = 50µm.