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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Pregnancy-specific glycoprotein 1 (PSG1) promotes wound closure in animal models

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KEYWORDS

Pregnancy-specific glycoprotein, wound therapy, TGFβ1, keratinocyte, epithelialisation

1. INTRODUCTION

Pregnancy-specific glycoproteins (PSG) are immunoglobulin superfamily members secreted by placental trophoblasts at high concentrations into the maternal circulation during pregnancy. Human PSGs are encoded by a multigene family and are closely related to the CEACAM family of predominantly membrane-anchored proteins.[1,2] PSGs have evolved predominantly in mammals with haemochorial placentation, such as rodents and primates, in which maternal blood is in direct contact with placental trophoblast, and their proposed functions include pro-angiogenic activity, immunoregulation and thromboregulation.[1,2] These activities are mediated through various mechanisms including TGFβ1 activation,[3] and binding to various cellular receptors including syndecans and integrins.[4,5] At the cellular level, PSG activity leads to macrophage polarisation to the M2 phenotype, induction of Th2 cytokines, and T regulatory cell differentiation.[1,2]

In acute wounds, which includes surgical incisions, trauma and burns, the promotion of rapid wound closure and the prevention of scarring are key aims.[6] We hypothesised that PSG1 has potential to provide a novel therapeutic for wounds given its pro-angiogenic and immunomodulatory activities. Prompted by these observations, we investigated the efficacy of recombinant PSG1 (PSG1-V5-His) in cell line and animal models of wound healing.

2. METHODS

Animals

C57Bl/6J mice and Landrace pigs were housed under standard husbandry conditions in the Biological Services Unit, University College Cork. Ethical permissions for animal procedures were granted by the UCC Animal Experimentation Ethics Committee and procedures were conducted under a Health Products Regulatory Authority (www.hpra.ie) licence to J. Clover (AE19130/P024 Case no. 702095).

Cell culture

The human immortalised keratinocyte cell line, HaCaT,[7] was purchased from the German Cancer Research Center (DKFZ). Cells were grown in DMEM (D6429, Sigma-Aldrich, UK), supplemented with 10% FBS; 100 µg/ml streptomycin; 100 U/ml penicillin; 2 mM L-Glutamine; and cultured at 37 °C and 5% CO_2.

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FreeStyle™ 293-F HEK cell line was used for recombinant protein production. Cells were cultured in Freestyle 293 Expression medium (Thermo Scientific, UK) at 37 °C and 8% CO_2, by shaker culture, to a density of 1×10^6 cells per ml in 250 ml culture volume.

Recombinant PSG1 protein production

The pTT3-PSG1-V5-His expression vector was used to produce PSG1-V5-His (hereafter PSG1) protein. Briefly, the expression vector was transiently transfected into Freestyle 293-F cells using Freestyle MAX reagent (Thermo Scientific, UK). The plasmid DNA was diluted in OptiPRO Serum Free Medium at a ratio of 1 µg DNA in 20 µl OptiPRO for every 1 ml of cells. Freestyle MAX reagent was also diluted in OptiPRO at the same ratio (1 µl Freestyle MAX reagent in 20 µl OptiPRO per ml of cells). The diluted DNA and Freestyle MAX reagent were then combined, mixed gently and incubated at room temperature (RT) for 20 minutes (min). The mixture was added to the cell suspension and the cells were cultured for a further 72 hours (h). The culture was then centrifuged at 1,000 revolutions per minute (rpm) for 5 min at RT to separate the protein-containing medium from the cells.

Recombinant PSG1 protein was purified from cell culture medium using Qiagen Ni-NTA resin. Imidazole (Sigma, UK) was added to the culture medium to a final concentration of 10 mM to reduce non-specific binding. Ni-NTA resin was added to the medium at a ratio of 1 ml resin suspension (corresponding to 0.5 ml resin bed volume) to 100 ml medium. The medium and resin were batch bound overnight on a rotating wheel at 4 °C, passed through a disposable polypropylene column (Pierce, Thermo Fisher Scientific, Ireland), and washed with wash buffer (500 mM NaCl, 20 mM NaH_2PO_4, pH6) until the absorbance at 280 nm reduced to 0. Protein was then eluted from the column with increasing concentrations of imidazole in wash buffer, usually 4×1.5 ml 50 mM fractions, 5×1.5 ml 200 mM fractions, 4×1.5 ml 300 mM fractions and 3×1.5 ml 500 mM fractions. The fractions were pooled and concentrated to a volume of 1 ml using an Amicon Ultra Ultracel 10K centrifugal filter (Millipore, Ireland). The concentrate was then gel filtered using a HiLoad® 16/60 Superdex® 75 pg (Merck, UK) column and AKTA explorer FPLC, in phosphate buffered saline (PBS) at 4 °C. The resultant peak was fractionated and purified recombinant PSG1 protein was then further concentrated to a volume of 1 ml and sterile filtered using a 0.2 µm filter. Protein was quantified by Bradford Assay or UV Spectroscopy, checked by polyacrylamide gel electrophoresis, tested for LPS contamination (Limulus Amebocyte Lysate QCL-1000; Cambrex BioScience, Germany) aliquoted and frozen at minus 80 °C.

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Scratch wound assays

HaCaT cells (1×10^5 cells/ml) were seeded in 1 ml in 6-well plates, with one IBIDI Culture Insert per well. After 24 h, inserts and medium were removed and each well was treated with 1 ml of cell culture medium, supplemented with 50 µg of PSG1 in 50 µl PBS, or 50 µl PBS only. Scratch wounds were imaged 16 h post-treatment using an EVOS FL Auto and Wimasis WimScratch or ImageJ analysis was used to determine degree of wound closure. A selective inhibitor of TGFβRI (SB432542, R&D Systems, USA) was used to explore the mode of action of PSG1 in wound healing. HaCaT cells were treated with 10 µm/ml SB431542 for 24 h before PSG1 treatment.

Gene expression analysis

RT² Profiler™ PCR Array Human Wound Healing (330231, cat no. PAHS-121ZE-4; Qiagen, UK) was used with the ABI 7900HT 384 well Fast Block sequence detection system, as per manufacturer's instructions. HaCaT cells were grown as previously described, with cells seeded at a density of 1×10^5 cells/ml per well in 6-well plates. 80% confluent HaCaT cells were treated with 50 µg PSG1 for 24 h. Post-treatment, cells were harvested and total RNA was extracted. 1 µg RNA was used to synthesise cDNA using the RT² First Strand Kit as recommended by the manufacturer. cDNA template was mixed with RT² SYBR Green qPCR Mastermix and 10 µl applied to each well of a 384-well plate. Cycling conditions were as follows: initial denaturation (95 °C for 10 min); 40 cycles of 95 °C for 15 sec and 60 °C for one min; followed by a melting curve program (60 – 95 °C, with a heating rate of 1 °C per 30 sec and a continuous fluorescence measurement). Fluorescence data were collected, CT values were exported and analysed using the online GeneGlobal Data analysis centre (Qiagen).

qPCR of Wound Array Gene Candidates

First strand cDNA was synthesised using 1 µg total RNA in a 20 µl reaction using random hexamer priming and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative RT-PCR (qPCR) primers were designed to give unbiased amplification of candidate gene transcripts: *CXCL5* primer sequences were hCXCL5 F: 5'-ACAGACCACGCAAGGAGTTC-3'; hCXCL5 R: 5'-TCCTTGTTTCCACGGTCCAA-3'. *TNFα* primer sequences were hTNFα F: 5'-CAACCTCCTCTCTGCCAT-3'; hTNFα R: 5'-GAAGACCCCTCCCAGATA-3'. *COL3A1* primer sequences were hCOL3A1 F: 5'-

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TCCGGGTGAGAAAGGTGA-3'; hCOL3A1 R: 5'-GCAGGTCCAGAACCTCCAG-3'. The reference gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression was used to normalise mRNA input and cDNA synthesis efficiency using the primers: hGAPDH F: 5'-GAAGGTGAAGGTCGGAGT-3'; hGAPDH R: 5'-GAAGATGGTGATGGGATTTC-3'. qPCR was carried out in triplicate 20 µl reactions using SYBR Green PCR Master Mix (Applied Biosystems, UK), 1 µl cDNA and primers at 600 mM using the ABI PRISM 7900HT instrument. PCR cycle: initial denaturation (95 °C for 10 min); amplification and quantification repeated for 40 cycles (95 °C for 45 sec, 60 °C for 30 sec, and 72 °C for 60 sec with a single continuous fluorescence measurement); followed by a melting curve program (60 – 95 °C, with a heating rate of 1 °C per 30 sec and a continuous fluorescence measurement). HaCaT cell cDNA was used to produce the standard curve. PCR products were identified by generating a melt curve and results were expressed as mean gene of interest expression relative to mean GAPDH expression.

Mouse excisional skin wound model

Mice were anaesthetized with an intraperitoneal injection of Ketamine (90 mg/kg; Vetalar, Boehringer, Ingelheim Germany) and Xylazine (10 mg/kg; Sedaxylan, Bimeda, Dublin, Ireland). A 0.4 cm diameter circular full thickness excision wound was created in healthy or diabetic adult male C57Bl/6J mice and either 100 µl PBS or 50 µg PSG1 in 100 µl PBS was injected intradermally at four equidistant sites around the wound margin. A preliminary dose-response experiment indicated that the lowest effective dose that enhanced wound closure was 50 µg, and this dose was used in subsequent experiments. Wounds were measured and photographed immediately following PSG1 administration (day 0) and on day 4 using a Canon DSLR camera (Tokyo, Japan).

Diabetes was induced essentially as described.[8] Briefly, multiple intraperitoneal injections of streptozotocin (40 mg/kg) were administered to adult male C57Bl/6J mice on five consecutive days, along with 10% sucrose in drinking water, to induce type 1 diabetes. Development of hyperglycemia was confirmed using commercially sourced test strips.

Pig excisional skin wound model

Pigs were anaesthetized with Xylazine (2 mg/kg; Sedaxylan, Bimeda, Dublin, Ireland) and Ketamine (15 mg/kg; Vetalar, Boehringer, Ingelheim Germany), and maintained with 2% Isofluorane. Six 2×2 cm (4 cm^2) full thickness excisional wounds were created on each flank

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of 5 female landrace pigs and treated on day 0 with 1 ml PBS or 250 µg PSG1 in PBS by intradermal injection at eight sites around the wound margin, with a repeated treatment on Day 3. Dressings were changed on days 3 and 7, and wounds were photographed on days 0, 3, 7 and 10 using a Canon DSLR camera (Tokyo, Japan). Animals were sacrificed on day 10 and the wounds were excised for histological analysis, essentially as described.[9,10] Rate of wound healing was determined by comparing size of wound to original wound. Wound area was measured by tracing the wound margin using an image analysis programme (NIH ImageJ). The percentage of wound closure was calculated as: (area of original wound - area of actual wound) / (area of original wound) × 100.[11]

At day 10 the wounds were harvested for histological analysis. To provide a further measure of re-epithelialisation, wound sections were stained with hematoxylin and eosin (H&E), and several overlapping images were taken of the entire wound section at 4× magnification. The images were spliced together and the percentage of section epithelialised was calculated by blinded observers as: (total section length - unepithelialised length) / (total section length) ×100.[12]

Statistics

Cell migration, gene expression, and mouse wound closure data were analysed using GraphPad Prism 9.0. Pig data were analysed using Microsoft Excel.

3. RESULTS

We used cellular scratch wound assays in vitro to determine whether cell types relevant to wound healing respond to PSG1. Treatment with 50 µg/ml PSG1 enhanced migration of the human HaCaT keratinocyte cell line and this action was TGFβ dependent because addition of 10 µm/ml of the TGFβR1 inhibitor SB431542 for 24 h before PSG1 treatment inhibited cell migration(n=3) ($P<0.001$) (Fig. 1).

Changes in HaCaT gene expression following PSG1 treatment were analysed using qRT-PCR assays. Cells were seeded at 1×10^5 cells/ml in 1ml in 6-well plates and treated with PBS or 50 µg/ml PSG1 for 24 h, after which mRNA was extracted for analysis of gene expression. In two separate experiments, we observed regulation of multiple wound healing associated genes. There was good consistency of upregulated genes but not of downregulated genes

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(Tables 1A & 1B; Supplementary Tables 1A & 1B). Nevertheless, in a separate series of three replicated experiments, we validated increased expression of CXCL5 and TNFα and decreased expression of COL3A1 following treatment of HaCaT cells with 50 μg/ml PSG1, using custom primers in a qRT-PCR assay (n=3) ($P<0.05$) (Fig. 2A-C).

To determine whether PSG1 exhibits efficacy in vivo, 0.4 cm diameter circular full thickness excision wounds were created in adult male C57Bl/6J mice. Following a preliminary dose-response experiment (Supplementary Fig. 1), mice were treated with either PBS or PSG1 as described in the Methods section. PSG1 administration significantly enhanced wound closure at day 4 (n=15 PBS, n=14 PSG1) ($P<0.05$) (Fig. 3A). In a separate set of identical experiments on streptozotocin-induced diabetic mice, PSG1 administration similarly accelerated wound closure measured at day 4 compared to PBS treated wounds (n=19 PBS, n=19 PSG1) ($P<0.01$) (Fig. 3A).

We next evaluated PSG1 efficacy in a pig model of excisional wound healing. Pig skin is histologically similar to the human and heals primarily through re-epithelisation rather than wound contraction, as occurs in rodents.[13] PSG1 administration did not affect wound contraction at any of the time points examined but produced significantly enhanced total wound closure on day 10 (n=28 PBS, n=27 PSG1) ($P<0.0001$), which is explained by the pronounced effect on re-epithelialisation (n=28 PBS, n=27 PSG1) ($P<0.00001$) (Fig. 3B), as further confirmed by histological analysis of wound margin biopsies collected on day 10 ($P<0.05$) (Supplementary Fig. 2).

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4. DISCUSSION

We show that recombinant human PSG1, administered intradermally at wound margins, enhances skin wound healing in the mouse and pig, including in an acute diabetic mouse model. PSG1 action in the acute wound environment may be multimodal because PSG1 interacts with multiple cell types and influences several pathways relevant to wound healing such as angiogenesis, macrophage polarisation, and T regulatory cell differentiation.[1,2] In addition, we identified a strong effect on wound re-epithelialisation in the pig and, using the human HaCaT keratinocyte cell line, showed that PSG1 treatment regulates wound healing associated genes and enhances wound closure in cell monolayers *in vitro*. These observations are consistent with our hypothesis that clinical use of PSG1 may be beneficial in expediting

the closure of acute incisional and traumatic wounds and, by extrapolation, may enhance re-epithelialisation of burn injuries thereby reducing fluid loss and infection. A limitation of our study with respect to chronic wounds is the extent to which the streptozotocin treated diabetic mouse is a realistic model of the complex pathogenesis of human diabetic ulcer.[14,15] To comprehensively address this issue in future work, investigations in a range of other wound models is warranted.

The initial acute phase of wound healing is associated with a pro-inflammatory response and our observed upregulation of pro-inflammatory wound healing-associated cytokines in HaCaT cells may be consistent with enhanced re-epithelisation in the pig model.[6] However, persistent hyperinflammation contributes to failure of chronic wound resolution, including in diabetic foot ulcer.[16] Our observation of up-regulation of pro-inflammatory genes in treated HaCaT cells in vitro may be at odds with the proposed use of PSG1 to treat chronic wounds. However, the majority of PSG1 actions on multiple cell types appear to be anti-inflammatory, mediated through alternative activation of macrophages, TGFβ1 activation, and other mechanisms.[1,2] Therefore, the observed pro-inflammatory response observed here may be context or cell type specific, reflecting the action of PSGs on cultured keratinocytes.

In conclusion, we show that recombinant PSG1 promotes healing of acute excisional skin wound models in the mouse and pig, and we identify a possible pro-inflammatory response of PSG1 action on keratinocytes, possibly underpinning wound re-epithelialisation. Further work is required to explore PSG1 mechanisms of action in more depth in models of acute and chronic wound therapy.

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FIGURE LEGENDS

Figure 1

PSG1 enhances wound closure in cell line scratch wound assays. 16 h treatment with 50 µg/ml PSG1-V5-His of HaCaT cells with or without 10 µM SB431542 TGFβR1 inhibitor (n=3; each data point is the mean of 3 technical replicates; * $P<0.05$, ** $P<0.01$, **** $P<0.0001$, t-test).

Figure 2

PSG1 regulates wound healing associated genes. A - C. qRT-PCR analysis of CXCL5, TNFα and COL3A1 expression in HaCaT cells treated with 50 µg/ml PSG1-V5-His for 24 hours. Expression normalised to GAPDH (n=3; each data point is the mean of three technical replicates; * P<0.05, t-test).

Figure 3

PSG1 enhances wound healing in mouse and pig excisional wound models. A. Intradermal administration of 50 µg PSG1-V5-His to the wound margins of normoglycemic and diabetic mice enhanced wound closure at Day 4 (n=15 PBS, n=14 PSG1; * P<0.05, 95% CI [1.14, 13.26], t-test) and diabetic mice (n=19 PBS, n=19 PSG1; ** P<0.01, 95% CI [2.60,17.53], t-test). B. Intradermal administration of 250 µg PSG1-V5-His to the wound margins of pigs enhanced wound re-epithelialisation at Day 1 (n=28 PBS, 27 PSG1; ***** $P \leq 0.000004$, 95% CI [14.82, 33.70], t-test).

Table 1

Genes regulated following treatment of HaCaT cells with PSG1.

Supplementary Figure 1

PSG1 dose-response in the mouse.

Supplementary Figure 2

Re-epithelialisation measured in histological sections from pig wounds.

Supplementary Table 1

Genes (extended list) regulated following treatment of HaCaT cells with PSG1.

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AUTHOR CONTRIBUTIONS

TM: Conceptualization; formal analysis; data curation; funding acquisition; project administration; writing – review and editing original draft (lead); JMW: Formal analysis; data curation; writing – original draft. KBW: Formal analysis; data curation. DW: Formal analysis; data curation. JC: Conceptualization; formal analysis; data curation; funding acquisition; writing – review and editing original draft.

CONFLICT OF INTEREST

TM and JC are named as inventors on patent EP21168470.9 regarding use of PSG proteins in therapy.

ETHICS STATEMENT

Ethical permissions for animal procedures were granted by the UCC Animal Experimentation Ethics Committee and procedures were conducted under Health Products Regulatory Authority (www.hpra.ie) licence AE19130/P024 Case no 702095 to JC.

DATA SHARING

The data that support the findings of this study are available from the corresponding author (TM) upon reasonable request.

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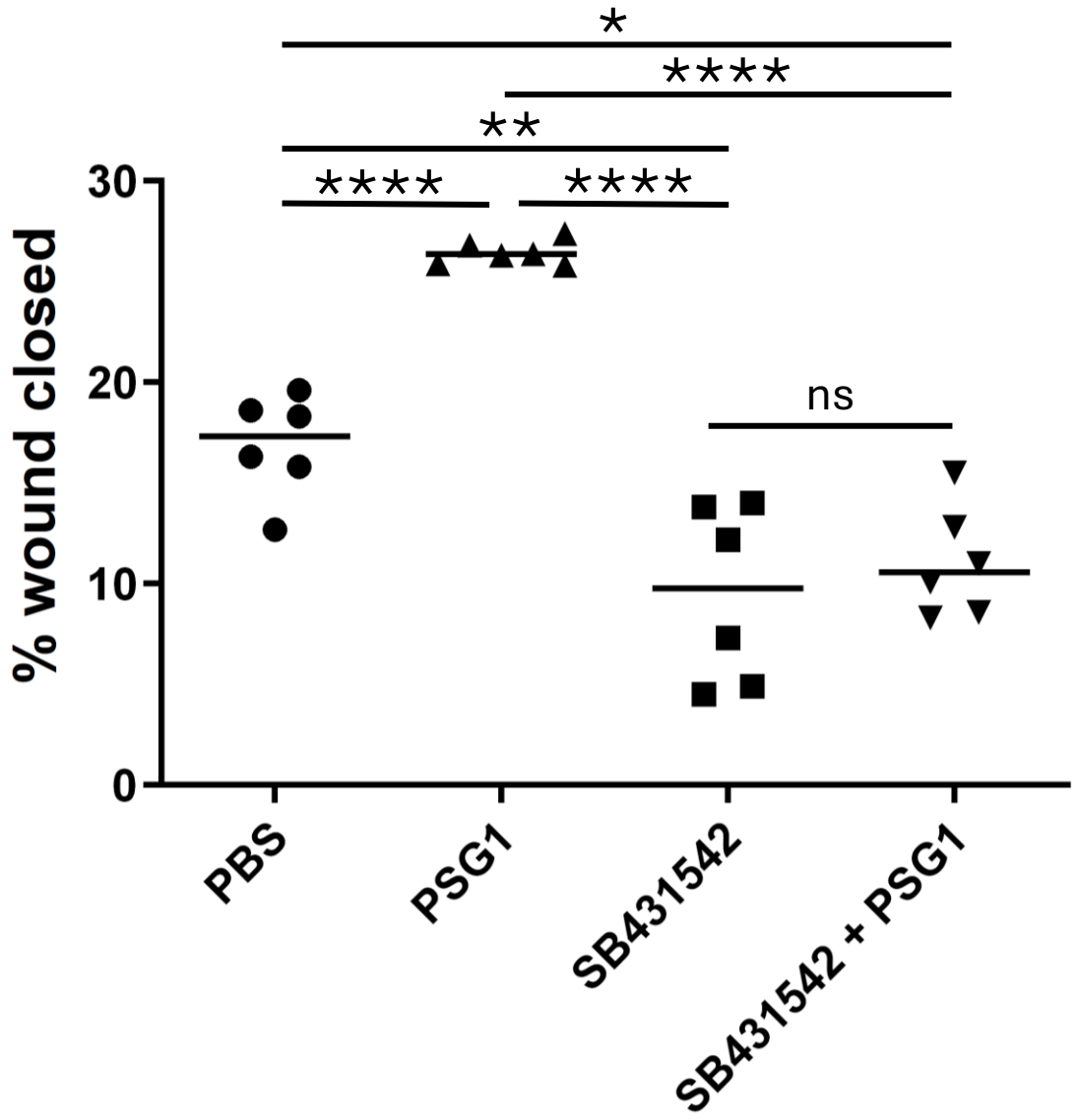
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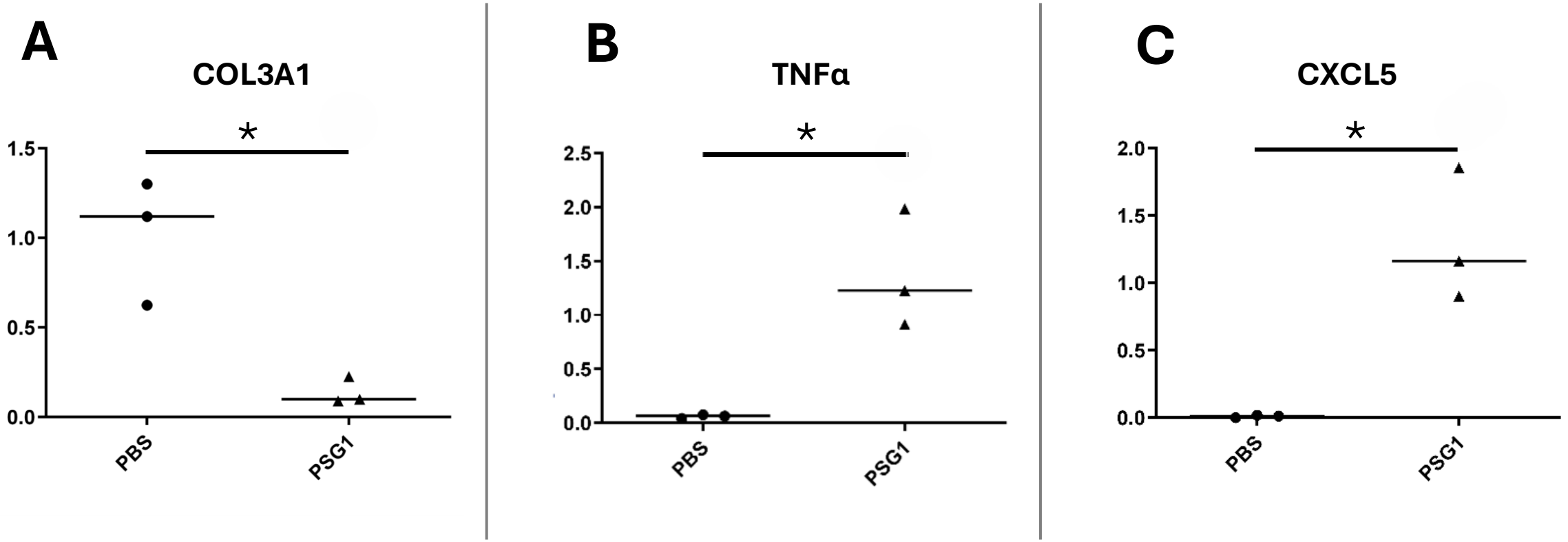
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*
****
**
****
****
ns
% wound closed
30
20
10
0
PBS
PSG1
SB431542
SB431542 + PSG1


A
COL3A1
*
1.5
1.0
0.5
0.0
PBS
PSG1
B
TNFα
*
2.5
2.0
1.5
1.0
0.5
0.0
PBS
PSG1
C
CXCL5
*
2.0
1.5
1.0
0.5
0.0
PBS
PSG1

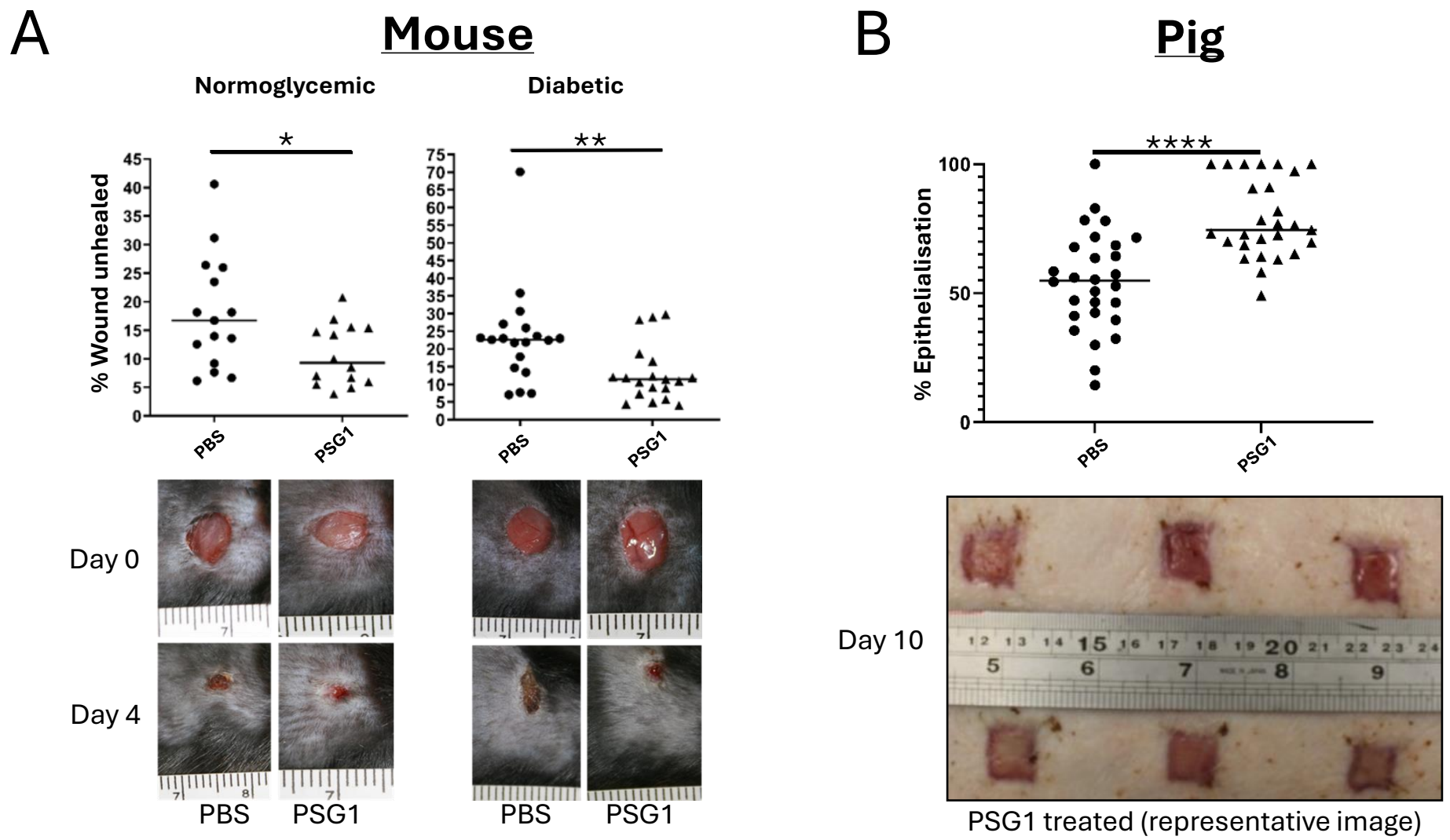

A
Mouse
Normoglycemic
Diabetic
*
**
% Wound unhealed
PBS
PSG1
Day 0
Day 4
PBS
PSG1
PBS
PSG1
B
Pig
****
% Epithelialisation
PBS
PSG1
Day 10
PSG1 treated (representative image)

Table 1A. Genes upregulated following treatment of HaCaT cells with PSG1

First experiment	Fold change	Replicate experiment	Fold change
CXCL5	27.21	*TNF*	11.05
CXCL11	22.88	*CXCL5*	10.15
CCL7	21.74	*CXCL11*	9.13
CCL2	10.65	*CCL2*	6.92
CXCL2	6.07	*MMP9*	6.88

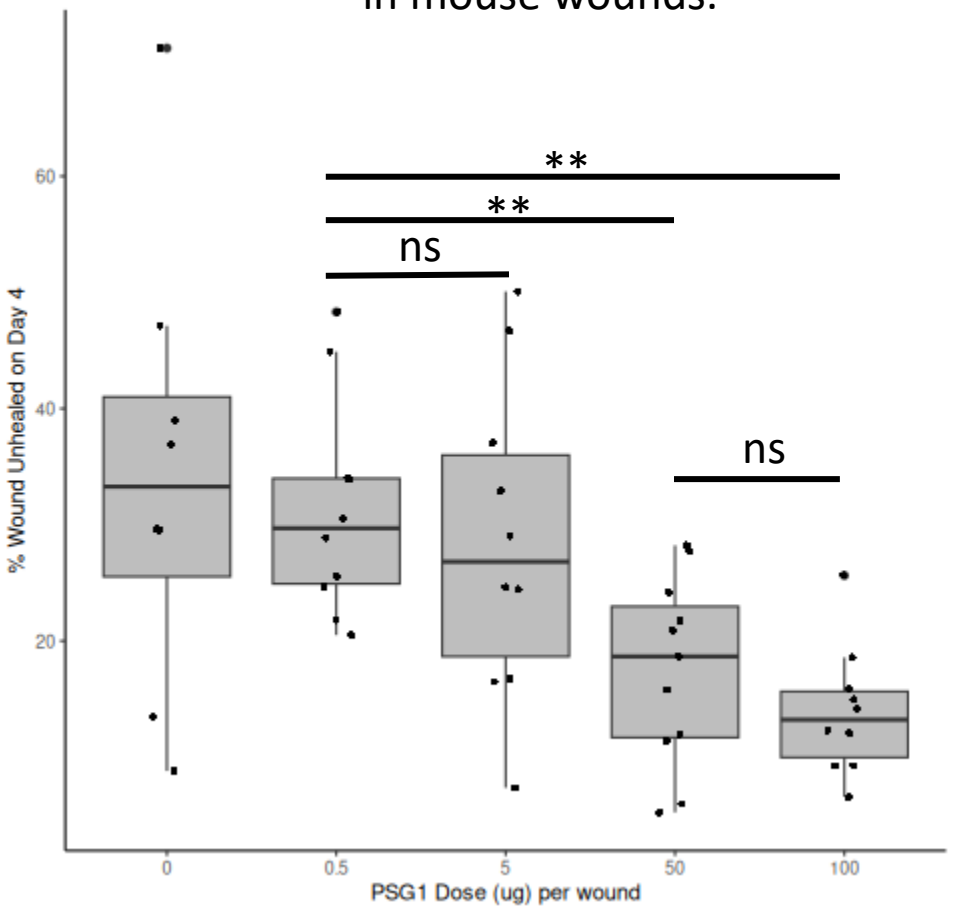
Table 1B. Genes downregulated following treatment of HaCaT cells with PSG1

First experiment	Fold change	Replicate experiment	Fold change
COL3A1	5.45	*IL4*	4.51
CSF3	5.43	*COL3A1*	4.16
COL4A1	2.89	*ITGB1*	3.10
EGF	2.86	*GAPDH*	2.82
WISP1	2.58	*RPLPO*	2.62

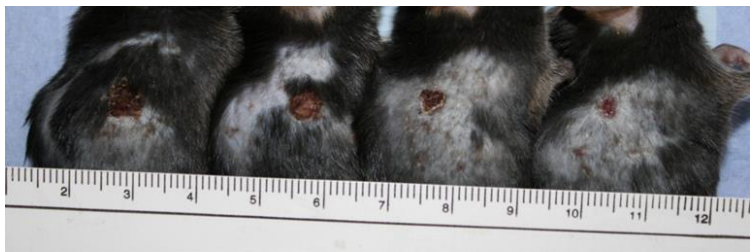
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Supplementary Figure 1. PSG1 treatment dose – response in mouse wounds.

A



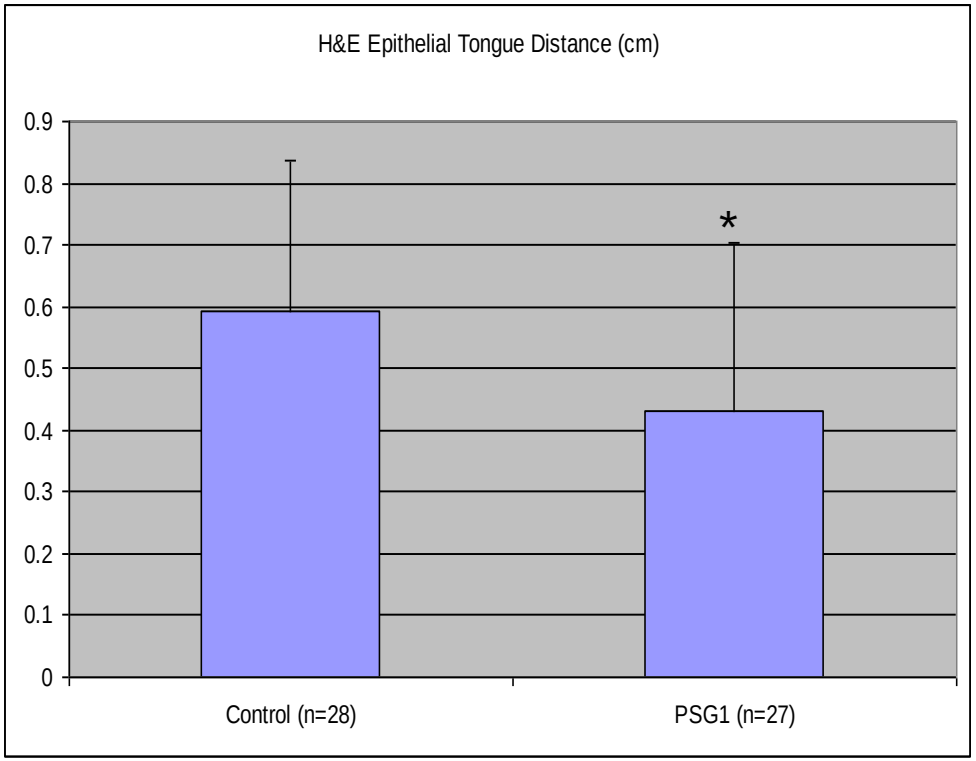
B



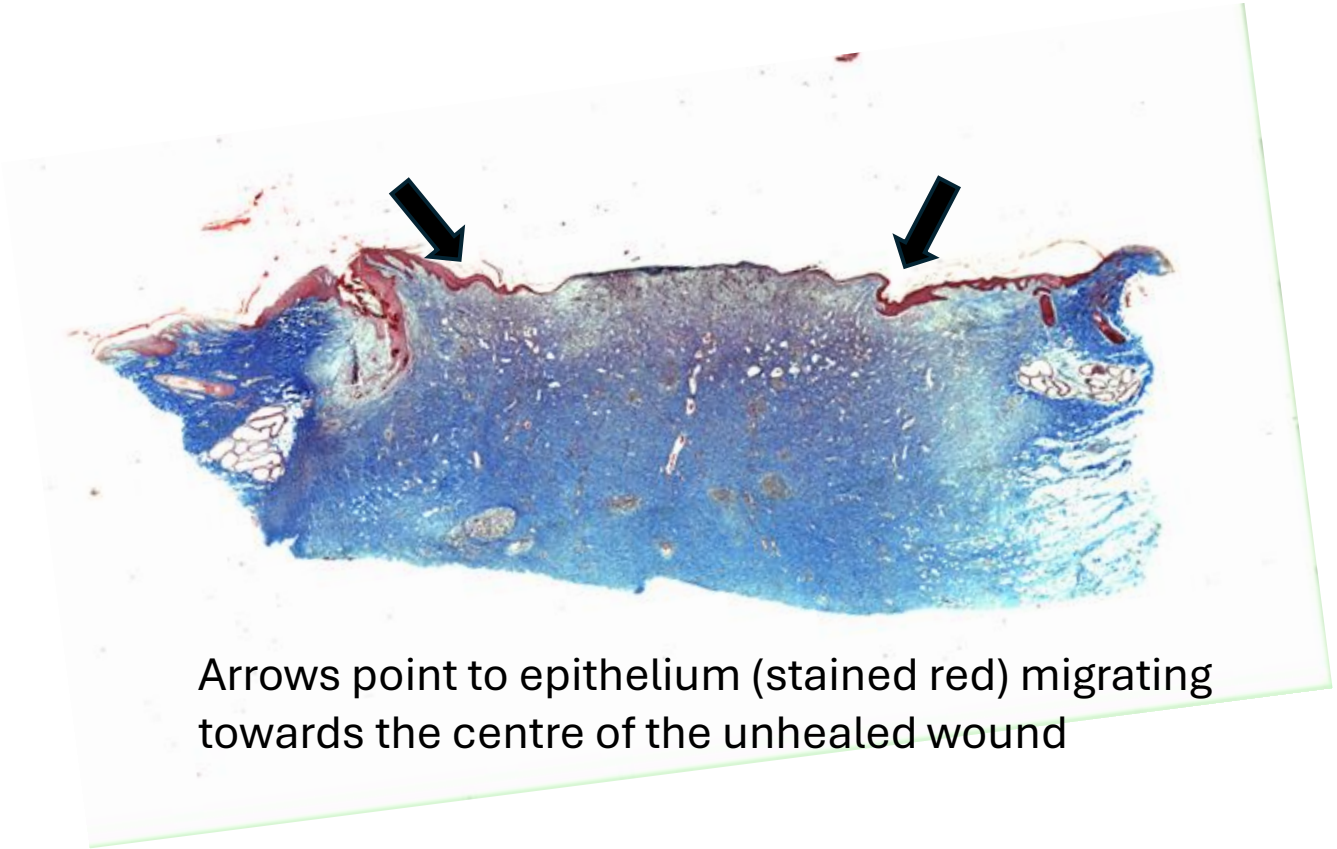
Dose (ug): 0.5 5 50 100

A) Dose-response analysis of PSG1 treatment of mouse wounds (two replicates of 5 animals per treatment group; n=50 mice in total). For the analysis, PBS control was excluded due to loss of two animals in replicate 2. Comparisons of the mean percentage of wounds unhealed on day 4 were carried out using the "pairwise.wilcoxon.test" function from the R statistical analysis environment (URL https://www.R-project.org/). The lowest effective dose (compared to 0.5 ug dose) was 50 ug, which was used in subsequent experiments. **B)** Representative images of mouse wounds on day 4.

Supplementary Figure 2. Re-epithelialisation measured in H&E sections: Distance between Epithelial Tongues



	H&E Epithe	stdev	ttest
Control (n=28)	0.592607	0.243568	0.024081 *
PSG1 (n=27)	0.431333	0.271119	
% difference (n=27)	16.12738		



Arrows point to epithelium (stained red) migrating towards the centre of the unhealed wound

Supplementary Table 1A. PSG1 upregulation of multiple wound healing associated genes in HaCaT cells. Genes common to both repliates highlighted in green.

Genes upregulated 1st replicate	Fold change	Genes upregulated 2nd replicate	Fold change
CXCL5	27.21	TNF	11.05
CXCL11	22.88	CXCL5	10.15
CCL7	21.74	CXCL11	9.13
CCL2	10.65	CCL2	6.92
CXCL2	6.07	MMP9	6.88
MMP7	4.61	CXCL1	4.15
MMP9	4.58	FGF7	4.14
IL6	4.01	MMP7	3.46
MMP1	3.76	CCL7	3.42
ITGB3	2.16	CXCL2	3.24
		IL10	3.05
		MMP1	2.63
		ITGA5	2.47
		IL6	2.41
		COL5A3	2.20

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Supplementary Table 1B. PSG1 mediated downregulation of wound healing associated genes in HaCaT cells. Genes common to both replicates highlighted in green.

Genes downregulated 1st replicate	Fold change	Genes downregulated 2nd replicate	Fold change
COL3A1	5.45	IL4	4.51
CSF3	5.43	COL3A1	4.16
COL4A1	2.89	ITGB1	3.10
EGF	2.86	GAPDH	2.82
WISP1	2.58	RPLPO	2.62
VTN	2.44	PTEN	2.42
ITGA3	2.27	TAGLN	2.40
MAPK3	2.13	COL1A2	2.33
PLAT	2.08	TGFBR3	2.29
PDGFA	2.06	CTNNB1	2.17
COL4A3	2.03	PDGFA	2.08
		ITGB5	2.06

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