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**Platelet-Released Growth Factors Induce Expression of Genes Involved in  
Extracellular Matrix Organization and Epidermis Development in Primary  
Human Keratinocytes and Fibroblasts**

Dissertation

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# Table of Contents

<b>Table of Contents .....</b>	<b>i</b>
<b>List of Abbreviations.....</b>	<b>iv</b>
<b>List of Figures .....</b>	<b>vi</b>
<b>List of Tables.....</b>	<b>vii</b>
<b>1 Introduction .....</b>	<b>1</b>
1.1 Human skin function and structure.....	1
1.2 Wound healing .....	3
1.2.1 The normal wound healing process.....	3
1.2.2 The role of platelets in wound healing .....	5
1.2.3 Chronic wounds.....	5
1.3 Thrombocyte concentrate products.....	6
1.4 Aim of the study .....	7
1.5 Hypothesis and approach.....	7
<b>2 Materials and methods.....</b>	<b>8</b>
2.1 Materials.....	8
2.1.1 Devices .....	8
2.1.2 Consumables .....	9
2.1.3 Chemicals and solutions.....	11
2.1.4 Cell culture media .....	12
2.1.5 Kits .....	13
2.1.6 Oligonucleotides.....	13
2.1.7 Cells.....	14
2.1.8 Software .....	14
2.2 Methods .....	15
2.2.1 Cell Culture .....	15
2.2.1.1 Keratinocyte culture .....	15
2.2.1.2 Fibroblast culture.....	16
2.2.1.3 Cryopreservation .....	16
2.2.1.4 Stimulation .....	17
2.2.1.5 <i>Ex vivo</i> stimulation .....	17
2.2.1.6 Scratch Assay .....	18
2.2.2 RNA Isolation .....	18
2.2.2.1 RNA Isolation with RNAmagic (TRIzol) .....	18

2.2.2.2 RNA isolation with Macherey-Nagel Nucleospin RNA kit .....	19
2.2.3 cDNA Synthesis .....	19
2.2.4 Quantitative real-time polymerase chain reaction (qPCR) .....	20
2.2.5 PRGF preparation .....	21
<b>3 Results .....</b>	<b>22</b>
3.1 Primary keratinocyte stimulations .....	22
3.1.1 Whole transcriptome sequencing of PRGF-stimulated keratinocytes revealed an induction of genes involved in extracellular matrix organization and epidermis development .....	22
3.1.2 Stimulation of primary human keratinocytes with PRGF verified the induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 .....	24
3.1.3 Stimulation of primary human keratinocytes with PRGF leads to a time-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 .....	25
3.1.4 Inhibition of Epidermal Growth Factor Receptor (EGFR) suppresses the PRGF-dependent induction of FN1, MMP9, KRT7, TGM2, COL22A1 and FERMT1 but amplifies induction of TGFBI, COL1A1 and COL1A2 in primary human keratinocytes.....	27
3.1.5 Inhibition of Interleukin-6 Receptor (IL-6R) has no influence on the PRGF-induced expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary human keratinocytes .....	29
3.2 Primary fibroblast stimulations .....	31
3.2.1 Stimulation of primary human fibroblasts with PRGF leads to induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1 and COL1A1 .....	31
3.2.2 Stimulation of primary human keratinocytes with PRGF leads to a time-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA .....	32
3.2.3 Inhibition of Epidermal Growth Factor Receptor (EGFR) suppresses the PRGF-dependent induction of MMP9 but amplifies induction of TGFBI, KRT7, TGM2, VEGFA, FN1, FERMT1, COL1A1 and COL1A2 in primary human fibroblasts.....	35
3.3 Stimulation of <i>ex vivo</i> human skin with PRGF leads to an increased expression of TGFBI, FN1, MMP9, FERMT1 and COL1A2.....	38
3.4 PRGF induces an increased migration rate in fibroblast scratch assay .....	40
<b>4 Discussion .....</b>	<b>42</b>
4.1 Induction of TGFBI expression.....	42
4.2 Induction of FN1 expression.....	42
4.3 Induction of MMP9 expression .....	43

4.4 Induction of KRT7 expression .....	44
4.5 Induction of TGM2 expression .....	44
4.6 Induction of COL22A1 expression .....	45
4.7 Induction of FERMT1 expression .....	45
4.8 Induction of COL1A1 and COL1A2 expression.....	45
4.9 Induction of Ki67 expression in fibroblasts .....	46
4.10 Induction of VEGFA expression in fibroblasts .....	46
4.11 EGFR as mediator of PRGF stimulation .....	46
4.12 <i>Ex vivo</i> stimulations .....	47
4.13 Time-dependency of gene induction .....	47
4.14 Conclusions .....	48
<b>5 Summary .....</b>	<b>50</b>
<b>6 References .....</b>	<b>51</b>
<b>7 Acknowledgements.....</b>	<b>56</b>

# List of Abbreviations

%	Percentage
-/-	Homozygous mutation
°C	Degree Celcius
μl	Microliter
AMP	Antimicrobial peptide
CaCl <sub>2</sub>	Calcium chloride
Cat.	Catalog
cDNA	Complementary DNA
CO <sub>2</sub>	Carbon dioxide
COL1A1	Collagen type I alpha 1 chain
COL1A2	Collagen type I alpha 2 chain
COL22A1	Collagen type XXII alpha 1 chain
DMEM	Dulbecco's modified eagle medium
dNTP	Deoxyribonucleoside triphosphate
DPBS	Dalbecco's phosphate puffered saline
dT	Deoxythymine
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
FERMT1	Fermitin family member 1
FIZZ-1	Finding in inflammatory zone-1
FN1	Fibronectin 1
g	Relative centrifugal force
GO	Gene ontology
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
hBD-2	Human beta-Defensin 2
hBD-3	Human beta-Defensin 3
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
KGM	Keratinocyte growth medium
KGM2	Keratinocyte growth medium 2
KRT7	Keratin 7
miR-155	MicroRNA-155
ml	Milliliter
mm	Millimeter
mM	Millimolar
MMP9	Matrix metalloprotease 9
NHEK	Normal human epidermal keratinocytes
No.	Number
PBS	Phosphate puffered saline
PCR	Polymerase chain reaction
PRF	Platelet-rich fibrin

PRGF	Platelet-released growth factor
PRP	Platelet-rich plasma
qPCR	Quantitative real-time polymerase chain reaction
Ref.	Reference
RNA	Ribonucleic acid
RP38	Ribosomal protein L38
RPMI1640	Roswell Park Memorial Institute 1640 medium
RT	Reverse transcriptase
SEM	Standard error of the mean
βIG-H3	Transforming growth factor-beta-induced protein ig-h3
T/E	Trypsin/EDTA
TGFBI	Transforming growth factor beta-induced
TGF-β	Transforming growth factor beta
TGM2	Transglutaminase 2
U/ml	Units per milliliter
UKSH	Universitätsklinikum Schleswig-Holstein
VEGFA	Vascular endothelial growth factor A

# List of Figures

Figure 1: Epidermis structure.....	3
Figure 2: A whole transcriptome analysis of PRGF-stimulated keratinocytes .....	23
Figure 3: PRGF induces expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.....	24
Figure 4: Time kinetic of the PRGF-mediated induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes .....	25
Figure 5: Effect of Epidermal Growth Factor Receptor (EGFR) inhibition by Cetuximab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.....	27
Figure 6: Effect of Interleukin-6 Receptor (IL-6R) inhibition by Tocilizumab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.....	29
Figure 7: PRGF induces expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary fibroblasts .....	31
Figure 8: Time kinetic of the PRGF-mediated induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA in primary fibroblasts .....	32
Figure 9: Effect of Epidermal Growth Factor Receptor (EGFR) inhibition by Cetuximab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA in primary fibroblasts.....	35
Figure 10: PRGF induces TGFBI, MMP9, FERMT1 and COL1A2 <i>ex vivo</i> .....	38
Figure 11: PRGF induces an increased migration rate in fibroblast scratch assays .....	40
Figure 12: Comparison between control and PRGF-stimulated fibroblasts in scratch assay experiment .....	41



# List of Tables

Table 2.1.1: Devices.....	8
Table 2.1.2: Consumables .....	9
Table 2.1.3: Chemicals and solutions.....	11
Table 2.1.4: Cell culture media .....	12
Table 2.1.5: Kits .....	13
Table 2.1.6: Oligonucleotides .....	13
Table 2.1.7: Cells .....	14
Table 2.1.8: Software .....	14

# 1 Introduction

## 1.1 Human skin function and structure

Human skin functions as a protective barrier between the body and the external environment. It acts as physical, chemical and antimicrobial barrier between our body and the external environment. It plays an important role in thermoregulation [1], prevents pathogens and harmful substances from infiltrating the body and hinders unregulated loss of water and solutes [2].

The skin comprises three layers. The deepest layer is the subcutis, mainly made up of fat and connective tissues [3]. The middle layer is a connective tissue layer called the dermis that contains sebaceous glands, sweat glands and a high amount of blood vessels, as well as various cells such as fibroblasts, mast cells, and infiltrating leucocytes [1]. The epidermis is the upper layer of the skin and is the key component of the skin barrier function [2].

The epidermis consists of several layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (Figure 1). It comprises mostly keratinocytes, which go through a differentiation process known as cornification as they travel from the lower to the upper layer of the epidermis, resulting in layers of dead cells in stratum corneum. The keratinocytes are attached to each other mostly by desmosomes, but other types of adhesion can also be found, such as gap junctions and tight junctions. Other cells found in the epidermis are melanocytes, Langerhans cells and Merkel cells [1,3].

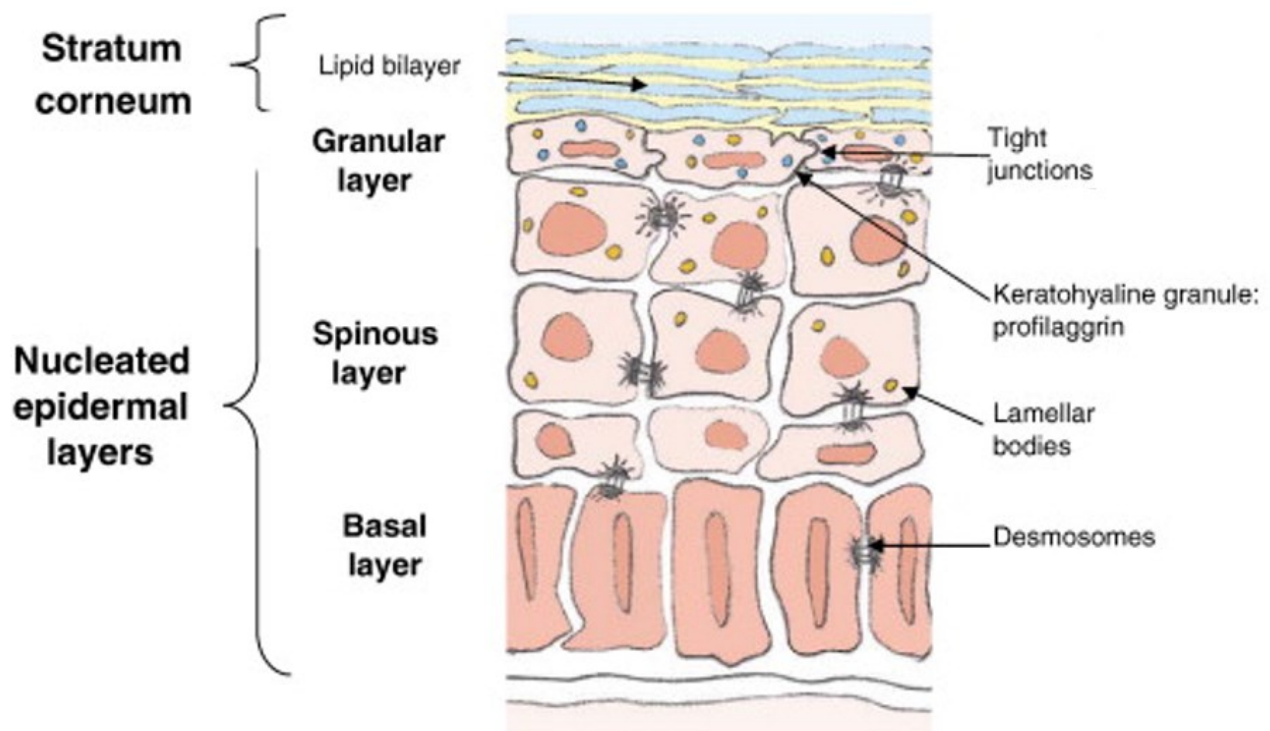
Stratum basale is the deepest layer of the epidermis. It consists of epidermal stem cells and transiently amplifying cells, which, through mitosis, deliver new cells to the upper layers [1,3]. Stratum basale is also the only layer where melanocytes, the pigment producing cells of the skin, can be found. The keratinocytes in this layer are anchored by hemidesmosomes and focal contacts to the basal lamina, a part of the basement membrane that separates the dermis from the epidermis. Some constituents of the hemidesmosomes include integrins, collagen XVII, collagen VII and collagen III, and an important component of the focal contacts is kindlin-1 plaque protein [3].

Stratum spinosum is about two to five layers thick and sits directly above stratum basale. In this layer, the keratinocytes begin differentiating and contain more cellular keratin filaments

compared to the basal layer. This layer also houses most of the Langerhans cells, which are antigen-presenting cells that play a role in the T-cell mediated immune response [3].

Stratum granulosum, which is above stratum spinosum, is about three layers thick, and the keratinocytes in this layer contain keratohyalin granules. These granules are aggregates of cellular keratin filaments, such as keratin 1 and 10, and proteins, such as profilaggrin and loricrin. The keratohyalin granules increase in size as it travels from the lower part to the upper part of stratum granulosum, indicating that the keratinocytes are progressively becoming more and more differentiated [1]. Keratinocytes in the middle layer of stratum granulosum are connected by tight junctions, making it an important component of the skin's diffusion barrier [3].

The uppermost layer of the epidermis is the stratum corneum. This layer ranges between 25 to 100 layers of cells, depending on the location of the skin on the body. In this layer, the keratinocytes lose their nucleus and organelles, hence becoming essentially dead. However, these terminally differentiated keratinocytes, also known as corneocytes, are crucial to the epidermis' barrier function. As keratinocytes enter the stratum corneum, profilaggrin proteins are cleaved by caspase-14 into small filaggrin fragments [3]. These filaggrin fragments allow even more aggregation of cellular keratin filaments, so that the keratin filaments are completely aggregated into tight bundles, allowing the cells to take a flattened shape [2,3]. The corneocytes are attached to each other by corneodesmosomes, which, compared to normal desmosomes, are strengthened by corneodesmosin protein. This gives the stratum corneum an especially high mechanical durability. As the corneocytes move up within the stratum corneum, the amount of corneodesmosomes gradually decreases, and the corneocytes eventually undergo desquamation at the top layer [3]. The intercellular space between corneocytes is filled with polar lipids known as ceramides, which create a seal that, just like the tight junctions in stratum granulosum, provides a diffusion barrier for the epidermis [2,3].



**Figure 1: Epidermis structure** [4]. A simplified illustration of the four layers of epidermis from superficial to deep: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale.

## 1.2 Wound healing

Since the skin is such an important organ for survival, the body needs to repair any wounds on the skin. Our body achieves this through a complex healing mechanism that requires the collaboration of many cell types, structural proteins, growth factors, and proteinases [5-7].

### 1.2.1 The normal wound healing process

The wound healing process can generally be divided into four phases: hemostasis, inflammation, proliferation, and remodeling/maturation phases [8].

Hemostasis occurs within the first few minutes after the wound is formed. It can be divided into primary and secondary hemostasis. During primary hemostasis, the wounded blood vessel walls contract to slow down blood flow, and platelets attach themselves to the damaged blood vessel walls. The platelets are activated by several factors such as collagen, adrenalin and many more, causing them to change form and express receptors on their surface that allow platelet aggregation at the location of the injury. During secondary hemostasis, a coagulation cascade occurs, which leads to the production and polymerization of fibrin. Thrombocytes and

erythrocytes are embedded in the fibrin mesh, creating a thrombus that clogs the wound site to prevent further bleeding [8].

During the inflammation phase, neutrophils infiltrate the wound site to remove foreign particles, bacteria, and necrotic cells. They also release factors that draw monocytes into the wound site. The monocytes transform into macrophages once they have reached the injured area, which further clean the wound site from bacteria by phagocytosis and secrete cytokines that trigger recruitment and activation of even more leukocytes. The macrophages are also responsible for inducing apoptosis of inflammatory cells and phagocytizing the remnants at the end of the inflammatory phase. Furthermore, macrophages release growth factors that promote proliferation and migration of fibroblasts and keratinocytes, as well as angiogenesis [5,6,8,9].

48 to 72 hours following onset of wound, somewhat overlapping with the inflammation phase, proliferation phase commences. Fibroblasts enter the wound, producing crucial substances for wound healing, such as proteoglycans and glycosaminoglycans (GAG), which are components of the ground substance of granulation tissue and collagens, which grant tensile strength to the wound [5]. Meanwhile, the proliferation of endothelial cells also occurs, allowing new blood vessels to infiltrate further into the wound, providing nutrients and cytokines for fibroblasts that are expanding into the wound and blood vessels. Additionally, keratinocytes create a new epithelial layer over the wound by proliferating and migrating across the wound. As levels of hyaluronic acid in the ground substance diminish and chondroitin sulfate levels increase, fibroblast proliferation and migration are gradually suppressed, and fibroblasts differentiate into myofibroblasts. Myofibroblasts are able to contract and are therefore responsible for the contraction of the wound, resulting in a decrease in wound size [5,6,8].

In the remodeling phase, the granulation tissue is replaced by scar tissue [8]. New collagen is synthesized in balance with degradation of old collagens by collagenases and matrix metalloproteases. Type III collagens are replaced with type I collagens until a ratio of 4:1 is reached, similar to the environment in normal skin. Water is resorbed from the scar, fibronectin begins to vanish, and hyaluronic, and glycosaminoglycans are replaced by proteoglycans, allowing collagen fibers to be packed closer together, hence enabling the alignment and cross-linking of collagen fibers. Consequently, the volume of the scar decreases, and the tensile strength of the tissue increases [5,8].

### **1.2.2 The role of platelets in wound healing**

Besides their primary function in hemostasis, various studies have shown that platelets are an important component of various processes in wound healing. By releasing growth factors, cytokines and extracellular matrix modulators, they are able to interact with other cells and promote wound-healing mechanisms such as angiogenesis through induction of migration, proliferation, differentiation, and stabilization of endothelial cells in new blood vessels, and connective tissue restoration through migration, proliferation and activation of fibroblasts [7,9].

### **1.2.3 Chronic wounds**

Occasionally, wound recovery may be delayed or does not occur entirely, resulting in chronic wounds. Factors that could lead to this phenomenon include local factors, such as hypoxia and the presence of necrotic tissues, as well as systemic factors, such as diabetes mellitus.

Hypoxia aids wound healing in the acute setting by activating angiogenesis and promoting the release of growth factors. However, prolonged hypoxia interferes with the wound healing process, resulting in chronic wounds [10, 11]. This occurs as too much or too little oxygen leads to increased production of reactive oxygen species, such as hydrogen peroxide ( $H_2O_2$ ) and superoxide ( $O^2$ ). While reactive oxygen species can aid wound healing, excessive amounts will cause further tissue damage instead. Additionally, fibroblasts cannot replicate when there is too little oxygen.

Necrotic tissue is often present in chronic wounds. Due to its presence, infections, which hinder wound healing, are more likely to occur as bacteria are able to use it as their growth medium. The prolonged inflammatory reaction caused by necrotic tissue is also disadvantageous for wound healing, as the resultant inflammatory factors may damage endogenous growth factors and glycosaminoglycan matrix required for normal wound healing. Furthermore, necrotic tissue may also hinder migration of keratinocytes and fibroblasts to the wound site by releasing endotoxins. [11]

People with diabetes mellitus have an increased risk of suffering from chronic wounds due to several reasons, such as hypoxia due to insufficient angiogenesis, hyperglycemia, and increased levels of metalloproteases. Hypoxia and hyperglycemia increase the amount of reactive oxygen species in wounds. An excess of metalloproteases causes tissue dismantling.

### 1.3 Thrombocyte concentrate products

While there are already established treatments and interventions used to treat chronic wounds, such as the use of antibiotics [11], wound recovery is not always easily achievable through these methods. Hence, there have been many attempts to find new ways to heal chronic or hard to heal wounds. Considering the importance of platelets in normal wound healing and their ability to release growth factors, thrombocyte-concentrate products have quickly become one of the most researched methods to aid wound healing [12].

Platelet-rich plasma (PRP) is a first-generation thrombocyte concentrate product. It is obtained from autologous blood and is produced by mixing the blood with anticoagulants, followed by centrifugation to create a thrombocyte concentrate. PRP can therefore supply wounded regions with thrombocytes to promote wound healing [12,13].

Platelet-rich fibrin (PRF) is a second-generation thrombocyte concentrate product. One advantage it has over PRP is that it does not require mixing autologous blood with anticoagulants or bovine thrombin, which are necessary to make PRP. Its production is simpler and more cost-effective than PRP while providing similar, if not more, benefits for wound healing. One of its disadvantages is that the blood needs to be processed quickly [12,13].

Platelet-released growth factor (PRGF) is the main subject of this study. It is similar to PRF in that it is a thrombocyte concentrate product, but it contains only the contents of thrombocytes such as chemokines, cytokines, and growth factors, without actually containing thrombocytes cells, as the thrombocytes are destroyed during preparation and the cell remnants are discarded [14, 15, 16]. PRGF can therefore be regarded as a thrombocyte concentrate lysate.

So far, a few studies have investigated the effects of PRGF on the skin. A study has shown that PRGF might lower the proliferation rate of keratinocytes by suppressing the expression of Ki67 [14] while promoting differentiation of keratinocytes by inducing transglutaminase-1 and involucrin [15]. Another study demonstrated that PRGF induces the expression of antimicrobial peptides (AMPs) human beta-Defensin 2 (hBD-2) and human beta-Defensin 3 (hBD-3) in human keratinocytes. AMPs hBD-2 and hBD-3 are known to have potent antimicrobial activities, suggesting the likely utility of PRGF in preventing infections in chronic wounds, thereby promoting wound healing [16, 17].

## 1.4 Aim of the study

Platelet-released growth factors (PRGF) is a thrombocyte concentrate lysate. Similar to its clinically equivalent variations (e.g. Vivostat PRF), it is widely known to be able to support the healing of chronic and hard-to-heal wounds. However, the exact mechanisms of action of PRGF are not completely known. To date there have only been limited studies investigating the effect of PRGF or other thrombocyte concentrate products on keratinocytes or fibroblasts remain scarce. This study therefore aims to fill the gap in knowledge by investigating the working mechanisms of PRGF through the identification of genes in keratinocytes and fibroblasts influenced by PRGF.

## 1.5 Hypothesis and approach

Based on the results of previous studies, we hypothesized that PRGF could aid skin wound healing by regulating various genes in cells involved in skin wound healing, such as keratinocytes and fibroblasts. Therefore, we performed a whole transcriptome analysis of PRGF-stimulated keratinocytes and used the results as a starting point. This revealed that PRGF-stimulated keratinocytes have an increased expression of genes involved in extracellular matrix organization. Accordingly, we selected a few genes in this group that were greatly induced and likely to benefit wound healing. These are transforming growth factor beta-induced (TGFB1), fibronectin 1 (FN1), matrix metalloprotease 9 (MMP9), transglutaminase 2 (TGM2), collagen type XXII alpha 1 chain (COL22A1), fermitin family member 1 (FERMT1), collagen type I alpha 1 chain (COL1A1) and collagen type II alpha 2 chain (COL1A2). Additionally, we also investigated Keratin 7 (KRT7), which was not categorized as a gene involved in extracellular matrix organization in the gene ontology analysis but plays a role in epidermis development and was among the top 100 genes in the whole transcriptome analysis with the highest fold change (ratio of gene expression between stimulated and unstimulated samples).

In this study, PRGF influence on the expression of TGFB1, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in keratinocytes and fibroblasts will be investigated through *in vitro* stimulation experiments of primary keratinocytes and fibroblasts, and *ex vivo* experiments. The analysis will be done using a real-time polymerase chain reaction (PCR). Through scratch assays, the effects of PRGF on the migration of fibroblasts will also be examined in this study.



## 2 Materials and methods

### 2.1 Materials

#### 2.1.1 Devices

Device Type	Product Name	Manufacturer
Centrifuge	Eppendorf 5417R	Eppendorf (Hamburg)
	Eppendorf 5810R	Eppendorf (Hamburg)
	Eppendorf 5424	Eppendorf (Hamburg)
	Eppendorf 5415D	Eppendorf (Hamburg)
	Mega Star 600R	VWR (Daramstadt)
	Spectrafuge C1301B	Labnet (Edison, USA)
CO <sub>2</sub> -incubator	CB 150	Binder (Tuttlingen)
	Formascientific 3164	Thermo Fisher Scientific (Schwerte)
Cryogenic freezing container	Nalgene Cryo 1°C Freezing Container, (Cat. No. 5100-0001)	Thermo Fisher Scientific (Schwerte)
Cryogenic storage vessel	Arpege 70	Air Liquide (Düsseldorf)
Fluid aspiration system	BVC Professional	Vacuubrand
Fume cupboard	-	Köttermann (Ütze)
Homogeniser	SpeedMill Plus	Analytik Jena (Jena)
Microscope	Leica DMI3000 B	Leica (Wetzlar)
	Leitz DiaVert	Leica (Wetzlar)
	Wilovert 30	Hund (Wetzlar)
	Zeiss Axio Vert. A1	Zeiss (Jena)
Photometer	NanoDrop 1000 Spectrophotometer	Peqlab (Karlsruhe)
	Multiskan Sky Microplate	Thermo Scientific
	Spectrophotometer (with µDrop Plate)	(Schwerte)

Pipette	Eppendorf Research (0.5-10, 10-100, 100-1000 µl)	Eppendorf (Hamburg)
	Eppendorf Reference (0.5-10, 10-100, 100-1000 µl)	Eppendorf (Hamburg)
	Eppendorf Reference 2 (0.5-10, 10-100, 100-1000 µl)	Eppendorf (Hamburg)
	Pipetus	Hirschmann (Eberstadt)
Real-Time PCR system	StepOne Plus	Applied Biosystems (Carlsbad, USA)
Sequencing system	HiSeq 4000	Illumina (San Diego, USA)
Sterile workbench	Cytosafe 2004	Gelaire (Seven hills, Australia)
	Köttermann 8511	Köttermann (Ütze)
Thermocycler	T3 Thermocycler	Biometra (Göttingen)
Vortexer	Vortex Genie 2 (Model G-560-E)	Scientific Industries (New York, USA)
	IKA VF2	IKA (Staufen)
	Reax top	Heidolph (Schwabach)
Water bath	GFL 1004	GFL (Burgwedel)
	GFL 1002	GFL (Burgwedel)

### 2.1.2 Consumables

Item Type	Product Name	Manufacturer
Biopsy punch	KAI Standard biopsy punch (6mm)	pfm medical (Köln)
Cell culture flask	Corning® 175cm <sup>2</sup> Angled Neck Cell Culture Flask with Vent Cap (Ref: 431080)	Corning (Kaiserslautern)
	Corning® 75cm <sup>2</sup> U-Shaped Canted Neck Cell Culture Flask with Vent Cap (Ref: 430641U)	Corning (Kaiserslautern)

	Tissue culture flask T-75 (Ref: 83.3911.002)	Sarstedt (Nümbrecht)
Cell culture plate	Costar flat bottom cell culture plate:	
	- 6 Well (Ref: 3506)	Corning
	- 12 Well (Ref: 3513)	(Kaiserslautern)
	- 24 Well (Ref: 3524)	
	Falcon clear flat bottom cell culture plate:	Corning
	- 12 Well (Ref: 353043)	(Kaiserslautern)
	- 24 Well (Ref: 353047)	
	Cellstar multiwell plate (6 Well; Ref: 657160)	Greiner Bio-One (Kremsmünster, Austria)
Cell strainer	Falcon cell strainer (100 µm)	BD Bioscience (Heidelberg)
		Corning (Kaiserslautern)
Centrifuge tubes	Sarstedt screw cap tubes (15/50 ml)	Sarstedt (Nümbrecht)
Cryogenic vials	CryoTubes	Nunc (Wiesbaden)
Disposal bags	Sarstedt disposal bags (200 x 300 mm)	Sarstedt (Nümbrecht)
Lysis tube	innuSPEED Lysis Tubes W	Analytik Jena (Jena)
Micro centrifuge tubes	Sarstedt Micro tube 2 ml	Sarstedt (Nümbrecht)
Micro tubes	Biosphere SafeSeal Tube (0.5/1.5/2 ml)	Sarstedt (Nümbrecht)
Neubauer counting chamber (hemocytometer)	Assistent Neubauer counting chamber	Karl Hecht (Sondheim vor der Röhn)
Pasteur pipettes	Assistent pasteur pipettes (230 mm)	Karl Hecht (Sondheim vor der Röhn)
	Labsolute pasteur pipettes (150 mm)	Th. Geyer (Renningen)
Petri dish	Sarstedt petri dish (92 x 16 mm)	Sarstedt (Nümbrecht)

Pipette tips	Biosphere filter tips (0.5-20/2-100/100-1000 µl)	Sarstedt (Nümbrecht)
	Sarstedt tips (0.5-20/2-100/100-1000 µl)	Sarstedt (Nümbrecht)
qPCR adhesive film	MicroAmp Optical Adhesive Film	Applied Biosystems (Carlsbad, USA)
qPCR reaction plate	MicroAmp Fast 96-Well Reaction Plate (0.1 ml)	Applied Biosystems (Carlsbad, USA)
qPCR reaction tubes	MicroAmp Fast Reaction Tubes	Applied Biosystems (Carlsbad, USA)
qPCR tube-caps	MicroAmp Optical 8-Cap Strip	Applied Biosystems (Carlsbad, USA)
Scalpels	Feather standard scalpels (no. 23)	pfm medical (Köln)
Serological pipette	Sarstedt serological pipette (5/10/25/50 ml)	Sarstedt (Nümbrecht)

### 2.1.3 Chemicals & solutions

Name	Manufacturer
Accutase	Capricorn Scientific (Ebsdorfergrund)
Ampuwa Spüllösung 1000ml Plastipur (Water)	Fresenius Kabi (Bad Homburg)
Cryo-SFM	Promocell (Heidelberg)
SYBR Premix Ex Taq II (Takara TB Green Premix Ex Taq II + Rox)	Takara Bio Europe (St.-Germain-en-Laye, France)
my-Budget RNAmagic	Bio-Budget Technologies GmbH (Krefeld)
Gibco DPBS (Dalbecco's Phosphate Puffered Saline)	Thermo Fisher Scientific
2-Mercaptoethanol	Carl Roth GmbH (Karlsruhe)
Chloroform	Sigma-Aldrich (Taufkirchen)
Isopropanol	Carl Roth GmbH (Karlsruhe)
Ethanol	Carl Roth GmbH (Karlsruhe)

Penicilline/Streptomycine (10000 U/ml)	Gibco (Eggenstein)
5x PrimeScript Buffer (for Real Time)	Takara Bio Inc.
PrimeScript RT Enzyme Mix I	Takara Bio Inc.
Oligo dT Primer	Takara Bio Inc.
Fetal calf serum (FCS)	Gibco (Eggenstein)
L-Glutamin (200 mM)	Gibco (Eggenstein)
Trypsin/EDTA (0.5%/0.2%)	Sigma-Aldrich (Taufkirchen)
Calcium chloride (CaCl <sub>2</sub> ) (Cat. No.: C-34005)	Promocell (Heidelberg)
Dulbecco's Modified Eagle Medium (DMEM) with 1g/l glucose, without L-glutamine (cat. No.: 12-707F)	Lonza (Walkersville, USA)
Keratinocyte Growth Medium 2 (KGM2) (Cat. No.: C-20011)	Promocell (Heidelberg)
Keratinocyte Growth Medium 2 (KGM2) supplement mix (Cat. No.: C39016)	Promocell (Heidelberg)
Roswell Park Memorial Institute (RPMI) 1640 Medium without L-Glutamine (Ref: 31870-025)	Gibco (Eggenstein)

#### 2.1.4 Cell culture media

Name	Contents
KGM (+++)	500 ml KGM2
	KGM2 Supplement mix
	5 ml Penicillin/Streptomycin
KGM (++-)	500 ml KGM2
	KGM2 Supplement mix
KGM (-+-)	500 ml KGM2
RPMI (+++)	500 ml RPMI 1640
	50 ml FCS
	5 ml L-Glutamin

	5 ml Penicillin/Streptomycin
	500 ml RPMI 1640
RPMI (++-)	5 ml L-Glutamin
	50 ml FCS
	500 ml RPMI 1640
RPMI (-+-)	5 ml L-Glutamin
	500 ml DMEM
DMEM (+++)	50 ml FCS
	5 ml L-Glutamin
	5 ml Penicillin/Streptomycin
	500 ml DMEM
DMEM (++-)	50 ml FCS
	5 ml L-Glutamin
	500 ml DMEM
DMEM (-+-)	5 ml L-Glutamin

### 2.1.5 Kits

Name	Manufacturer
Macherey-Nagel NucleoSpin RNA	Macherey-Nagel (Düren)

### 2.1.6 Oligonucleotides

Name	Sequence (5'→3')
<b>Reverse Transcriptase:</b>	
Oligo (dT)	(T)18
<b>qPCR:</b>	
HBD-2-for1	GCC TCT TCC AGG TGT TTT TG
hBD-2-ra	GAG ACC ACA GGT GCC AAT TT
hBD3 f3	TGT TTG CTT TGC TCT TCC TGT
hBD3 r3	CGC CTC TGA CTC TGC AAT AA
TGFBi-f1	ACC CAG AAG CCC TGA GAG
TGFBi-r1	TGC AGC CCA CCT CCA GTG
FN1-f1	ACA ACG TCA TAG TGG AGG CA

FN1-r1	CAT CCG TAG GTT GGT TCA AG
MMP9-f1	GAC ACG CAC GAC GTC TTC CA
MMP9-r1	CAC TGC AGG ATG TCA TAG GTC A
KRT7-f1	CTG CTG GAG GGC GAG GA
KRT7-r1	GCC ACC AGT GGA ATT CAT CA
TGM2-f1	CTC AAC CTG GAG CCT TTC TC
TGM2-r1	AGG GCC CGC ACC TTG ATG A
COL22A1-f1	CAG GAG AGA AAG GAG TCC C
COL22A1-r1	TGC CCC AGG CTG GCC TTT TC
FERMT1-f1	GAT TCC AGT GAC AAC ATG GAG
FERMT1-r1	TCA AAC TCG ATG ACC ACC TG
COL1A1-f1	CTG GAA GAG TGG AGA GTA CTG
COL1A1-r1	GTC TCC ATG TTG CAG AAG AC
COL1A2-f1	TGA AGG CTC TAG AAA GAA CCC
COL1A2-r1	TCC AGT AGT AAC CAC TGC TC
EGFR-f1	TCC CTC AGC CAC CCA TAT GTA C
EGFR-r1	GTC TCG GGC CAT TTT GGA GAA TTC
EGFR-v1-4-f1	GGG CTC TGG AGG AAA AGA AA
EGFR-v1-4-r1	TCC TCT GGA GGC TGA GAA AA
RP38-f1	TCA AGG ACT TCC TGC TCA CA
RP38-r1	AAA GGT ATC TGC TGC ATC GAA
VEGFA-f1	CTA CCT CCA CCA TGC CAA GT
VEGFA-r1	AGC TGC GCT GAT AGA CAT CC
Ki-67-f1	TGA CTT CCT TCC ATT CTG AAG AC
Ki-67-r1	TGG GTC TGT TAT TGA TGA GCC

### 2.1.7 Cells

Name	Manufacturer
NHEK	Promocell (Heidelberg)
Fibroblasts	self-production (the cells were isolated from donated adult human skin)

### 2.1.8 Software

Name	Description
Microsoft Word 2010	Word processor
Microsoft Excel 2010	Spreadsheet
GraphPad Prism 5.0	Graphing & statistical software
NanoDrop 1000 (Version 3.6.0)	Software for NanoDrop 1000 Spectrophotometer
StepOnePlus™ Software (Version 2.1)	Software for StepOne Plus real-time PCR system
AxioVision LE 4.2.8.0	Software for analysis of scratch assays
AxioVision Microscope Software	Software for capturing photos through the microscope

## 2.2 Methods

### 2.2.1 Cell Culture

All activities involving cell culture were conducted under a sterile workbench.

#### 2.2.1.1 Keratinocyte culture

The keratinocytes used in this study were NHEK (normal human epidermal keratinocytes) cells, a cell pool of primary keratinocytes isolated from juvenile foreskins of three to five donors.

The NHEK cells were grown in Corning® cell culture flasks in KGM2 (++- or +++) medium and incubated in an incubation chamber with a water-saturated atmosphere with 5% CO<sub>2</sub> and at 37°C. The medium in the flask was replaced with a fresh medium every two days.

The cells were observed daily under a microscope, and when a confluency of 90% had been reached, they were subcultivated into multiple flasks or plates. To do this, the cells were washed with PBS and given a 0.05% trypsin/EDTA (T/E) solution. After incubating for 5-10 minutes, the cells were observed under a microscope to ensure they were detached from the bottom of the flask. The suspension was then mixed with KGM2 (++-) to let the FCS in the medium neutralize the trypsin and centrifuged for five minutes at 200 g afterwards. The supernatant was thrown away, and the cells were resuspended in KGM2 (++- or +++) . They were then transferred into new flasks or plates.



### **2.2.1.2 Fibroblast culture**

The fibroblasts used in this study were obtained from healthy abdominal skin donated by the surgery center of UKSH Kiel. The isolation of fibroblasts from the skin is a 2-day process.

On the first day, the skin was removed from the PBS solution in which it was transported and placed on a sterile petri dish. The skin was cleaned from subcutaneous fats using sterile tweezers, scissors, and scalpels and then cut into 0.5 mm squares and transferred into a 50 ml centrifuge tube containing a prepared solution of 1 ml 2.5% trypsin and 25ml PBS. This was then stored at 4°C overnight.

On the second day, 20ml DMEM (+++) or RPMI1640 (+++) was added into the incubated tube to neutralize the trypsin. On a sterile petri dish, the skin's epidermis was separated from the dermis using sterile tweezers and scalpels. The dermis, which contained fibroblasts, were then placed skin-side up in (Costar) 6-well cell culture plates, with each well containing about 6 dermis pieces. RPMI1640 (+++) or DMEM (+++) medium was then given into each well with a pipette, being careful not to detach them from the bottom of the well, as this would prevent fibroblasts from successfully growing on the plate.

The medium was replaced with a fresh medium twice in the first week and then only once a week in the following weeks as long as there were still only a few cells. Once the cells had started to become confluent, they were given a new medium every two days. The dermis pieces were removed after a week. When a confluency of 70-90% had been achieved, the fibroblasts were transferred into flasks using a similar method to the subcultivation of keratinocytes, with the only difference being RPMI1640 (+++) or DMEM (+++) medium being used instead of KGM2 (++-).

### **2.2.1.3 Cryopreservation**

Cryopreservation was used to store cells for a long period. Like the cell subcultivation process, once the cells had reached a 90% confluency, they were washed with PBS and given Trypsin so they would detach from the bottom of the flask or plate. The suspension was then mixed with KGM2 (++-) for keratinocytes or either RPMI1640 (++-) or DMEM (++-) for fibroblasts to let the FCS neutralize the trypsin. After centrifuging for 5 minutes at 200 g, the supernatant was thrown away, and the cells were resuspended in 1000 µl Cryo-SFM and transferred into a cryogenic vial. The vials were put in a cryogenic freezing container and stored overnight in a -80°C Freezer so that they would freeze at a rate of 1-3°C per minute. Afterwards, the vials were

transferred to a cryogenic storage vessel containing liquid nitrogen and stored there until it needs to be used.

#### **2.2.1.4 Stimulation**

Stimulation of keratinocytes & fibroblasts was done using similar methods, with the only differences being the medium and the cell culture plate used.

Before stimulation, keratinocytes were given full medium without antibiotics (KGM2 ++-) until a 90-100% confluency was reached. Fibroblasts were given DMEM (++) or RPMI1640 (++-), which are also full media without antibiotics. Costar cell culture plates were used for keratinocytes because they provided a surface with better adhesion than the Falcon cell culture plates. For fibroblasts, the Costar and Falcon cell culture plates and the Cellstar multiwell plate were used.

For the stimulation, growth media without FCS or supplements and antibiotics (KGM +/- for keratinocytes and RPMI1640 +/- or DMEM +/- for fibroblasts) were used to minimize the possibility of additional stimulation by FCS or supplements. The stimulus, which was PRGF, was diluted in the medium at a 1:5, 1:10, 1:20, 1:40 or 1:50 ratio, depending on the design of the experiment. As a control, a KGM2 medium that had gone through the same processes as in PRGF production was used. This was also diluted in medium, generally at a 1:10 ratio. The cells were given the diluted stimulus and incubated in an incubation chamber for a period determined beforehand, which ranged between 4 to 48 hours.

The stimulation was ended by taking the supernatant away at the end of the incubation period. The supernatant from each well was transferred into separate Biosphere safe seal tubes (typically a 1.5 ml tube) and stored at -20°C. The cells on the plate were then washed once with PBS before they enter the RNA-Isolation process.

#### **2.2.1.5 Ex vivo stimulation**

*Ex vivo* stimulations were done on healthy human skin donated by the surgery center of UKSH Kiel.

To prepare the skin for stimulation, the dermis of the skin was separated from subcutaneous fats and subdermal tissues using sterile scissors and scalpels, and then cut into circles using 6mm biopsy punches. Ten skin punches were used for the stimulation.

As stimulus, PRGF was diluted in KGM2 (-+-) at a 1:5 or 1:10 ratio. As a control, a KGM2 medium that had gone through the same processes as in PRGF production was diluted in normal KGM2 (-+-) at a 1:5 or 1:10 ratio. In 10 Biosphere safe seal tubes (1.5 ml), 5 punches were submerged in 300µl control solution and 5 punches in 300µl stimulus solution. They were then incubated for 24 hours in an incubation chamber.

The stimulation was ended by moving the skin punches from the solution into 10 new Biosphere safe seal tubes (1.5 ml), each containing 300 µl RNAlater. These were stored at -20°C overnight and were thawed the next day for RNA isolation. The remaining solutions in the incubated tubes were centrifuged at 12000 g for 5 minutes, and the supernatant was then transferred into fresh 1.5 ml tubes and stored at -20°C.

#### **2.2.1.6 Scratch assay**

A scratch assay was done on fibroblasts to investigate whether stimulation with PRGF leads to increased cell migration.

As a stimulus, PRGF was diluted in DMEM (-+-) at a 1:10 ratio. As a control, a KGM2 medium that had gone through the same treatment as in PRGF production was diluted in DMEM (-+-) at a 1:10 ratio.

In a 12-well plate, fibroblasts were fed with DMEM (++-) until 90 - 100% confluence was reached. All 12 wells were then scratched once using 2 – 100 µl pipette tips. Two lines were drawn on the plate for each well as reference points to make sure that an observed spot could be found again at the next observations. Three wells were given 500 µl control solution, and for each tested PRGF, 3 wells were given a 500 µl stimulus solution containing the specific PRGF.

Photos of the cells were taken immediately after the stimulation, then again after around 6, 24, 30, and 48 hours. Analysis of the photos was done using AxioVision LE 4.2.8.0 software by measuring the gap where no cells were present. By comparing the size of the gap at different times of observation, the migration progress was assessed. Statistical analysis was done using GraphPad Prism.

### **2.2.2 RNA isolation**

#### **2.2.2.1 RNA isolation with RNAmagic (TRIzol)**

The cells were washed once with PBS, lysed using RNAmagic, and transferred into a micro centrifuge tube (2ml). After mixing with chloroform and centrifugation, the mixture became

separated into 3 phases: a clear phase at the top (RNA), a white middle phase (protein), and a red bottom phase (DNA). The clear, upper phase was transferred into a fresh 1.5 µl Biosphere safe seal tube and given Isopropanol to precipitate the RNA, which, after centrifugation, became a pellet at the bottom of the tube. The pellet was then washed twice with 75% ethanol. This pellet was the RNA. The pellet was resuspended in 12 µl RNase-free water, and the RNA-concentration and purity were measured using a spectrophotometer.

#### **2.2.2.2 RNA isolation with Macherey-Nagel NucleoSpin RNA kit**

RNA-isolation of some fibroblast stimulation and all *ex vivo* stimulation experiments were done using the Macherey-Nagel NucleoSpin RNA kit.

The stimulated skin was cut into small pieces and transferred into a lysis tube containing ceramic and steel beads and the lysis buffer (a pre-prepared solution of 600 µl RLT plus Buffer or RA1 Buffer and 10 µl β-Mercaptoethanol). The tubes were shaken using SpeedMill to further shred the skin and subsequently centrifuged, so that the skin pieces settled. The lysate was filtered through a NucleoSpin Filter and given ethanol. By centrifuging the lysate through the NucleoSpin RNA Column, the RNA would be bound to the membrane of the column. The membrane was desalted by adding MDB (Membrane Desalting Buffer) to the membrane to allow a more effective rDNase digestion and then dried by centrifugation. A DNase reaction mixture (10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase) was prepared and applied directly to the membrane and incubated at room temperature for 15 minutes to allow the DNA digestion to take place. The membrane was then washed once with RAW2 Buffer and twice with RA3 Buffer. Finally, the RNA was eluted in RNase-free water, and the RNA-concentration and purity were measured using a spectrophotometer.

#### **2.2.3 cDNA synthesis**

Single strand complementary DNA (cDNA) was synthesized from the RNA obtained from an experiment by reverse transcription using PrimeScript RT Reagent Kit.

In a 0.5 ml micro tube, 0.5 µg RNA was mixed with a reaction mixture consisting of 2 µl reaction buffer made up of dNTP (10 mM) and RNase inhibitor (40 U/µl), 0.5 µl Oligo dT primer (20mM) and 0.125 µl PrimeScript RT Enzyme Mix I. RNase-free water was then added, so that the total volume of the mixture would be 10 µl. Afterwards, the tube was placed in a Thermocycler, where the mixture would go through an incubation process for 5 minutes at 65°C, a synthesis process for 30 minutes at 42°C, another incubation process for 5 minutes at

85°C and then finally be cooled down to 4°C. 40 µl RNase-free water was then added to the mixture so that the cDNA would have a concentration of 10 ng/µl.

The cDNA was stored at -20°C.

#### **2.2.4 Quantitative real-time polymerase chain reaction (qPCR)**

For quantitative real-time PCR, SYBR Green I fluorescence dye, which only emitted fluorescence once it had intercalated with the PCR-products, was used. Adding another fluorescence dye, ROX (6-Carboxy-X-rhodamine), to SYBR Green functioned as a passive reference to the volume contained in a sample.

For each sample, a reaction mixture consisting of 5.2 µl SYBR Premix Ex Taq II with the addition of ROX, 3 µl RNase free water, and 0.8 µl of primer (0.4 µl of each forward and reverse primers with 10 µM concentration) was prepared.

1 µl of the cDNA to be measured was pipetted into each well of a 96-Well PCR plate or an 8-Well PCR strip, and then 9 µl of the reaction mixture was pipetted into each well, creating a total volume of 10 µl. The PCR plate was then sealed with a plastic foil and centrifuged at 200 g for 1 minute or, if using a PCR strip, the PCR strip was sealed using its lid and centrifuged for a few seconds. The plate or strip was then inserted into StepOne Plus Real-Time PCR System.

The gene amplification was based on a two-step touchdown program consisting of 46 cycles. It began with a 30-second denaturation process at 95°C and a subsequent 30-second elongation process at 66°C. The second until sixth cycles consisted of a 5-second denaturation process at 95°C and, again, a 30-second elongation process. However, the temperature used in the elongation process is progressively reduced by 1°C in every cycle, so that the temperature would reach 60°C at the end of the sixth cycle. The remaining 40 cycles consisted of a 5-second denaturation process at 95°C and a 30-second elongation process at a constant 60°C. The intensity of the fluorescence was measured at the end of each cycle.

To examine whether any unspecific PCR product was present, a melting curve was created at the end of the amplification by increasing the temperature at a rate of 1.2°C/min from 60°C to 95°C while measuring the fluorescence every 15 seconds.

The analysis of the qPCR data was done using Microsoft Excel, where the amount of a gene was compared to the amount of the housekeeping gene, which was the ribosomal protein L38 (RP38). This gene was used as our housekeeping gene because it was unregulated and

constantly expressed in epithelial cells. For all primers, a calibration curve was created and considered in the analysis. Statistical analysis was done using GraphPad Prism.

### **2.2.5 PRGF preparation**

The initial material used for PRGF preparation in this study were thrombocyte concentrates of healthy human donors obtained from the Institute of Transfusion Medicine, University of Schleswig-Holstein, Campus Kiel.

The thrombocyte concentrates (7 ml each) were centrifuged at 2000 g for 10 minutes. The centrifugation created a pellet, which was then washed twice with 7 ml sodium citrate buffer (0.011 mol/l, pH 5.5, 37°C). After centrifuging again at 2000 g for 10 minutes, the thrombocyte pellet was resuspended in 3.5 ml KGM  $-+ -$ . The solutions were then transferred to 2 ml tubes.

The thrombocyte suspensions and control KGM  $(-+ -)$  that contained no thrombocytes were placed on ice and lysed by ultrasound. Each 2 ml tube received five waves of 10 ultrasound shocks. During the whole ultrasound treatment process and while resting, the tubes were always kept on ice. After the ultrasound lysis process, the suspensions and control KGM  $(-+ -)$  were stored at -80°C for 24 hours.

After 24 hours, the suspensions and control KGM  $-+ -$  were thawed and went through the same ultrasound lysis process again. They were then stored at -80°C for another 24 hours.

After 24 hours, the suspensions and control KGM  $-+ -$  were thawed and centrifuged at 18000 g and -10°C for 3 minutes to separate cell debris. The supernatants, which were the PRGFs, were stored as 500 µl aliquots and stored at -20°C. The control KGM  $-+ -$  were also stored as 500 µl aliquots at -20°C.

## 3 Results

### 3.1 Primary keratinocyte stimulations

Real-time PCR analyses on keratinocytes in this study were done on remaining frozen cDNA from previous studies as well as cDNA collected from new experiments.

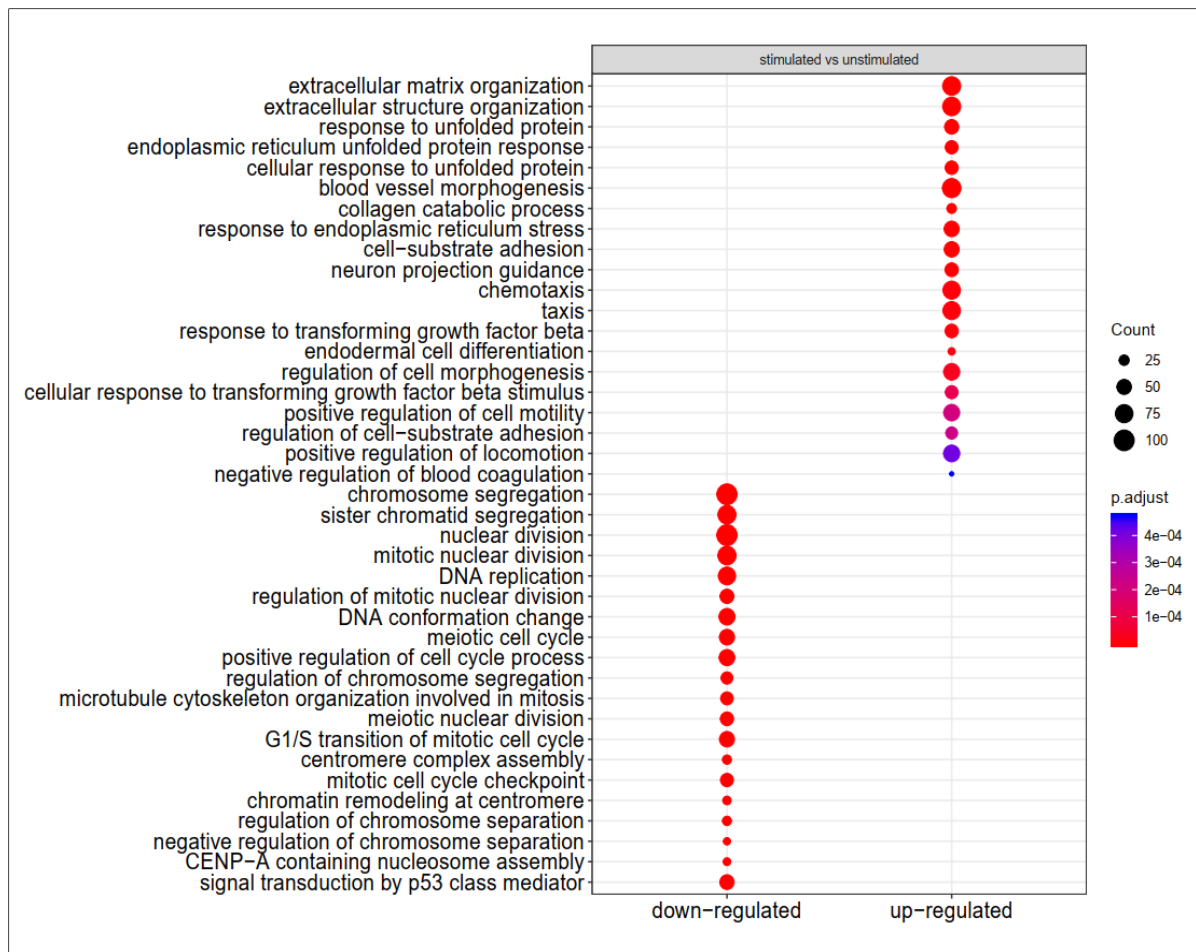
#### **3.1.1 Whole transcriptome sequencing of PRGF-stimulated keratinocytes revealed induction of genes involved in extracellular matrix organization and epidermis development.**

As a starting point of the study, human primary keratinocytes were treated with PRGF obtained from 5 different donors, and their RNAs were analyzed by whole transcriptome sequencing to create an overview of PRGF effects on keratinocytes.

The whole transcriptome analysis (RNA-Seq) was done on a HiSeq 4000 (Illumina) by the Institute of Clinical Molecular Biology of Kiel University. The processes involved in this analysis were as described in the paper titled “Platelet-Released Growth Factors and Platelet-Rich Fibrin Induce Expression of Factors Involved in Extracellular Matrix Organization in Human Keratinocytes” [18].

The data of one unstimulated sample was excluded from the analysis due to principal component analysis showing considerable variance from the other unstimulated samples on transcriptome-wide level.

The analysis revealed 1459 upregulated and 1398 downregulated genes in stimulated keratinocytes compared to unstimulated keratinocytes. Gene ontology (GO) enrichment analysis revealed a substantial induction of genes involved in extracellular matrix (ECM) organization, epidermis development, and cell adhesion (Figure 2). Downregulated genes were generally components of DNA replication and cell cycle.

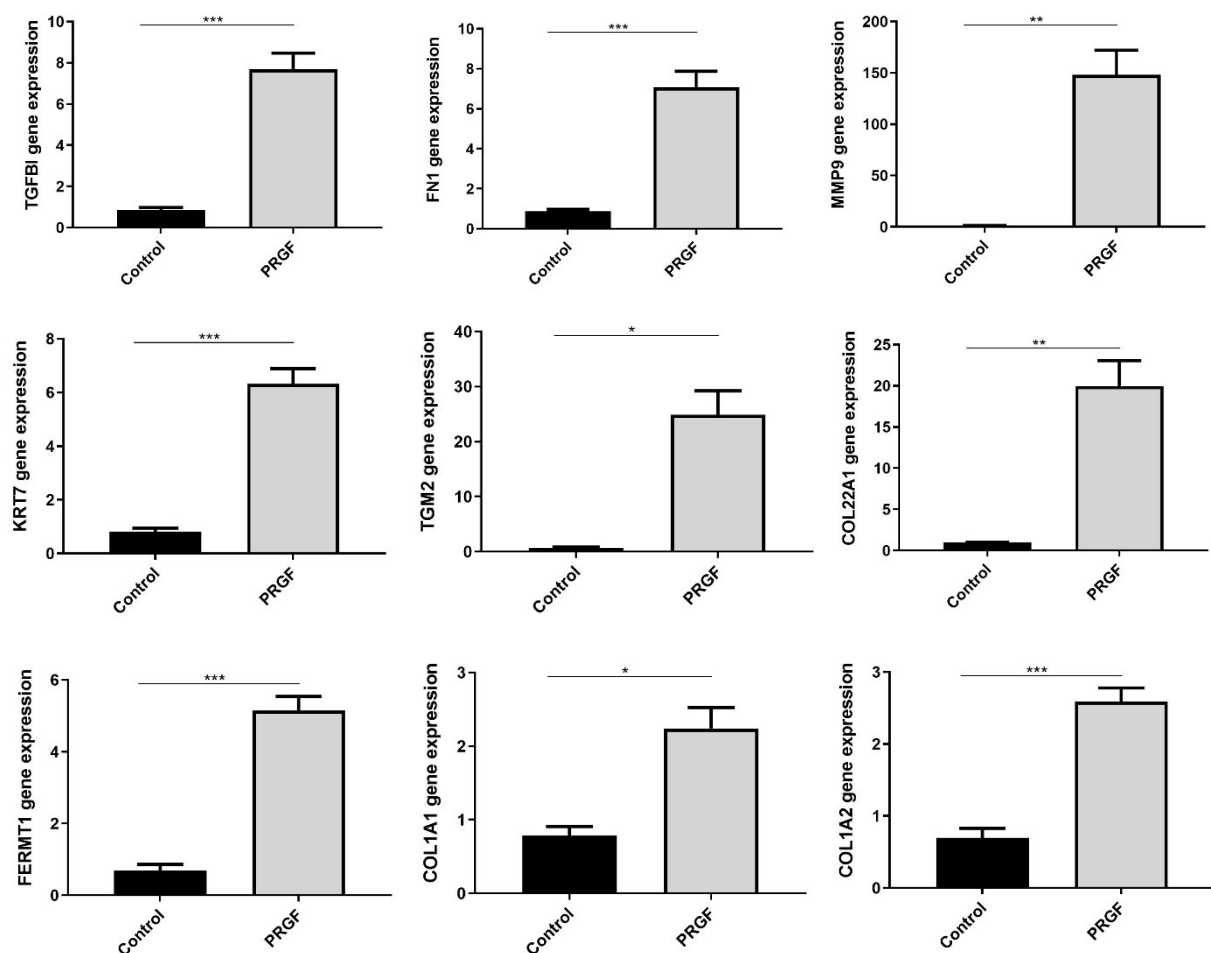


**Figure 2: Whole transcriptome analysis of PRGF-stimulated keratinocytes.** Gene Ontology (GO) analysis revealed biological processes where genes are significantly affected in PRGF-stimulated keratinocytes in comparison to unstimulated keratinocytes. The diagram shows the top 20 (according to p-value) biological processes where genes are significantly up- and down-regulated respectively. The size of the dots represents the amount of significantly affected genes within a particular biological process. The colors represent the adjusted p-values.



### 3.1.2 Stimulation of primary human keratinocytes with PRGF verified the induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2

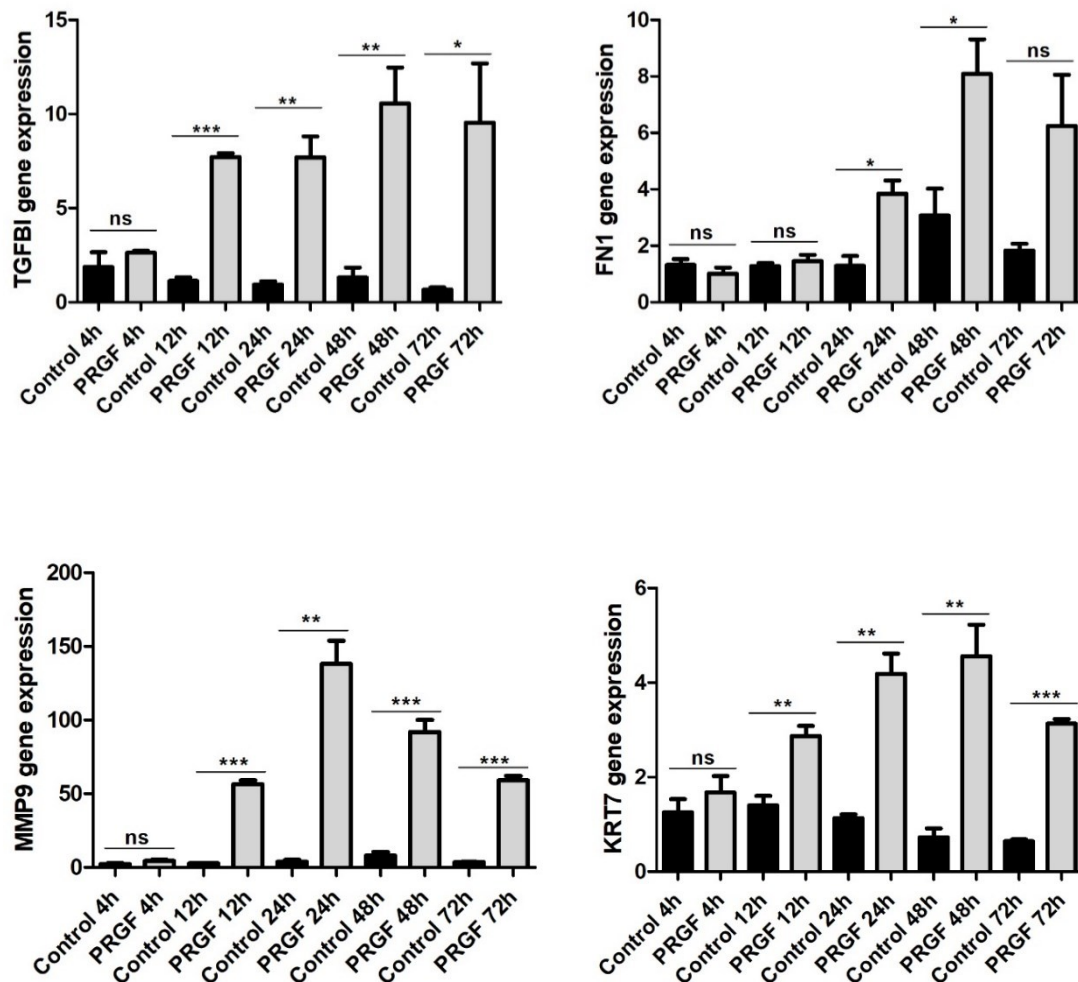
To confirm that PRGF could induce the expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary human keratinocytes, we stimulated keratinocytes with PRGFs from ten different donors at a concentration of 1:10 for 24 hours. This was done in two separate experiments, in each of which five of the ten PRGFs were used. We excluded the data of two stimulations from the second experiment due to considerable divergence from the other stimulations. Real-time PCR analysis showed that PRGF caused induction of these genes in primary human keratinocytes (Figure 3).

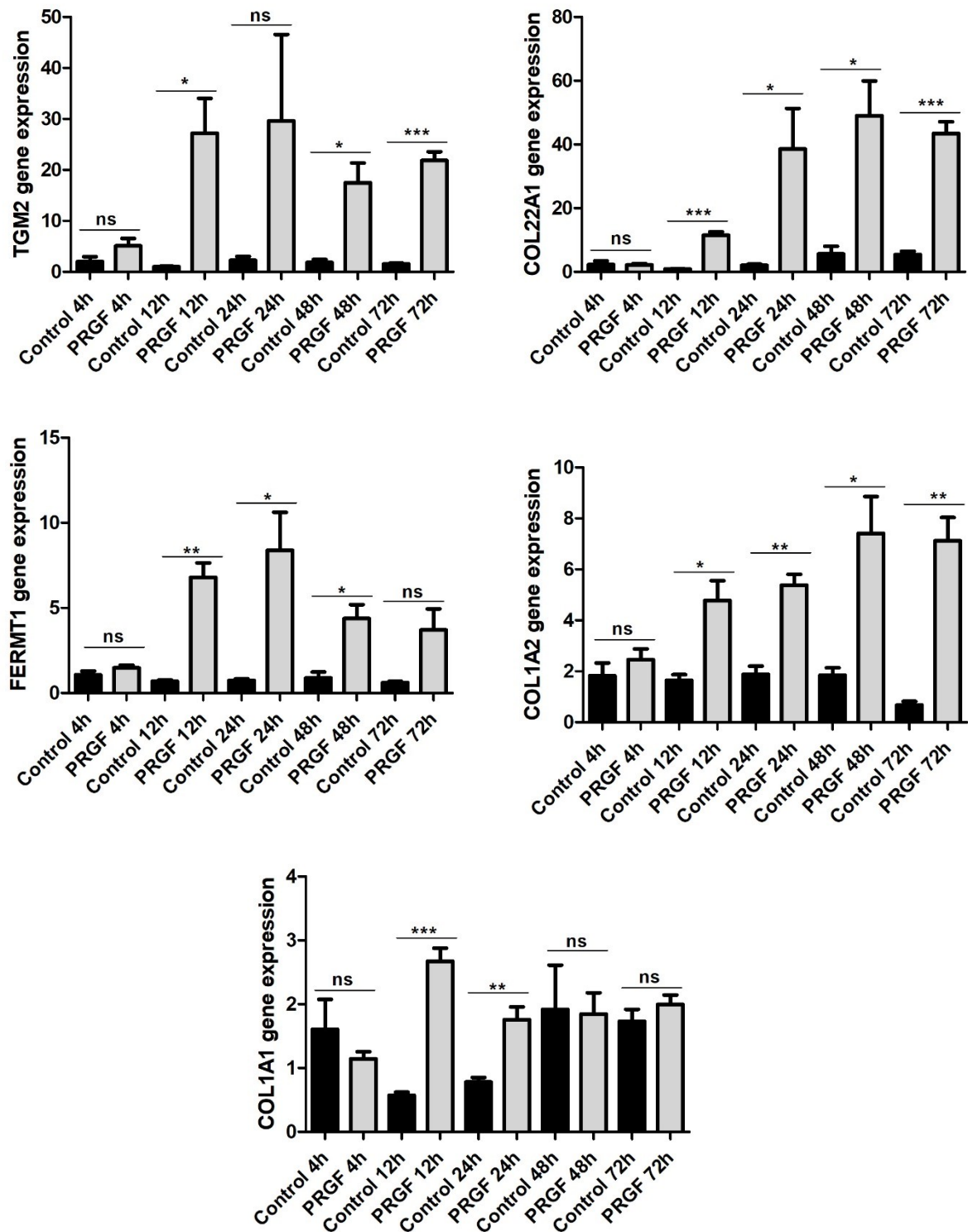


**Figure 3: PRGF induces expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.** Keratinocytes were stimulated in two independent experiments with a total of 10 different PRGFs at 1:10 dilution for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of cumulative data of an experiment with 5 different PRGFs consisting of duplicates and another experiment with 5 different PRGFs consisting of triplicates (Student's t-test \* $p < 0.05$  \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ).

### 3.1.3 Stimulation of primary human keratinocytes with PRGF lead to a time-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2

To assess the time kinetic of the PRGF-dependent induction of all selected genes, primary human keratinocytes were stimulated with PRGF at a concentration of 1:10 for 4, 12, 24, 48, and 72 hours. The analysis showed that all genes, besides FN1, started to be significantly induced by PRGF after 12 hours, while FN1 was significantly induced after 24 hours. TGFBI, FN1, KRT7, COL22A1 and COL1A2 reached a peak at 48 hours. MMP9, FERMT1 and TGM2 reached a peak at 24 hours, although TGM2's result was not significant at 24 hours due to strong variance between values. COL1A1 reached a peak at 12 hours (Figure 4).

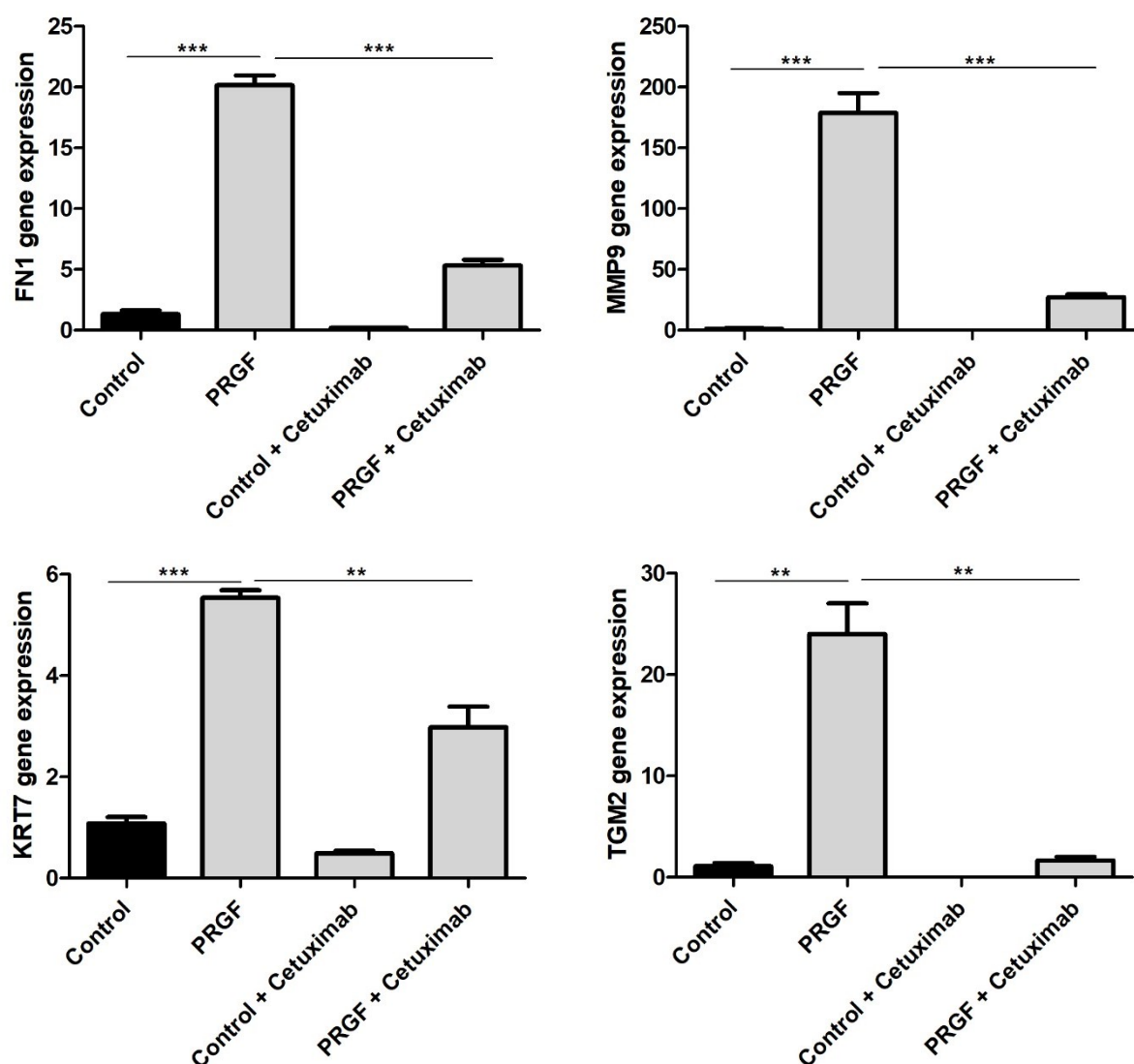


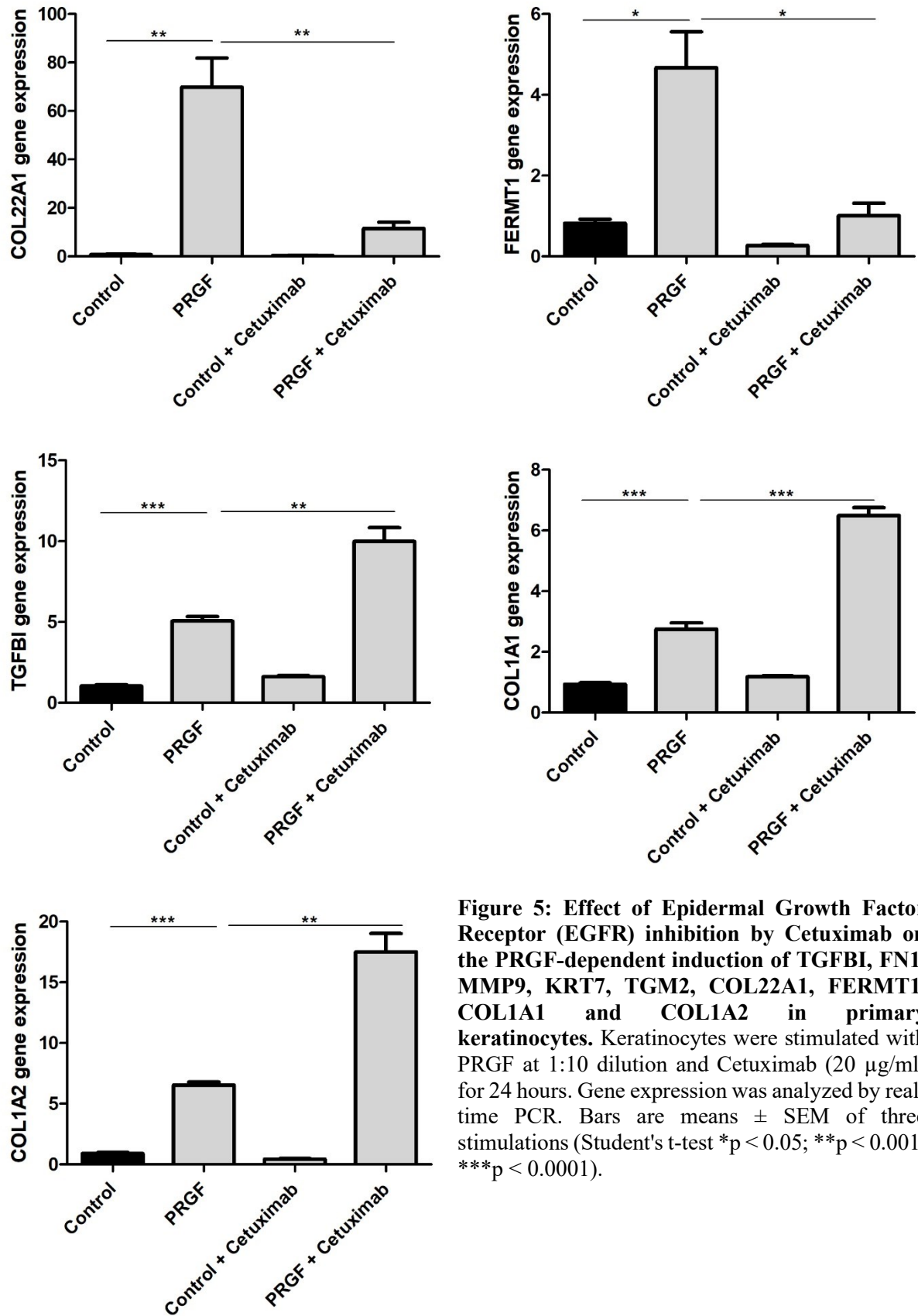


**Figure 4: Time kinetic of the PRGF-mediated induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.** Keratinocytes were stimulated with PRGF at 1:10 dilution for 4, 12, 24, 48 and 72 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of three simulations (Student's t-test \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ; ns = not significant).

### 3.1.4 Inhibition of Epidermal Growth Factor Receptor (EGFR) suppressed the PRGF-dependent induction of FN1, MMP9, KRT7, TGM2, COL22A1 and FERMT1 but amplifies induction of TGFBI, COL1A1 and COL1A2 in primary human keratinocytes

Past studies have shown that the induction of hBD-2 and hBD-3 in primary human keratinocytes through PRGF stimulation was suppressed by Cetuximab, a monoclonal antibody that specifically blocks EGFR. Therefore, to discover whether EGFR also regulated the PRGF-dependent induction of the selected genes, we stimulated primary human keratinocytes with PRGF at a concentration of 1:10 in the presence of Cetuximab (20  $\mu$ g/ml) for 24 hours. The results showed that the induction of FN1, MMP9, KRT7, TGM2, COL22A1 and FERMT1 by PRGF were reduced by Cetuximab, which indicated that EGFR mediated the PRGF-dependent induction of these genes. However, the induction of TGFBI, COL1A1 and COL1A2 was enhanced by the inhibition of EGFR by Cetuximab (Figure 5).

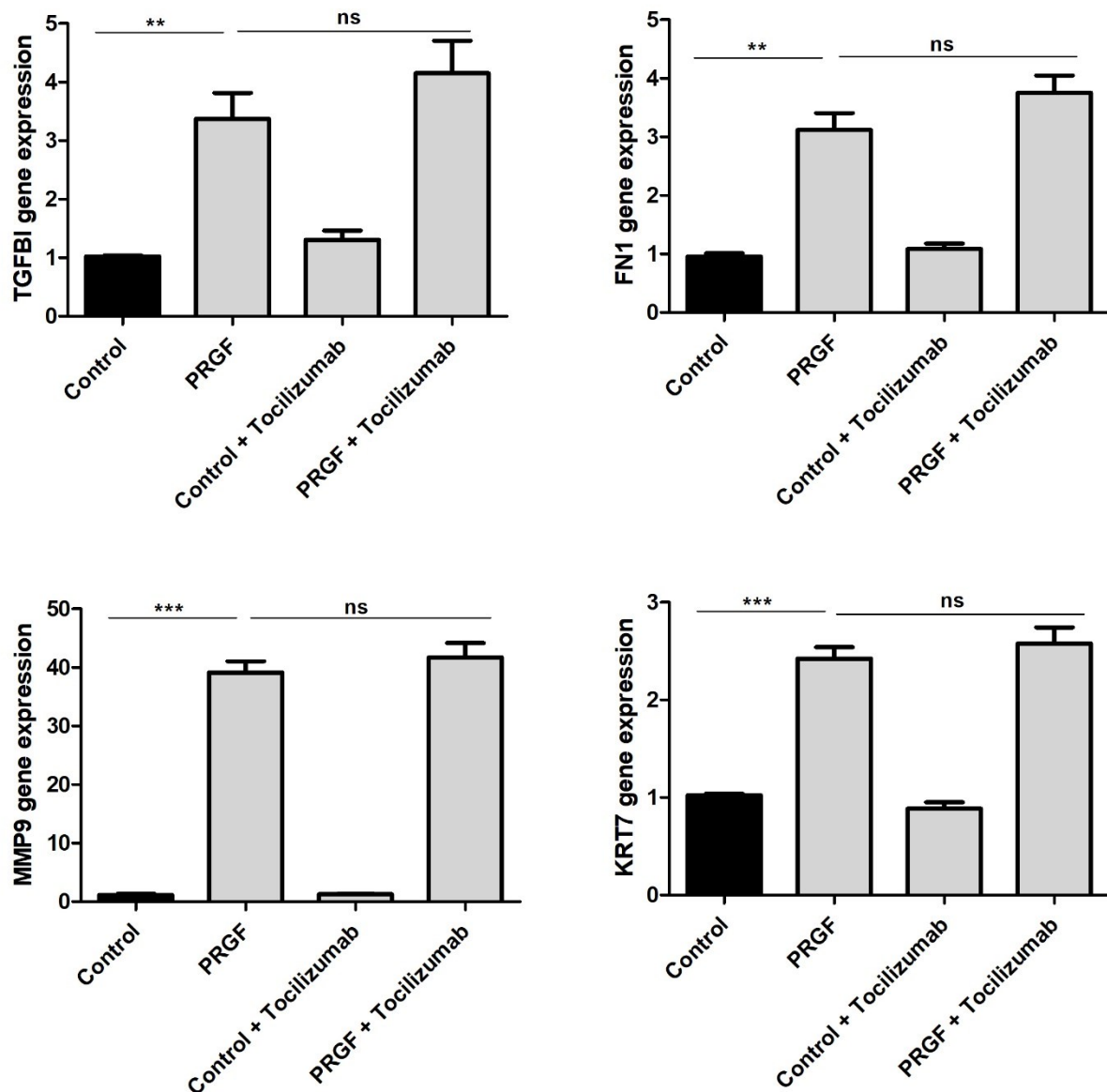


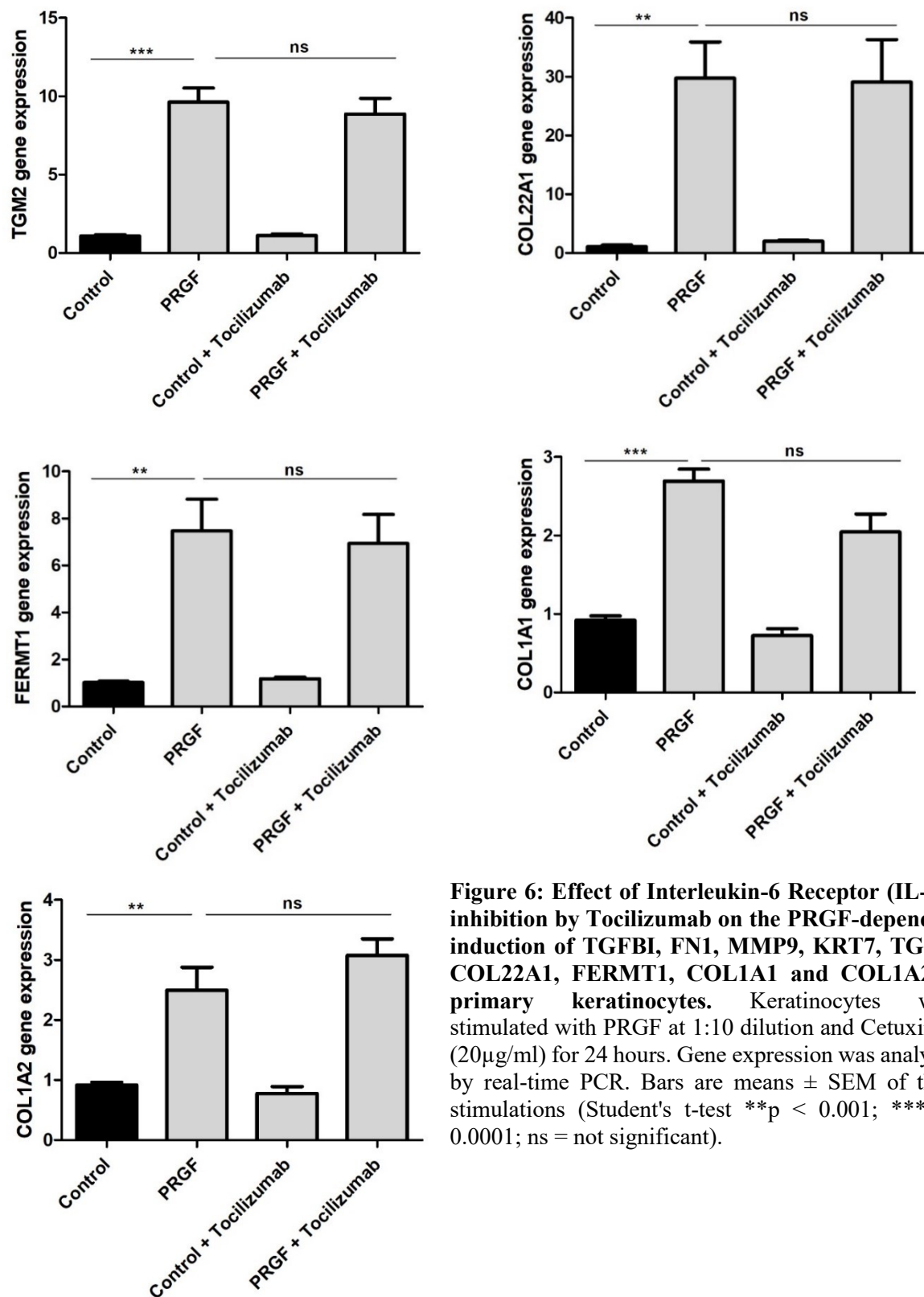


**Figure 5: Effect of Epidermal Growth Factor Receptor (EGFR) inhibition by Cetuximab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.** Keratinocytes were stimulated with PRGF at 1:10 dilution and Cetuximab (20  $\mu$ g/ml) for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of three stimulations (Student's t-test \* $p$  < 0.05; \*\* $p$  < 0.001; \*\*\* $p$  < 0.0001).

### 3.1.5 Inhibition of Interleukin-6 Receptor (IL-6R) had no influence on the PRGF-induced expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary human keratinocytes

In past studies, it was shown that Tocilizumab, a monoclonal antibody that blocks IL-6 receptors, was able to inhibit the PRGF-mediated induction of hBD-2 and hBD-3 in primary human keratinocytes. To find out whether this also applies to the genes in this study, we stimulated primary human keratinocytes with PRGF at a concentration of 1:10 in the presence of Tocilizumab (50 µg/ml) for 24 hours. The results showed that the induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 by PRGF were not enhanced or lessened by the IL-6 receptor inhibition by Tocilizumab (Figure 6).





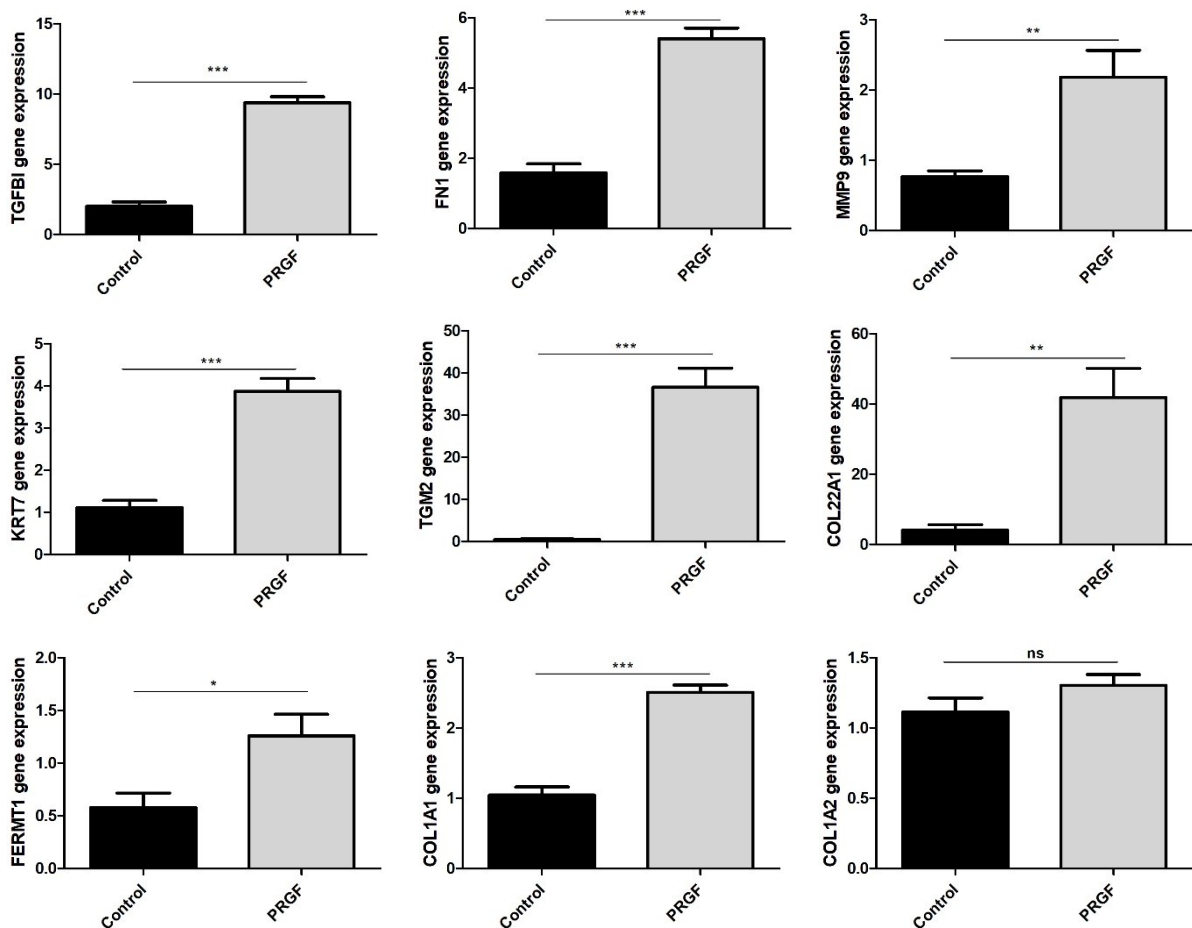
**Figure 6: Effect of Interleukin-6 Receptor (IL-6R) inhibition by Tocilizumab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes.** Keratinocytes were stimulated with PRGF at 1:10 dilution and Cetuximab (20µg/ml) for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of three stimulations (Student's t-test \*\*p < 0.001; \*\*\*p < 0.0001; ns = not significant).

## 3.2 Primary Fibroblast Stimulations

Considering that PRGF induced all chosen genes in primary human keratinocytes, we wanted to discover whether stimulating primary human fibroblasts with PRGF would lead to the same effect. Therefore, we carried out stimulations of primary human fibroblasts with PRGF.

### 3.2.1 Stimulation of primary human fibroblasts with PRGF lead to induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1 and COL1A1

We stimulated primary human fibroblasts with five different PRGFs at a concentration of 1:10 for 24 hours. The analysis showed that stimulation with PRGF lead to significantly increased expressions of all the tested genes except for COL1A2, which had only a slight, insignificant increase (Figure 7).

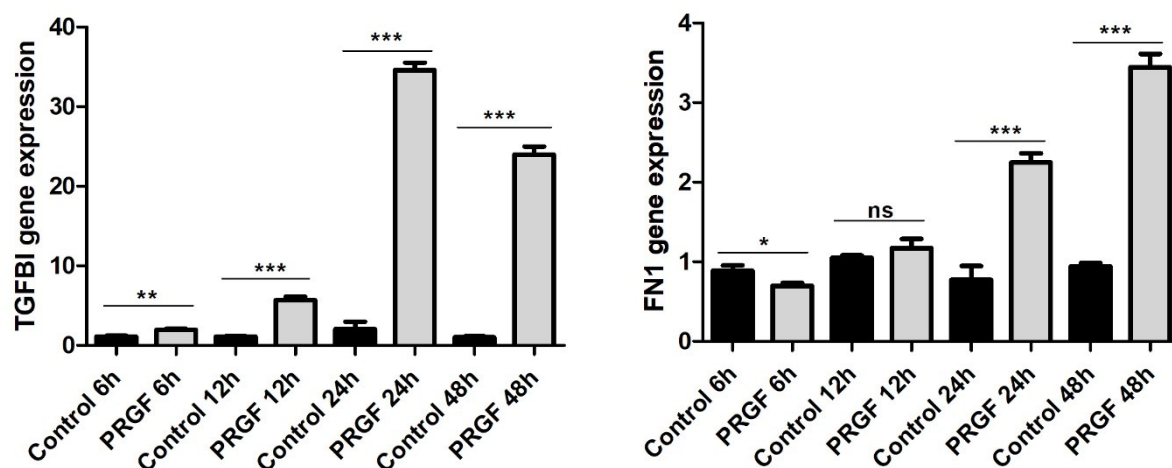


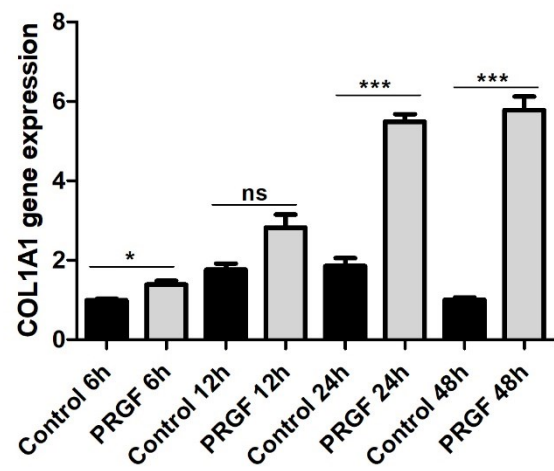
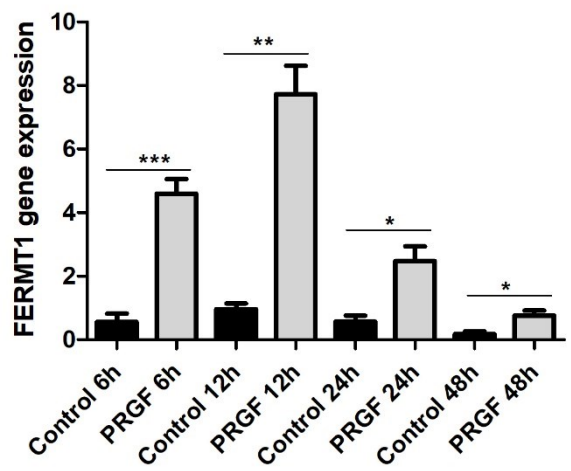
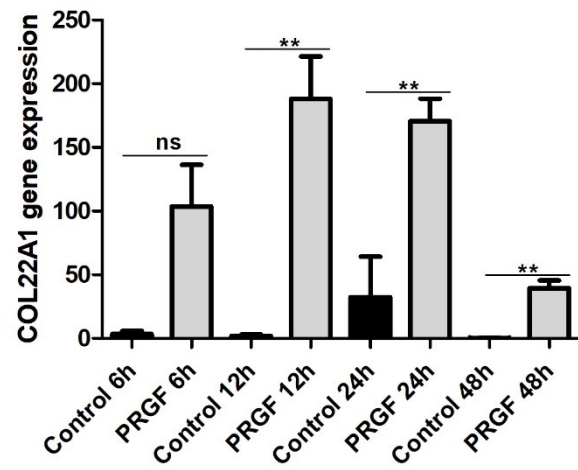
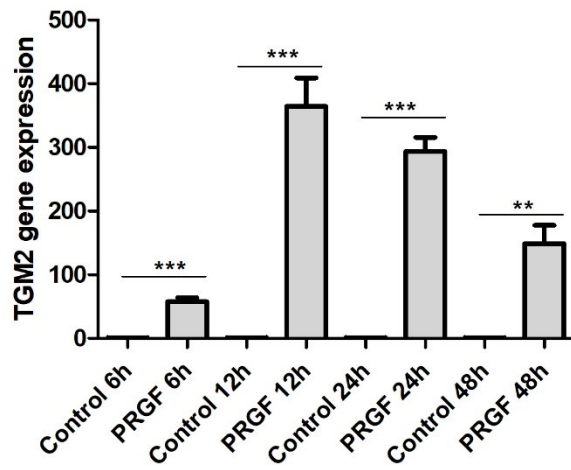
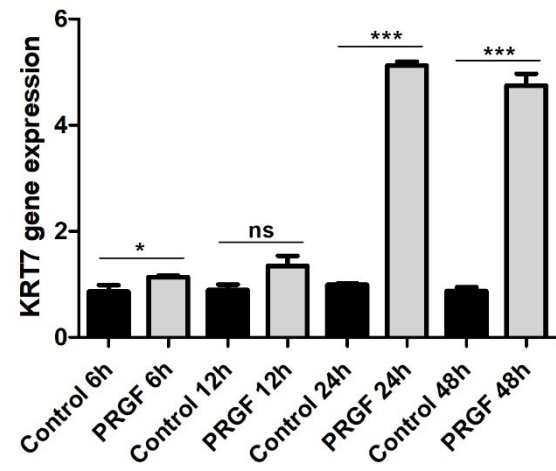
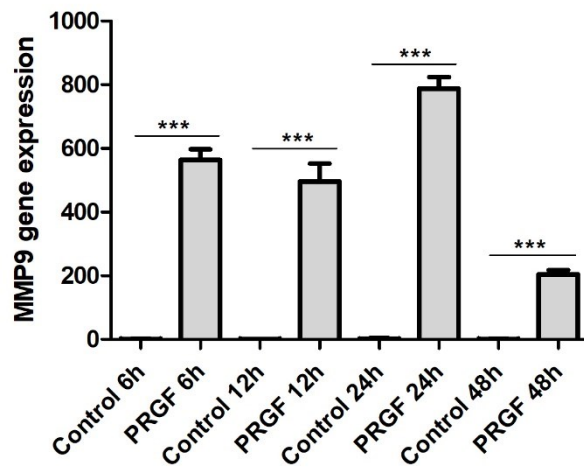
**Figure 7: PRGF induces expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary fibroblasts.** Fibroblasts were stimulated with 5 different PRGFs obtained from 5 different blood donors at 1:10 dilution for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM (Student's t-test \*p < 0.05; \*\*p < 0.001; \*\*\*p < 0.0001; ns = not significant).

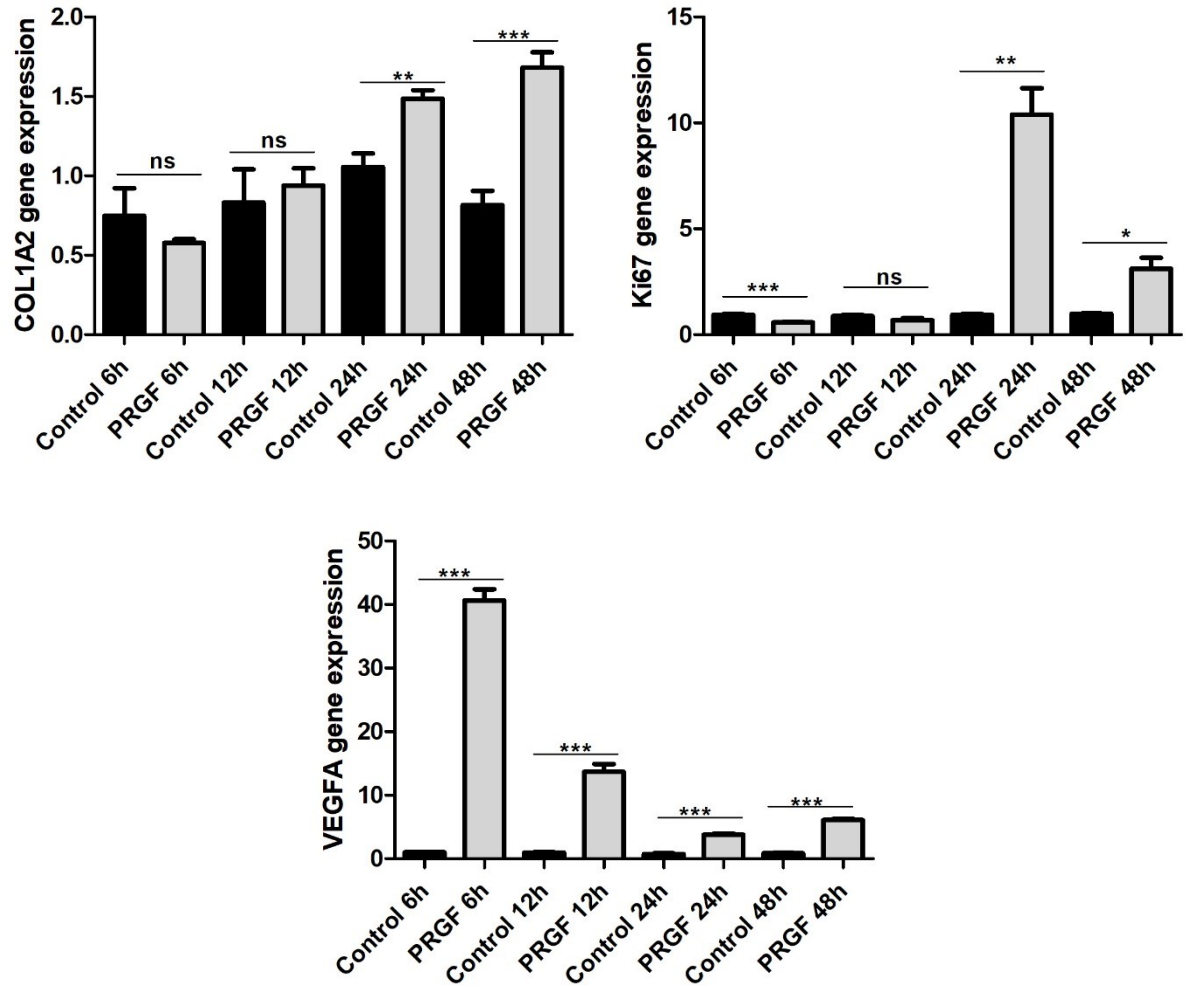


### 3.2.2 Stimulation of primary human fibroblasts with PRGF lead to a time-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA

To study the time kinetic of the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary human fibroblasts, we stimulated fibroblasts with 2 different PRGFs at a concentration of 1:10 and stopped the stimulations at different times (6, 12, 24, and 48 hours). The analysis showed that TGM2, COL22A1 and FERMT1 were most prominently expressed at the 12-hour mark, TGFBI, MMP9 and KRT7 at the 24-hour mark, and FN1, COL1A1 and TGM2 after 48 hours. COL1A2, which was not significantly induced in the previous experiment, was significantly induced in this experiment after 24 hours and reached a peak at 48 hours. Moreover, we analyzed the expression of the proliferation marker Ki67, since PRGF suppressed ki67 in primary human keratinocytes according to a past study, and on vascular endothelial growth factor A (VEGFA), as VEGFA plays a significant role in wound healing. The results showed that the expression of Ki67 and VEGFA were both induced by PRGF, with a peak at the 24-hour mark for Ki67 and the 6-hour mark for VEGFA (Figure 8).



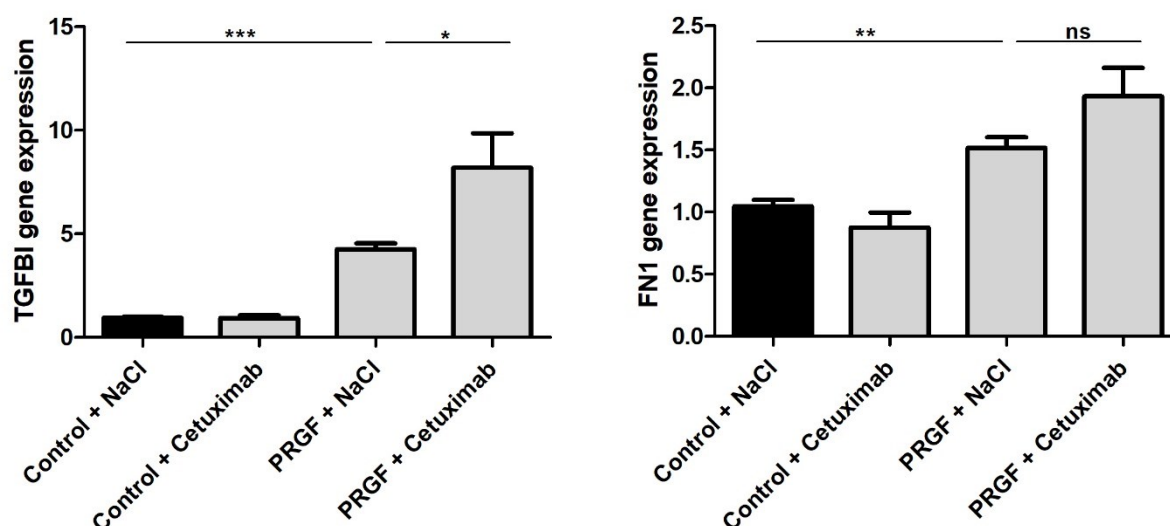


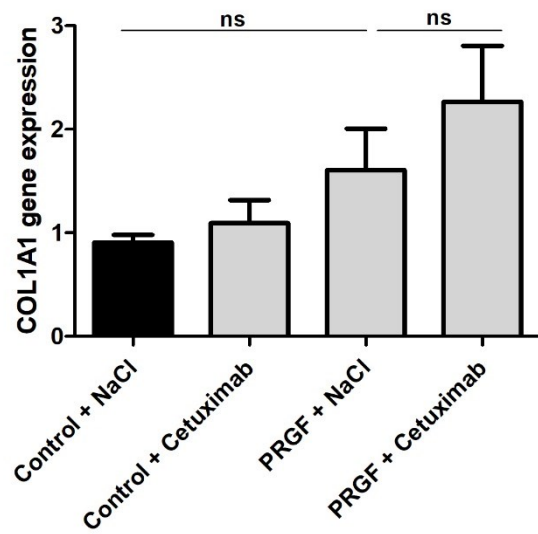
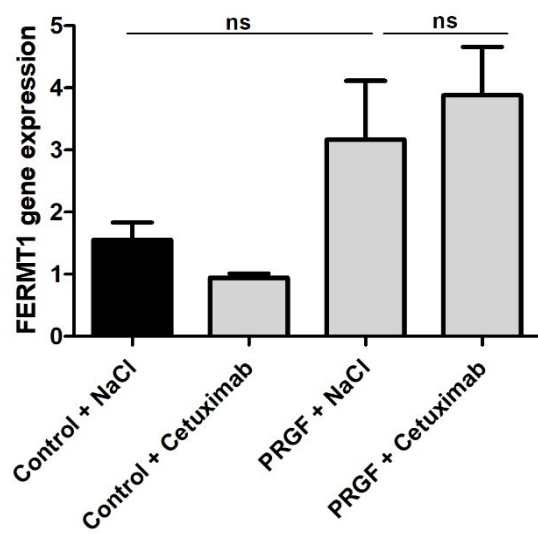
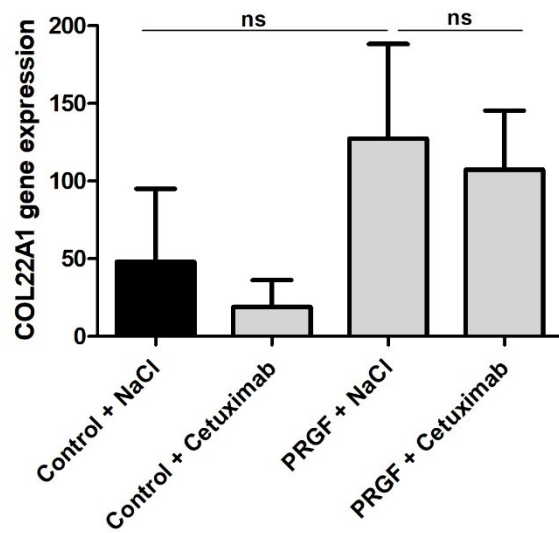
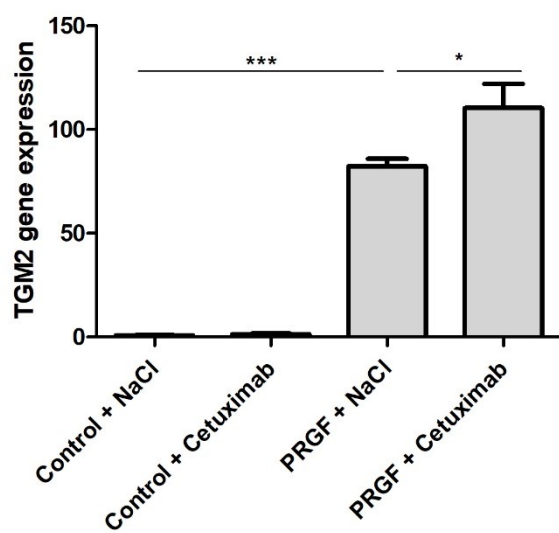
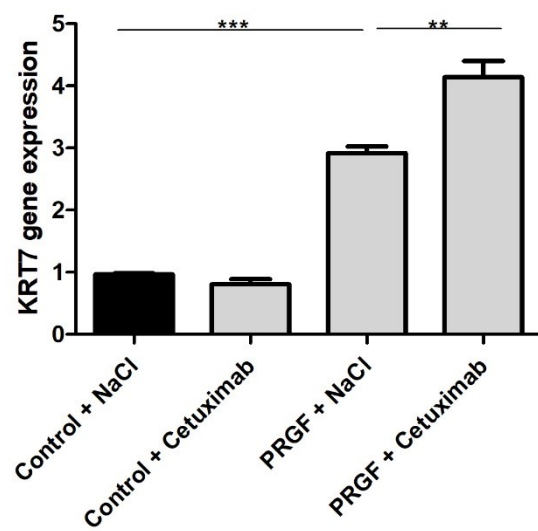
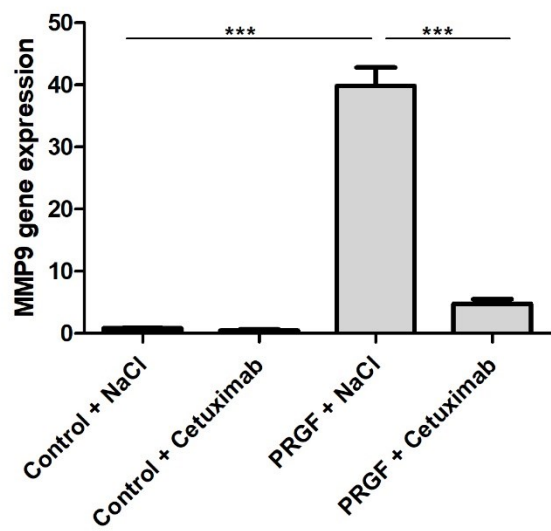


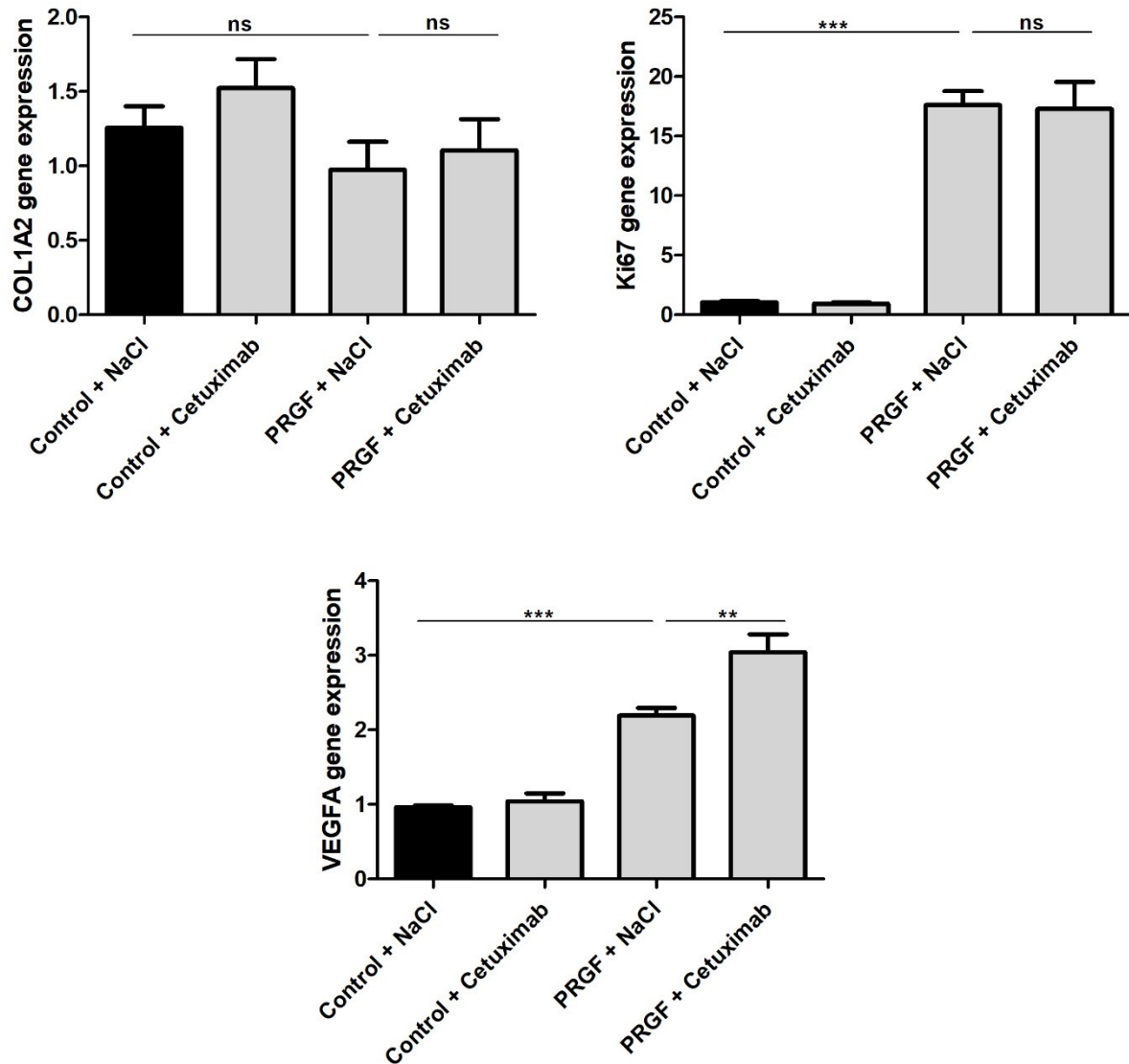
**Figure 8: Time kinetic of the PRGF-mediated induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA in primary fibroblasts.** Fibroblasts were stimulated with two different PRGFs at 1:10 dilution for 4, 12, 24 and 48 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of cumulative data of an experiment with 2 different PRGFs consisting of triplicates (Student's t-test \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ; ns = not significant).

### 3.2.3 Inhibition of Epidermal Growth Factor Receptor (EGFR) suppressed the PRGF-dependent induction of MMP9 but amplified induction of TGFBI, KRT7, TGM1, VEGFA, FN1, FERMT1, COL1A1 and COL1A2 in primary human fibroblasts

To find out whether the PRGF-dependent induction of the genes in primary human fibroblasts was mediated by Epidermal Growth Factor Receptor (EGFR), we stimulated primary human fibroblasts with two different PRGFs at a concentration of 1:10 in the presence of Cetuximab (20 µg/ml), an EGFR-blocking monoclonal antibody, for 24 hours. The results showed that the induction of MMP9 by PRGF were reduced when Cetuximab was present, which indicated that EGFR mediated the PRGF-dependent induction of MMP9 in primary human fibroblasts. On the other hand, the PRGF-mediated induction of TGFBI, KRT7, TGM2 and VEGFA was enhanced by the inhibition of EGFR by Cetuximab. The PRGF-induced expression of FN1, FERMT1, COL1A1 and COL1A2 also seem to be increased by the presence of Cetuximab; however, the increase was not significant. Ki67 expression was not altered by the inhibition of EGFR (Figure 9).



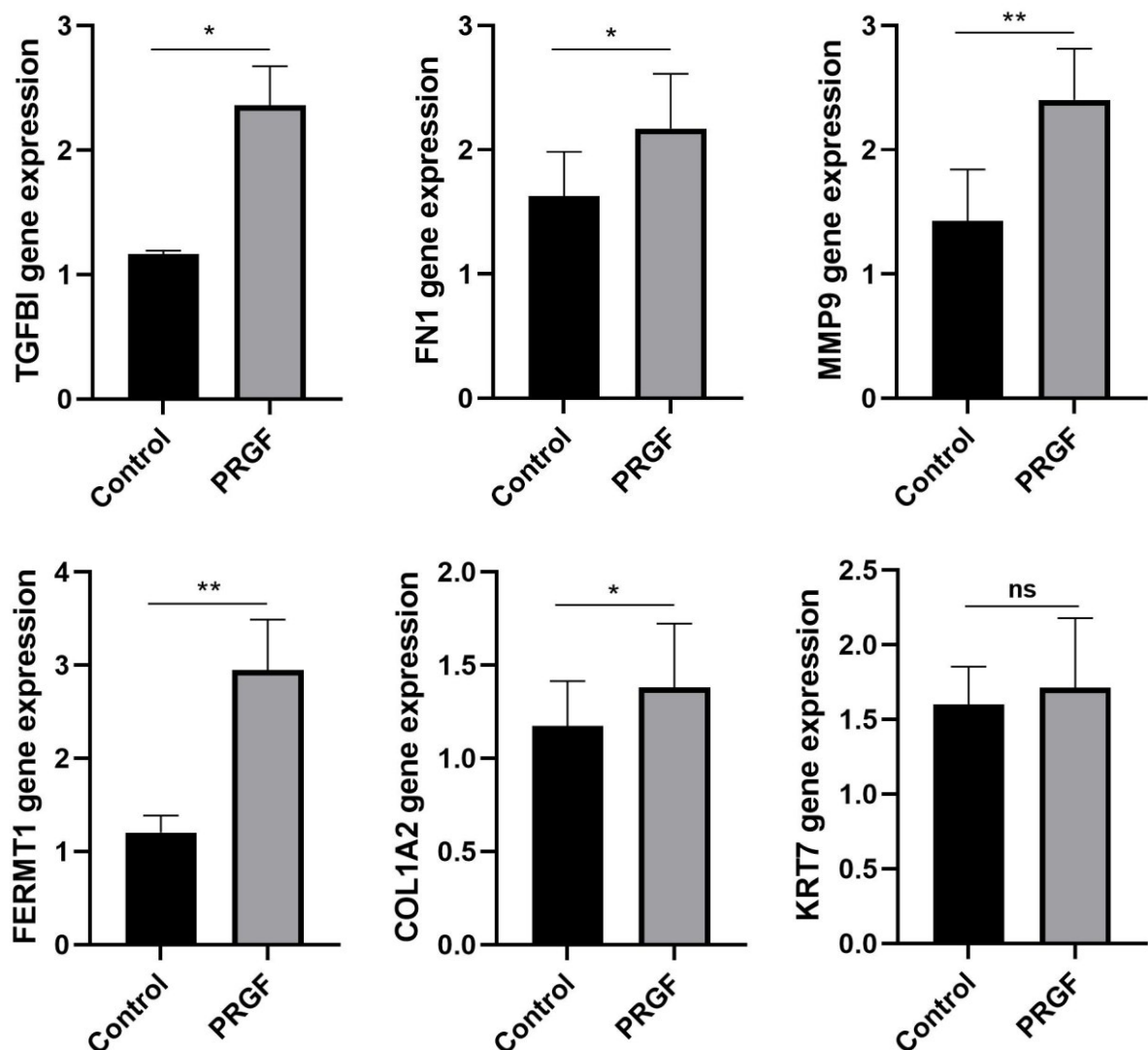


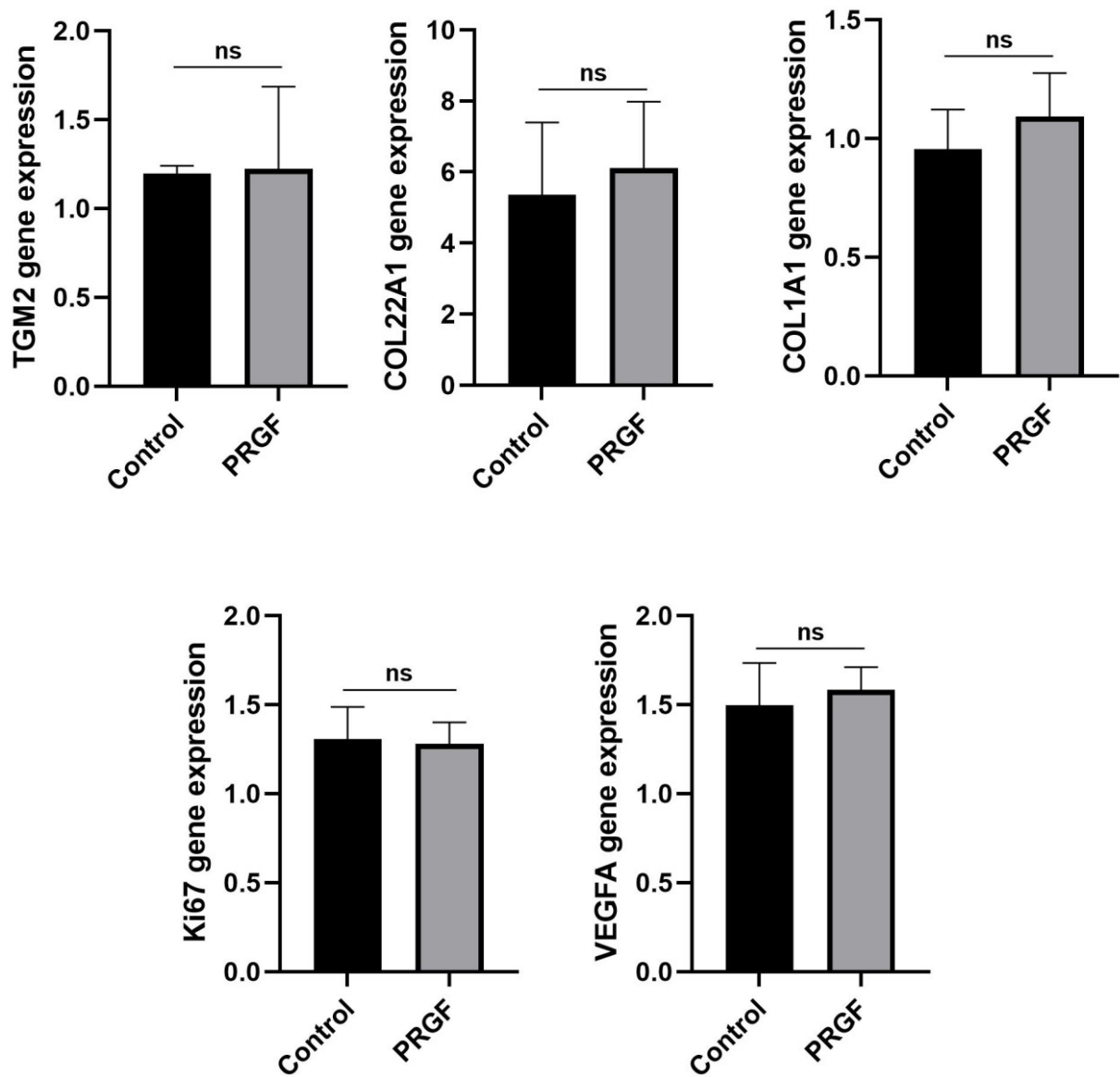


**Figure 9: Effect of Epidermal Growth Factor Receptor (EGFR) inhibition by Cetuximab on the PRGF-dependent induction of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2, Ki67 and VEGFA in primary fibroblasts.** Fibroblasts were stimulated with two different PRGFs at 1:10 dilution and Cetuximab (20 µg/ml) for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means ± SEM of cumulative data of an experiment with 2 different PRGFs consisting of triplicates (Student's t-test \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ; ns = not significant).

### 3.3 Stimulation of *ex vivo* human skin with PRGF lead to an increased expression of TGFBI, FN1, MMP9, FERMT1 and COL1A2

To investigate whether the observed increase of expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1, COL1A2 Ki67 and VEGFA *in vitro* would also be evident in an *ex vivo* setting, we stimulated pieces of healthy skin with PRGF, with a concentration of 1:5 on one occasion and with a concentration of 1:10 in three other separate stimulations. Each experiment consists of quintuplicates, except for one experiment where only 4 control samples were available due to 1 sample being lost during RNA extraction. The stimulation of *ex vivo* skin with PRGF significantly induced TGFBI, FN1, MMP9, FERMT1 and COL1A2. The remaining genes, apart from Ki67, also seemed to be induced, but the results were not significant (Figure 10).



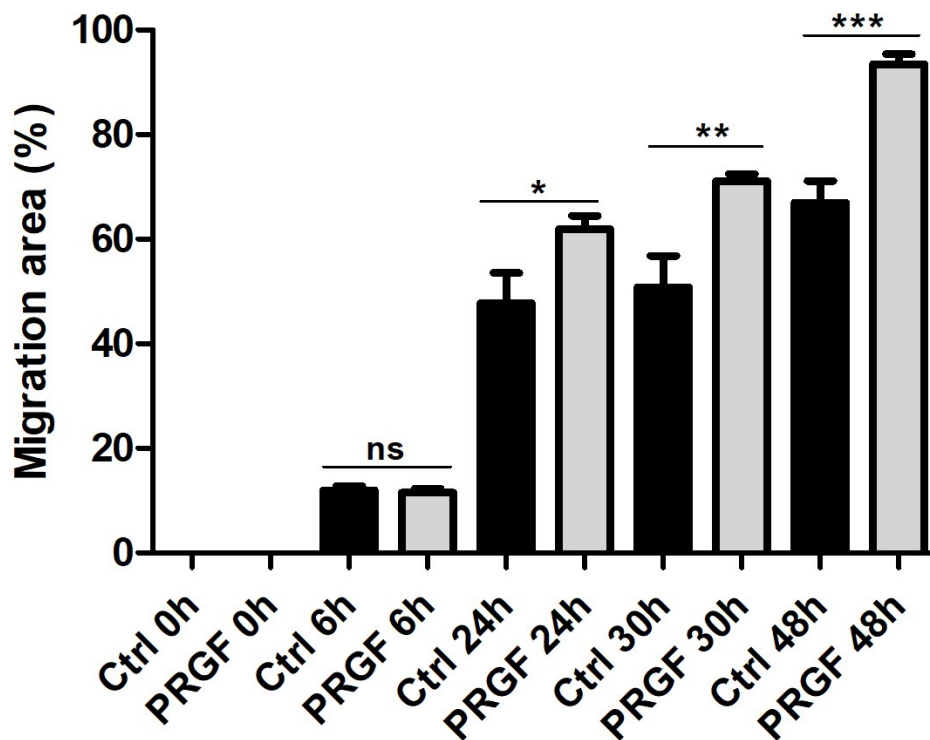


**Figure 10: PRGF induces TGFBI, MMP9, FERMT1 and COL1A2 *ex vivo*.** Healthy skin pieces were stimulated with PRGF in four independent experiments, one with PRGF at 1:5 dilution and three with PRGF at 1:10 dilution for 24 hours. Gene expression was analyzed by real-time PCR. Bars are means  $\pm$  SEM of cumulative data of four experiments mostly consisting of quintuplicates. (Paired student's t test \* $p < 0.05$ ; \*\* $p < 0.001$ ; ns = not significant).

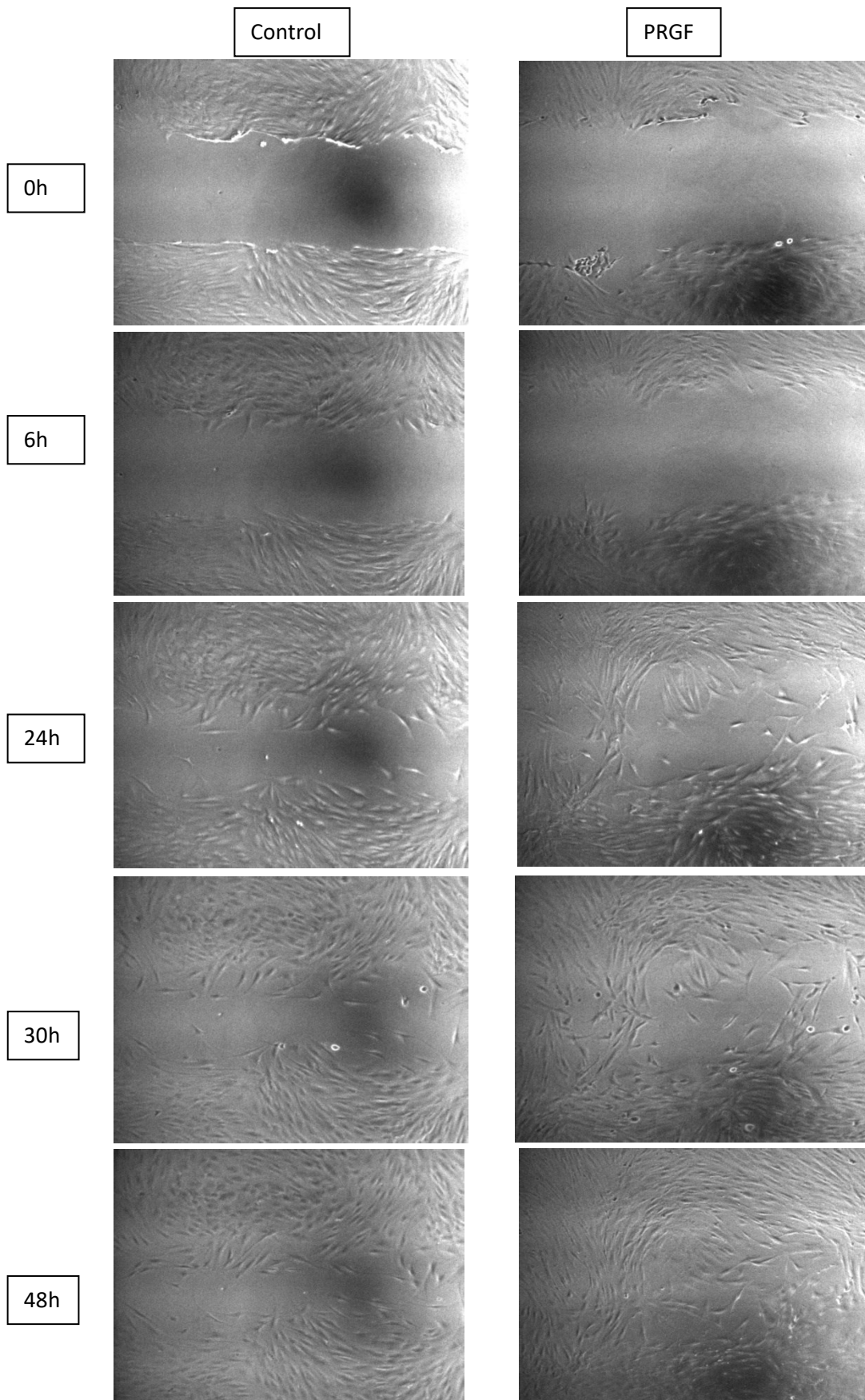


### 3.4 PRGF induced an increased migration rate in fibroblast scratch assay

Since PRGF induced the expression of the proliferation marker Ki67 in primary human fibroblasts (Figure 8), we hypothesized that PRGF-stimulated fibroblasts would be able to close an artificially generated wound in vitro faster than unstimulated fibroblasts. Therefore, we tested this hypothesis using scratch assay. We did two scratch assays on primary human fibroblasts with two different PRGFs. Photos of the fibroblasts were taken at the beginning of stimulation and after 6, 24, 30 and 48 hours (Figure 11). The photos were analyzed to calculate the area of the scratch site filled with fibroblasts after a certain period. The analysis of the photos showed that, corresponding to the observed increase of Ki67, the fibroblasts stimulated with PRGF had a significantly increased rate of migration after 24 hours (Figure 12).



**Figure 11: PRGF induces an increased migration rate in fibroblast scratch assays.** Confluent fibroblast cultures were wounded with a sterile pipette tip and stimulated with PRGF at 1:10 dilution for 48 hours. Pictures were taken at 0, 6, 24, 30 and 48 hours and fibroblast migration in the pictures was analyzed using AxioVision LE 4.2.8.0 software by calculating the area of the scratch site filled with fibroblasts after a certain period, where 0% indicates that no fibroblast has migrated to the scratch site, and 100% means that the scratch site is completely populated with fibroblasts. Bars are means  $\pm$  SEM of an experiment with two different PRGFs consisting of triplicates. (Student's t-test \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns = not significant).



**Figure 12: Comparison between control and PRGF-stimulated fibroblasts in scratch assay experiment.**

## 4 Discussion

Stimulation experiments on primary keratinocytes in this study confirmed that PRGF induces the expression of TGFBI, FN1, MMP9, KRT7, TGM2, COL22A1, FERMT1, COL1A1 and COL1A2 in primary keratinocytes. Further, stimulation experiments on primary fibroblasts showed that PRGF induced Ki67, VEGFA, as well as the aforementioned genes, except for COL1A2, in primary fibroblasts.

### 4.1 Induction of TGFBI expression

The TGFBI gene codes for transforming growth factor-beta-induced protein ig-h3 ( $\beta$ IG-H3), an extracellular matrix protein that is able to bind to other proteins found in the extracellular matrix such as fibronectin, proteoglycans, type I, II and IV collagens. Further,  $\beta$ IG-H3 contains motifs that act as a ligand recognition sequence for several types of integrin, a transmembrane protein. Due to its ability to bind to other extracellular matrix proteins and integrins,  $\beta$ IG-H3 provides a bridge between extracellular matrix components and many types of cells including keratinocytes and fibroblasts [19-21].

Past studies have shown that  $\beta$ IG-H3 plays an important role in cell adhesion and migration by modulating interactions between cells and extracellular matrix proteins, as well as cell-to-cell interactions [19-21]. Therefore, the upregulation of TGFBI gene by PRGF may enhance wound healing by increasing the influence of  $\beta$ IG-H3 on cell adhesion and migration. In particular, one study showed that  $\beta$ IG-H3 promoted the adhesion and spread of dermal fibroblasts *in vitro* [21]. This further supports the hypothesis that  $\beta$ IG-H3 may aid wound healing *in vivo* by promoting fibroblast adhesion and migration.

### 4.2 Induction of FN1 expression

FN1 is a protein coding gene for the glycoprotein fibronectin. FN1 encodes two types of fibronectin. The soluble type, also known as plasma fibronectin, is produced by hepatocytes and plays a role in the early wound healing process by assisting the blood clotting process. The insoluble type, also known as cellular fibronectin, is produced by many cell types, such as fibroblasts and endothelial cells, and contributes to the construction of the extracellular matrix and the regulation of cell adhesion, migration, proliferation and apoptosis during the late wound healing process. Cellular fibronectin builds a three-dimensional fibrillar structure in the extracellular matrix, to which components of the extracellular matrix can be deposited, such as

collagen types 1 and 3, fibrinogen and laminin. This structure is also able to regulate various signaling processes by sequestering growth factors such as TGF- $\beta$  and VEGF [22].

The induction of FN1 in primary keratinocytes and fibroblasts by PRGF indicates that PRGF may aid wound healing by increasing the production of insoluble fibronectin, which supports extracellular matrix structure.

### **4.3 Induction of MMP9 expression**

MMP9 is a gene that codes for the proprotein of the Matrix Metalloproteinase 9 enzyme, which is activated through proteolysis of the propeptide [24-25]. This enzyme plays a role in the proteolysis of extracellular matrix during tissue remodeling by degrading type IV and V collagens, allowing MMP9 to influence cell migration [24]. A study also showed that the expression of MMP9 protein on the surface of murine mammary carcinoma cells stimulated angiogenesis. Furthermore, the same study demonstrated that MMP9 was able to proteolytically cleave TGF- $\beta$ , therefore activating TGF- $\beta$ . Hence, MMP9 may be able to stimulate angiogenesis by activating TGF- $\beta$  [25].

Although MMP9 is needed for the reepithelization process in skin wound healing by allowing keratinocyte migration, an *in vivo* study showed that both a deficiency and an excessive amount of MMP9 enzyme could impair wound healing. The study showed that wounds in diabetic patients had an increased level of MMP9 compared to wounds in non-diabetic patients due to induction of MMP9 by high glucose levels in diabetic patients. The study demonstrated that FOXO1 mediated the observed induction of MMP9, and that a FOXO1 deletion in keratinocytes improved wound healing in diabetic patients, as this prevents overexpression of MMP9 [26].

Therefore, the meaning of MMP9 induction by PRGF in wound healing is unclear, since extremely high MMP9 concentrations could impair some of MMP9 functions in wound healing instead of improving them. There may be other factors induced by PRGF that come into play in real wounds that prevent the increased expression of MMP9 from impairing wound healing, or perhaps the increased expression does not translate into an overly increased secretion of MMP9 in keratinocytes and fibroblasts after all. Since MMP9, like other MMPs, is secreted as a proenzyme, it would also be possible that the secreted MMP9 proenzymes are not matched with an increase in activation through proteolysis so the amount of activated MMP9 after PRGF treatment is not excessive.

#### **4.4 Induction of KRT7 expression**

The protein coded by the KRT7 gene is keratin 7, a type II cytokeratin. It is expressed in various epithelial tissues, including epidermal cells [27]. Our gene ontology analysis revealed that KRT7 plays a role in cornification process, which is the differentiation process of keratinocytes, and therefore a part of the epidermis development.

Studies have also shown that in several cancer cell types, KRT7 takes part in regulating cell migration and adhesion [28]. For example, a study showed that an increased expression of KRT7 correlated to an increased migration of ovarian cancer cells [30]. Even though the observation was made on cancer cells instead of normal human keratinocytes or fibroblasts, it could be inferred that KRT7 might serve a similar function of regulating cell migration and adhesion during extracellular matrix organization.

A particular study on an antioxidant wound dressing that utilizes a laminin-derived twelve-amino-acid-sequence peptide A5G81 as a ligand showed that human epithelial keratinocytes cultured on A5G81-presenting monolayers displayed an increase in KRT7 expression in comparison to keratinocytes cultured on the more commonly used fibronectin-derived arginyl-glycyl-aspartic acid adhesion sequence. Considering that the study also showed that the antioxidant wound dressing significantly accelerated wound healing in wounds of diabetic mice, it seems that an increased level of KRT7 expression is correlated to better wound healing [30].

Therefore, an increased expression of KRT7 could be beneficial for wound healing in two ways. Firstly, it could support the differentiation of keratinocytes in the late wound healing process and therefore accelerate the restoration of the physical barrier of the epidermis. Secondly, it could increase cell migration and adhesion during extracellular matrix organization, thereby accelerating the wound healing process.

#### **4.5 Induction of TGM2 expression**

TGM2 gene codes for a protein known as transglutaminase 2. Transglutaminase 2 assists the cross-linking between various intracellular and extracellular proteins, therefore supporting cell adhesion during tissue remodeling in the wound healing process [31]. Hence, the increase of TGM2 resulted from PRGF stimulation may be able to boost wound healing by supporting cell adhesion.

#### **4.6 Induction of COL22A1 expression**

COL22A1 is a protein-coding gene for the collagen type XXII alpha 1 chain. This collagen acts as a cell adhesion ligand for skin epithelial cells and fibroblasts [32]. Hence, the PRGF-mediated increase of COL22A1 expression could benefit wound healing by providing more collagen type XXII alpha 1 chain as a ligand for cell adhesion.

#### **4.7 Induction of FERMT1 expression**

FERMT1 gene codes for the kindlin-1 protein, which is a focal adhesion protein. It is located within epithelial cells and is able to bind and activate integrin, which can then bind to other extracellular matrix proteins such as fibronectin and laminin. Therefore, it is essential for cell-matrix adhesion. This protein is required for normal cell proliferation and cell migration to the wound site [33,34,35].

A defect in FERMT1 gene could lead to Kindler Syndrome. The clinical manifestations of this syndrome include several skin defects such as blistering, epidermal atrophy and impaired wound healing [33,34].

Considering the functions of FERMT1, the induction of FERMT1 expression due to PRGF stimulation in keratinocytes and fibroblasts could benefit wound healing by assisting the cell migration process.

#### **4.8 Induction of COL1A1 and COL1A2 expression**

COL1A1 and COL1A2 are genes for the production of two type-1 collagens, the alpha-1 (COL1A1) and alpha-2 (COL1A2). Type-1 collagens are the most abundant collagen and are important structural proteins in the extracellular matrix. They can be found in many organs such as ligament, cornea and blood vessels but are functionally most prominent in bone and skin [36].

A study revealed that mice lacking in microRNA-155 (miR-155) had higher expressions of finding in inflammatory zone-1 (FIZZ-1) gene, which caused increased deposition of type-1 collagens. These miR-155<sup>-/-</sup> mice also displayed better wound healing capabilities in comparison to wild type mice. Therefore, an increase in type-1 collagens seem to assist wound healing [37].

Hence, the induction of COL1A1 and COL1A2 in keratinocytes and fibroblasts by PRGF should be beneficial in wound healing, as it indicates that PRGF might be able to increase type-1 collagen levels in wound site.

#### **4.9 Induction of Ki67 expression in fibroblasts**

Ki67 is a cellular proliferation marker [38]. Therefore, induction of Ki67 expression by PRGF in primary fibroblasts indicates that PRGF stimulates fibroblast proliferation. This was supported by the scratch assay, as it showed that PRGF-stimulated fibroblasts had a higher rate of wound closure in vitro. Hence, PRGF seems to increase fibroblast proliferation while decreasing keratinocyte proliferation [16].

#### **4.10 Induction of VEGFA expression in fibroblasts**

The VEGFA gene produces vascular endothelial growth factor A, which plays a role in angiogenesis [39]. Normal, unwounded skin does not express VEGFA, but once the skin is injured, VEGFA expression would be rapidly upregulated [40]. Therefore, VEGFA expression seems to be important for angiogenesis during normal wound healing. The induction of VEGFA in primary fibroblasts indicates that PRGF might promote angiogenesis by stimulating VEGFA production in primary fibroblasts. This would be greatly beneficial for wound healing, especially for chronic wounds of patients with impaired angiogenesis capabilities.

#### **4.11 EGFR as a mediator of PRGF stimulation**

EGFR is an important regulator of various processes in wound healing, such as re-epithelialization and dermal maturation [41]. EGFR signaling is known to mediate the activation of cell migration and proliferation [42], and absence of EGFR has been shown to lead to a delayed cutaneous wound healing [43]. Hence, we hypothesized that PRGF influence on wound healing might be mediated by EGFR.

The expression of some genes was suppressed by the inhibition of EGFR by Cetuximab, while some other genes were induced. This occurred in both keratinocytes and fibroblasts, but to different genes, respectively. In fibroblasts, only the expression of MMP9 was suppressed by the inhibition of EGFR by Cetuximab and in keratinocytes, decreased expression of FN1, MMP9, KRT7, TGM2, COL22A1 and FERMT1 was observed. The genes induced by EGFR-inhibition in fibroblasts were TGFBI, KRT7, TGM2, VEGFA, FN1, FERMT1, COL1A1 and COL1A2, and in keratinocytes the induced genes were TGFBI, COL1A1 and COL1A2.

This suggests that EGFR might partially mediate PRGF effects on factors of extracellular matrix organization, epidermis development and cell adhesion. Therefore, it is possible that EGFR would also mediate PRGF influence on other genes related to extracellular matrix reorganization that are not investigated in this study. Furthermore, the level of EGFR expression in patients during treatment with PRGF might influence the effects of PRGF on wound healing.

#### **4.12 *Ex vivo* stimulations**

Out of all the genes tested in this study, five genes were significantly induced by PRGF in *ex vivo* skin, which are TGFBI, FN1, MMP9, FERMT1 and COL1A2. This indicates that their expressions may not only be induced by PRGF in vitro, but also in the actual wound healing process. Hence, these genes offer strong prospects to be further investigated in an *in vivo* study.

Ki67 expression in *ex vivo* skin was not induced by PRGF, even though it was induced in fibroblasts. This might be due to PRGF suppressive effects on Ki67 expression in keratinocytes [16]. Since the *ex vivo* skin contained both fibroblasts and keratinocytes, it is possible that the induction in fibroblasts and suppression in keratinocytes canceled each other out.

#### **4.13 Time-dependency of gene induction**

Time kinetic experiments in this study showed that while some genes were induced quickly after stimulation with PRGF, it took others more time until noticeable changes became visible. The genes induced rapidly also tend to reach a peak early and began showing a gradually decreasing level of induction as time went by. This implies that when PRGF is being used to treat actual wounds, it would induce the expression of the genes at different times as well, and therefore their effects would unfold at different times. One possible explanation for this phenomenon is that the genes induced quickly were likely directly induced by PRGF, while genes which were induced after a delay might have been activated in a secondary manner via paracrine or autocrine signaling.

Considering that PRGF and other thrombocyte concentrate products have shown that they are able to aid wound healing, it is possible that this variety of peak expression times among the genes is necessary for successful wound healing. For example, if we consider a gene like MMP9, which is necessary for extracellular matrix organization but could also have negative effects on wound healing if it is excessively expressed, it is possible that, since it reaches its highest level of induction at 24 hours and begins falling afterwards, it is able to contribute positively to wound healing within the first 24 hours, and then, once it has fallen, does not affect



the wound healing process in a meaningful way anymore. In this case, the point at which MMP9 would affect wound healing negatively may not be reached during treatment with PRGF. Alternatively, perhaps other factors that could reduce MMP9's effects begin being upregulated by PRGF or secondarily through paracrine or autocrine signaling after 24 hours, so that MMP9 does not excessively affect wound healing once it has finished doing its function.

On the other hand, it is plausible that if induction of certain genes could be extended and some others could be reduced, the efficacy of PRGF for wound healing may be increased through more efficient extracellular matrix organization. It is, however, also possible that this would instead lead to a less effective wound healing or other undesirable effects.

In simpler terms, PRGF may work in a way in which it induces the expression of certain genes involved in extracellular matrix organization only at the right time, so that it would be beneficial for wound healing. Nevertheless, it is possible that prolonging or decreasing the length of upregulation of certain genes would lead to better wound healing effects.

#### **4.14 Conclusions**

In this study, we identified a number of genes induced by PRGF, both in primary keratinocytes and primary fibroblasts in vitro. TGFBI, FN1, FERMT1, COL1A1 and COL1A2 are crucial genes for the organization of the extracellular matrix structure and the induction of these genes by PRGF in primary keratinocytes and fibroblasts shown in this study might be a mechanism in which PRGF is able to enhance wound healing in chronic wounds. MMP9 is also an important gene for the organization of the extracellular matrix, however it is unclear whether the increase of MMP9 expression observed in this study would contribute to the wound healing effects of PRGF, as some studies have shown that, while MMP9 is definitely crucial for wound healing, a too high or low concentration of MMP9 could also impair wound healing. The study additionally shows that PRGF is able to increase levels of TGM2 and COL22A1 expressions, which play important roles in cell adhesion. KRT7 is also induced by PRGF, which stimulates the cornification process and support cell migration and adhesion. Furthermore, induction of Ki67 and VEGFA expression in fibroblasts conveys that PRGF might also promote fibroblast proliferation and angiogenesis. In an *ex vivo* setting, TGFBI, FN1, MMP9, FERMT1 and COL1A2 were further induced by PRGF. Additionally, scratch assay revealed that PRGF is able to increase fibroblast migration in vitro.

Overall, the study has strongly demonstrated that ECM organization may be a factor through which thrombocyte concentrate lysates establish their beneficial wound healing effects, as

PRGF is able to induce ECM-related genes in both keratinocytes and fibroblasts. Further, cell adhesion and the cornification process were increased by PRGF, suggesting that these may be mechanisms supported by thrombocyte concentrate lysates in wound healing. Additionally, angiogenesis, another key process in wound healing, and proliferation of fibroblasts might also be facilitated by PRGF.

The direction for future studies is to further explore other genes that may be influenced by PRGF, given that there are still a number of other genes involved in wound healing not yet investigated in this study. This would provide a clearer picture of how the mechanisms of action of PRGF in promoting wound healing.

## 5 Summary

Platelet-released growth factors (PRGF) is a thrombocyte concentrate lysate. Similar to its clinically equivalent variations (e.g. Vivostat PRF), it is widely known to be able to support the healing of chronic and hard-to-heal wounds. However, the exact mechanisms of action of PRGF are not completely known. Therefore, this study investigated the working mechanisms of PRGF through the identification of genes in keratinocytes and fibroblasts influenced by PRGF.

Whole transcriptome analysis and gene ontology (GO) enrichment of PRGF-stimulated keratinocytes revealed an increased expression of genes involved in extracellular matrix organization. This study particularly focused on transforming growth factor beta-induced (TGFB1), fibronectin 1 (FN1), matrix metalloprotease 9 (MMP9), transglutaminase 2 (TGM2), collagen type XXII alpha 1 chain (COL22A1), fermitin family member 1 (FERMT1), collagen type I alpha 1 chain (COL1A1), collagen type II alpha 2 chain (COL1A2) and Keratin 7 (KRT7). Real-time polymerase chain reaction (PCR) confirmed the in-vitro PRGF-mediated induction of the selected genes in keratinocytes and revealed induction of these genes in fibroblasts. TGFB1, FN1, MMP9, FERMT1 and COL1A2 were further induced by PRGF in an *ex vivo* setting. Additionally, PRGF promotes Ki67 and Vascular endothelial growth factor A (VEGFA) expressions in fibroblasts in vitro, indicating support of angiogenesis and fibroblast proliferation. A scratch assay further shows that PRGF increases fibroblast migration in vitro.

Overall, this study has demonstrated that PRGF influence on extracellular matrix organization in keratinocytes and fibroblasts may be a significant factor of its wound healing capabilities. Additionally, PRGF may support wound healing by promoting cell adhesion, cornification and fibroblast proliferation.

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