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The role of TREM2 protein in skin fibroblast and keratinocyte proliferation and migration

Bachelor's Thesis (12 ECTS)
Curriculum Science and Technology

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Tartu 2021

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Abstract:

Skin is the first physical barrier of the body as it protects the body from external environment and lets the organism to sense pain, touch and temperature. Therefore, it is important to restore the skin as quicky as possible after damage. This research project aimed to investigate whether the protein Triggering receptor expressed on myeloid cells-2 (TREM2) contributes to skin fibroblast and keratinocyte proliferation and migration that could potentially promote wound healing. The expression of TREM2 in human skin was characterized and the effect of soluble recombinant TREM2 protein in cell proliferation and migration were investigated. It was found that TREM2 is expressed in the dermis and in extracellular matrix of dermis of healthy human skin, but it probably inhibits cell proliferation and has no overall effect on the migration of the cells.

Keywords: skin, migration, proliferation, fibroblasts, keratinocytes, TREM2

CERCS: B210 (Histology, cytochemistry, histochemistry, tissue culture)

TREM2 valgu roll naha fibroblastide ja keratinotsüütide proliferatsioonis ja migratsioonis

Lühikokkuvõte:

Nahk on keha esimene füüsiline tõke, kuna see kaitseb keha väliskeskkonna eest ja laseb organismil tunda valu, puudutust ja temperatuuri. Seetõttu on oluline kahjustuste korral nahk taastada võimalikult kiiresti. Käesolev töö uurib, kas TREM2 valk aitab kaasa naha fibroblastide ja keratinotsüütide proliferatsioonile ja migratsioonile kiirendades seejuures haava paranemist. Selleks iseloomustati TREM2 ekspressiooni nahas ning uuriti TREM2 rolli rakkude proliferatsioonis ja migratsioonis. Leiti, et TREM2 ekspresseerub küll dermises ja dermise rakuvälises maatriksis, kuid see arvatavasti pärsib rakkude proliferatsiooni ning sellel ei ole efekti rakkude migratsioonile.

Võtmesõnad: nahk, migratsioon, proliferatsioon, fibroblastid, keratinotsüüdid, TREM2

CERCS: B210 (Histoloogia, tsütokeemia, histokeemia, koekultuurid)

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TERMS, ABBREVIATIONS AND NOTATIONS

BCIP – 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

BSA – bovine serum albumin

DMEM – Dulbecco's modified Eagle medium

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

FBS – fetal bovine serum

ITS – insulin, transferrin and selenium solution

KLC-TREM2 – kappa light chain signal peptide and TREM2 fusion protein

LB – Luria-Bertani broth

mQ – highly purified water

NBT - nitro-blue tetrazolium chloride

NP-40 - nonyl phenoxypolyethoxylethanol

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PEG 40000 - polyethylene glycol 40000

Pen-Strep – penicillin-streptomycin solution

PVDF – polyvinylidene fluoride

SDS – sodium dodecyl sulfate

sTREM2 – soluble TREM2

TBS – Tris-buffered saline

TREM2 – triggering receptor expressed on myeloid cells-2

INTRODUCTION

Skin is the first physical barrier of the body as it protects the body from external environment and lets the organism to sense pain, touch and temperature (Lopez-Ojeda *et al.*, 2020). Skin has 3 layers: epidermis, dermis and hypodermis (Wong *et al.*, 2016). These 3 layers each contribute to skin's resistance to damage. Epidermis protects the body form ultraviolet damage, contributes to mechanical protection, maintains hydration and regulates permeability (Murphey *et al.*, 2020). Dermis contributes most of the mechanical strength of the skin providing its pliability, elasticity and tensile strength (Freinkel and Woodley, 2001). Hypodermis functions as an energy reserve and as a protective padding and support (Scott and Miller, 2003).

Even though skin is resistant, sometimes it can be damaged, which results in wound healing and skin regeneration process. It is important to restore the skin layers as quickly as possible, because skin acts as a protective shield from the external environment and is constantly exposed to possible injuries (Takeo *et al.*, 2015). This is why the present Bachelor's thesis research project investigated whether the protein Triggering receptor expressed on myeloid cells-2 (TREM2) contributed to skin fibroblast and keratinocyte proliferation and migration that could potentially promote wound healing. TREM2 is an extracellular immunomodulatory receptor that has a central role of myeloid cell activation and survival; in addition, it has several other functions, such as cell maturation, proliferation, phagocytosis and the regulation of inflammation (Kober and Brett, 2017). sTREM2 is a soluble form of TREM2 that is found in supernatants of mouse and human cell cultures and in the cerebrospinal fluid and peripheral blood (Li and Zhang, 2018). sTREM2 enhances microglial proliferation, migration and clustering in the vicinity of amyloid plaques (Zhong *et al.*, 2017). To author's knowledge the role of TREM2 protein in skin fibroblast and keratinocyte proliferation and migration has not been researched before.

This BSc project is part of a larger research theme in Dr. Viljar Jaks' laboratory (Institute of Molecular and Cell Biology, University of Tartu) which aims to characterise extracellular matrix proteins that could promote skin regeneration. Hypothesis stated in this research project is that TREM2 has a role in skin fibroblast and keratinocyte proliferation and migration that could potentially promote wound healing.

1 LITERATURE REVIEW

1.1 Skin

Skin is considered as one of the organs of an organism, since it consists of cells and tissues, has a differentiated structure and performs specific functions (Freinkel and Woodley, 2001). Skin has three layers: epidermis, dermis and hypodermis (Wong *et al.*, 2016). Skin's main function is protection of the organism. It is the first physical barrier of the body against the external environment, and it protects against microorganisms, mechanical damage, ultraviolet light and dehydration (Lopez-Ojeda *et al.*, 2020).

Skin allows the organism to sense pain, touch, deep pressure and temperature. There are several skin receptors that give the ability to do that. For example Merkel receptors (associated with Merkel cells located in the basal layer in epidermis) respond to sustained light touch induction over the skin and free nerve endings (located in the epidermis) respond to light touch, pain and temperature variations. (Lopez-Ojeda *et al.*, 2020)

Furthermore, skin's other functions include the endocrine and exocrine activity. Skin initiates the biochemical processes, that are involved in production of hormones, such as vitamin D (Lopez-Ojeda *et al.*, 2020). Skin also secretes sweat, sebum (produced in sebaceous glands) and pheromones and other bioactive substances (for example cytokines) (Lopez-Ojeda *et al.*, 2020; Morganti *et al.*, 2021). The secretion of sweat is important for another function of skin – thermoregulation. This is done by the sweat glands (Figure 1) (Baker, 2019; Lopez-Ojeda *et al.*, 2020).

Hair is also very important as it aids skin in some of its functions such as protection and thermoregulation. It also functions as sensory organ. (Murphey and Agarwal, 2020) Hair bulb is the part that actively produces the hair. It is located in the hair follicle (Figure 1) (Buffoli *et al.*, 2014). Arrector pili muscle connects the hair follicle to basement membrane, it also raises the hair follicles to trap body heat and express emotions (Figure 1) (Fujiwara *et al.*, 2011; Morganti *et al.*, 2021).

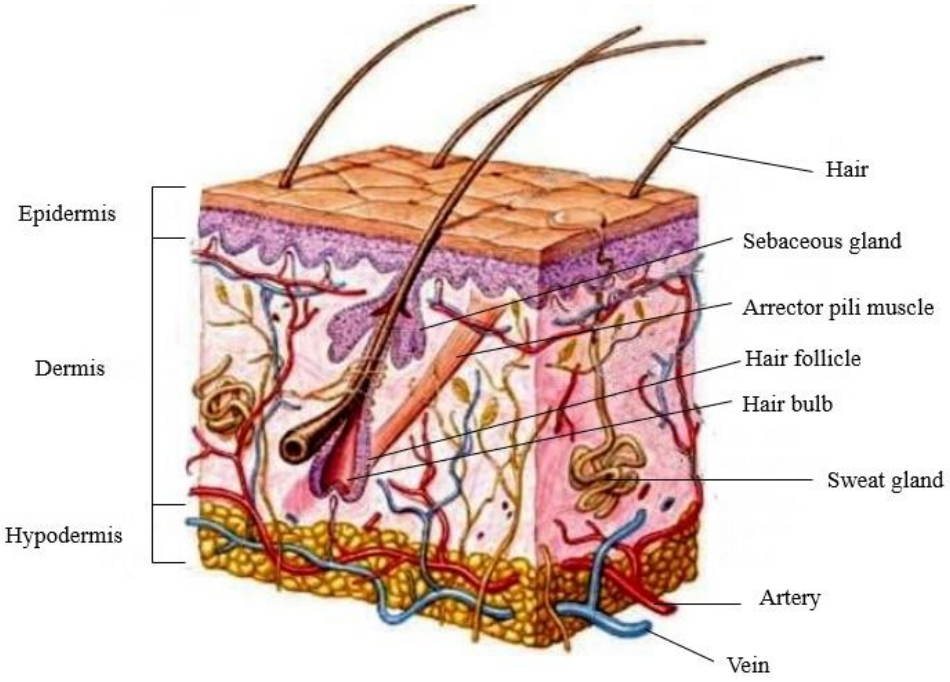

Figure 1. Structure of the skin. Figure has been changed according to MacNeil, 2008.

1.1.1 Epidermis

The outermost layer of the skin is the epidermis (Wong *et al.*, 2016). Epidermis is between 75 and 150 μm thick (on palms and soles up to 600 μm) and consists of four layers: the basal cell layer (*stratum basale* or *stratum germinativum*), spinous layer (*stratum spinosum*), granular layer (*stratum granulosum*) and overlying *stratum corneum* (Freinkel and Woodley, 2001; Wong *et al.*, 2016). In some areas of the body, where the epidermis is very thick, there is a fifth layer called hyalin layer (*stratum lucidum*) (Montagna and Parakkal, 1974). Epidermis consists primarily of keratinocytes. The structure of the cells depends on the position within the epidermis and the state of differentiation. All keratinocytes contain keratin intermediate filaments, that have the diameter of 8-10 nm. Keratins together with the microtubules and microfilaments provide the structural integrity. They are organised into two families – the acidic family, which are also called as type I (K10-K20) and the basic-neutral family, which are called type II (K1-K9). (Freinkel and Woodley, 2001) In addition, keratinocytes express integrins. Integrins (for example ITGB4/α6β4) are receptors that have many functions, such as intercellular adhesion, adhesion to extracellular matrix and regulation of terminal differentiation (Lipscomb and Mercurio, 2005; Watt and Jones, 1993).

The innermost layer of epidermis is the basal layer. This layer consists of keratinocytes and immigrant cells. Keratinocytes have a columnar shape and contain fine bundles of K5 and K14 keratin filaments. These filaments provide a cytoskeleton that is flexible enough to enable cell

division and migration. The basal keratinocytes are functionally and structurally associated with the components underlying the basement membrane zone (BMZ, also known as dermal-epidermal junction) via hemidesmosomes. This contributes to epidermal structural integrity and provides regulatory signals controlling keratinocyte differentiation, proliferation and migration. Basal keratinocytes are divided into three groups based on their clonogenicity[1]: stem cells, transit-amplifying cells and post-mitotic differentiating cells. Somatic skin stem cells, that take up approximately 10% of the basal keratinocytes, are long-lived cells that are slow-cycling and have a short S-phase. Stem cells have high clonogenic potential and divide producing one daughter cell that differentiates and another cell that remains a stem cell. The cell that differentiates is referred to as a transit amplifying cell. Transit amplifying cells are the majority of the basal keratinocytes. These cells will undergo a limited number of augmentation divisions. The last group is the post-mitotic differentiating cells. These cells take up about 5-10% of the basal keratinocytes and express some of the early differentiation markers, such as involucrin and K10. (Freinkel and Woodley, 2001) *Stratum basale* also contains melanocytes. Melanocytes produce melanin, which protects the skin against UV radiation. The other type of cells that are in the basal layer are the Merkel cells. Merkel cells are oval-shaped epidermal cells that serve mainly sensory function. (Yousef *et al.*, 2020)

The next layer is the spinous layer. Spinous layer is 8-10 cells thick (Yousef *et al.*, 2020). Cells in this layer have many focal junctions (desmosomes) between adjacent keratinocytes resulting in formation of so-called spines due to shrinkage artifact due to tissue processing. The overall structure, shape and subcellular properties of spinous cells depend on their exact location within the layer. The cells near the basal layer are polyhedral, while the cells in upper layers are more flattened, larger and contain lamellar granules. Spinous cells contain large bundles of keratin filaments, which are organized concentrically around the nucleus and inserted peripherally into desmosomes. The K5 and K14 keratin filaments are present as they continue following the synthesis in basal cells, but the K1 and K10 keratin filaments are also newly synthesised in spinous cells. (Freinkel and Woodley, 2001) Spinous layer also has Langerhans' cells (dendritic cells). These cells act as the defenders of the skin and play a significant role in antigen presentation. (Yousef *et al.*, 2020)

The granular layer is two to three cells thick (Freinkel and Woodley, 2001). Cells in this layer are flattened, have shrunken nuclei and contain basophilic keratohyalin granules in their cytoplasm. Keratohyalin granules are important in barrier function and keratinization since they

[1] Cell's ability to grow from a single cell into a colony (Franken *et al.*, 2006)

are precursors to the cornified cell envelope. (Scott and Miller, 2003) These granules do not have a membrane and are primarily composed of electron-dense protein, profilaggrin and keratin intermediate filaments (Freinkel and Woodley, 2001; Scott and Miller, 2003). Profilaggrin consists of tandem repeats of filaggrin monomers joined by small linker peptides (Freinkel and Woodley, 2001). Filaggrin serves as a matrix protein that promotes aggregation and disulfide bonding of keratin filaments and is also a source of free amino acids, which are important for normal hydration and barrier function of the granular layer (Freinkel and Woodley, 2001; Scott and Miller, 2003). Loricrin is also found in the granules. Loricrin is a protein of the cornified cell envelope, which means it is involved in the binding of keratin filaments together and anchoring them to the cross-linked envelope. (Scott and Miller, 2003)

As mentioned above, in places where the epidermis is thicker (palms and soles), there is a hyalin layer (Montagna and Parakkal, 1974; Yousef *et al.*, 2020). This is a layer between the *stratum corneum* and the granular layer (Montagna and Parakkal, 1974). The hyalin layer is about two to three cell layers thick and consists of eleidin, which is a transformation product of keratohyalin (Yousef *et al.*, 2020).

The *stratum corneum* is the outermost layer of the skin and of the epidermis. It is composed of layers of corneocytes. Corneocytes are keratinocytes, that are terminally differentiated and do not have nuclei. (Freinkel and Woodley, 2001) Diameter of these cells is about 30-40 μm and thickness is about 0.1-1.0 μm (Wong *et al.*, 2016). Corneocytes are organized to a system also known as brick and mortal model, where protein-rich corneocytes are surrounded by lipid-rich extracellular matrix. The two different components of *stratum corneum* have different functions. Corneocytes protect active cells from ultraviolet damage, contribute to mechanical protection, maintain hydration and regulated cytokine-mediated initiation of inflammation. The lipid-rich extracellular matrix on the other hand regulates permeability, has antimicrobial peptide activity, initiates corneocyte desquamation and excludes toxins, but at the same time allows selective chemical absorption. (Murphrey *et al.*, 2020) The thickness of *stratum corneum* varies extremely depending on the place of the body, for example the thickness is about 15 cells on the upper arm and can be hundreds on the palms and soles. The thickness also varies with age, disease and sex. (Freinkel and Woodley, 2001)

1.1.2 Dermis

Dermis (or corium) is the middle layer of skin layers (Brown and Krishnamurthy, 2020). The thickness of dermis ranges from 1.6mm to 6.1mm, but in some places, such as the tail region in

animals it can be up to 10.5mm (Scott and Miller, 2003). Dermis contributes most of the mechanical strength of the skin providing its pliability, elasticity and tensile strength and it also aids in thermoregulation, binds water and includes receptors of sensory stimuli, it modulates wound healing and the structure and function of the epidermis (Freinkel and Woodley, 2001; Scott and Miller, 2003).

Dermis is divided into two layers: the superficial papillary dermis and the deeper reticular dermis. Papillary dermis is composed of loose connective tissue that is highly vascular, while the reticular dermis forms a thick layer of dense connective tissue. The latter constitutes the bulk of dermis. (Brown and Krishnamurthy, 2020) Papillary dermis is made up of small diameter collagen fibres (mean 38 000nm) interspersed with elastic fibres. It interacts closely with rete ridge projections from the epidermis as well as surrounding hair follicles. The reticular dermis consists predominantly of large diameter collagen fibres (mean 80 000nm). These fibres are organized into large fibre bundles of branching elastic fibres that form a superstructure around the collagen fibres. (Wong *et al.*, 2016)

1.1.2.1 Cells in dermis

Dermis consists of a matrix of loose connective tissue composed of fibrous proteins, which are embedded in an amorphous ground substance (Montagna and Parakkal, 1974). There are also other extracellular components, such as vasculature, nerve endings, hair follicles and glands (Brown and Krishnamurthy, 2020). The dermal matrix consists predominantly of fibroblasts, which have the ability to produce most of the extracellular matrix (ECM) material and collagen, elastic and reticular fibres (Brown and Krishnamurthy, 2020). Among other proteins, fibroblasts express vimentin, which is fibroblast intermediate filament (Robinson-Bennett and Han, 2006). Vimentin contributes to the positioning and function of cellular organelles and provides architectural support for cells (Duarte *et al.*, 2019).

In addition to fibroblasts, there are other cell types that are also often found in the dermis, such as histiocytes, macrophages, melanocytes and extravasated leukocytes (Montagna and Parakkal, 1974). Macrophages are distributed throughout the body (Martinez *et al.*, 2008). Macrophages act as “pathogen sensors” as they are one of the first responders to the threat posed by pathogens (Plowden *et al.*, 2004). Among other proteins, macrophages express CD68. CD68 is exclusively expressed by macrophages, therefore it is the most widely used marker for this cell type (Chistiakov *et al.*, 2017). Macrophages also express ITGB4 (Evans *et al.*, 2019). Vimentin is expressed in macrophages as well (Robinson-Bennett and Han, 2006).

1.1.2.2 Skin extracellular matrix

Collagen is the principal component of dermis. Collagenous fibres account for approximately 90% of all dermal fibres and 80% of the dermal extracellular matrix making it the major dermal constituent (Scott and Miller, 2003). Type I and type III collagen are found in abundance, but type IV and other types have also been identified (Wong *et al.*, 2016). Collagen provides the tensile strength and elasticity of the skin. Collagen is a family of related molecules that have a lot of biological roles, such as morphogenesis, cellular adhesion, cellular migration, tissue repair, chemotaxis and platelet aggregation. (Scott and Miller, 2003)

The ground substance consists of glycosaminoglycans linked in vivo to proteins (proteoglycans). Glycosaminoglycans are fibroblast originated. Proteoglycans and glycosaminoglycans have several functions. They play a vital role in water storage and homeostasis. Although they only account for 0.1% of skin's dry weight, they can bind over 100 times their own weight in water. Proteoglycans and glycosaminoglycans also function in selective screening of substances and in collagen fibrillogenesis, orientation, growth and differentiation. They support dermal structure by resisting compression. The ground substance fills the spaces and surrounds other structures of dermis, but it still allows nutrients, electrocytes and cells to pass through it. (Scott and Miller, 2003)

1.1.3 Hypodermis

The hypodermis (or subcutis) is the deepest and usually the thickest layer of the skin, but there is no hypodermis in some parts of the body, such as cheeks, lips, anus, external ears and eyelids. In these areas the dermis in direct contact with musculature and fascia. (Scott and Miller, 2003) Hypodermis consists mainly of loose connective tissue that forms gliding layers of large pockets of adipose tissue. In addition to adipose cells, there are also fibroblasts and macrophages. (Wong *et al.*, 2016) The subcutis functions as an energy reserve and as a protective padding and support. It is important in thermogenesis and insulation and in maintaining surface contours. It also functions as a steroid reservoir and as the site of steroid metabolism and estrogen production. (Scott and Miller, 2003)

1.1.4 Skin homeostasis

Keratinocyte differentiation (keratinocyte cornification) is a slow coordinated process, that can take about 2 weeks (Lippens *et al.*, 2005). During this time, the basal cell travels from the inner layers of the skin to the skin surface where eventually it is sloughed and replaced by inner cells

continually differentiating outward, which means it occurs at the same time in different suprabasal layers of the epidermis (Fuchs, 1993; Lippens *et al.*, 2005). Structural proteins at the transition from the granular to cornified layer are crosslinked with the keratin filament network. During the cornification, keratinocytes become metabolically inactive and the organelles are degraded. As a result, the dead cells become flattened and consist of more than 80% keratins crosslinked to other cornified envelope proteins. During the terminal differentiation, the plasma membrane fuses with the membrane of the lamellar bodies, that contain lipids that eventually replace the original plasma membrane and play a role in water maintenance. The nuclei disappear completely and no nuclear fragments remain in the cells. Intercellular contacts are still maintained by desmosomes and adheres junctions, that continue to preserve the structural integrity of the keratin cytoskeleton and adhesion complexes for the epidermal structure. (Lippens *et al.*, 2005)

Interfollicular epidermal stem cells, also known as keratinocyte stem cells, are cells that can self-renew and are responsible for long-term maintenance of the tissue. These cells have high proliferative potential and are slow-cycling to conserve cell's proliferative potential and minimize DNA replication-repeated errors. As mentioned in the chapter above, these stem cells usually divide to one transit amplifying cell, which has limited proliferative potential, and one stem cell. (Lavker and Sun, 2000)

Hair follicle is a highly conserved sensory organ that is associated with thermoregulation, sebum production, neurogenesis, angiogenesis, wound healing and also immune response against pathogens. In addition, it contributes to physical protection, camouflage, sensory perception and social interactions. (Ji *et al.*, 2021) The hair follicle begins at the surface of the epidermis and the follicle that produces terminal hairs extends into deep dermis, sometimes even into subcutis, while the one that produces vellus hairs only extends to upper dermis (Martel *et al.*, 2020). Hair follicle morphogenesis and regeneration depend on intensive cooperation of epithelial (epidermal stem cells) and hair-inductive mesenchymal (dermal papilla) components, which is also known as epithelial-mesenchymal interaction. Hair follicle functioning is also affected by hair cycling, which means hair cycling regeneration plays an important role in functional hair follicle regeneration. Hair cycling is also regulated by the interaction between hair follicle stem cells and dermal papilla cells. (Ji *et al.*, 2021)

1.2 Wound healing

Wounds generally heal in 4 to 6 weeks. Wounds that take more time are called chronic wounds. There are many factors that can lead to chronic wounds, such as bacterial colonization, reperfusion injury and altered cellular response. Wound healing is divided into 3 phases: inflammatory phase, proliferative phase and remodelling phase. (Wallace *et al.*, 2019)

The inflammatory phase begins with hemostasis and inflammation (Kangal and Regan, 2020). An injury to the skin initiates an outpouring of lymphatic fluid and blood (Wallace *et al.*, 2019). During this the adequate hemostasis is achieved by launching clotting cascades that provide a temporary fibrin blood clot plug (Figure 2). The same fibrin plug forms a temporary matrix that serves as a scaffold structure for future healing processes and serves as a growth factor resource. (Kangal and Regan, 2020) Inflammatory cells, such as neutrophils, monocytes and endothelial cells adhere to the fibrin scaffold and within the first 24 hours neutrophils are recruited to decontaminate the wound and they stay there for 2 to 5 days (Figure 2) (Kangal and Regan, 2020; Wallace *et al.*, 2019). Macrophages arrive approximately 3 days after the initial injury (Figure 2). They release growth factors, chemokines and cytokines, which promote cell proliferation and synthesis of extracellular matrix. (Kangal and Regan, 2020)

The proliferative phase (granulation phase) is characterized by granulation tissue formation and vascular network restoration (Wallace *et al.*, 2019). This phase does not occur at a distinct time but is occurring all the time in the background, but it is still found to start approximately 3 to 10 days after the injury (Kangal and Regan, 2020; Wallace *et al.*, 2019). By the days 5 through 7, fibroblasts have started to lay down new collagen and glycosaminoglycans, which form the core of the wound and help to stabilize the wound (Wallace *et al.*, 2019). Epithelization begins after wounding which is stimulated by inflammatory cytokines and different growth factors. Stem cells in the bulbs of hair follicles differentiate into keratinocytes and these keratinocytes migrate across the wound from the edge until they reach into physical contact with each other. The contact inhibition ends the migration. After that the granulation tissue is formed, when fibroblasts migrate to the wound site and proliferate within the wound. In addition new vessels are formed by angiogenesis and vasculogenesis (Figure 2). (Kangal and Regan, 2020)

The remodelling (maturational) phase starts around week 3 and it can last up to 12 months. In this stage the synthesis and degradation of the new tissue needs to be strictly preserved. Any disruption in that ends up in chronic wound formation. In the remodelling phase the maturation of the wound begins as the granulation tissue formation ends. Extracellular matrix components

are exposed to some certain modifications to form a stronger and organized ECM. In addition, wound contraction beings. Fibroblasts differentiate into myofibroblasts, that contract bringing together the wound edges and enabling wound closure. After full epithelization of the wound, myofibroblasts undergo apoptosis. Finally, angiogenic responses cease and the blood blow deceases. Acute metabolic activity in the wound ends and the wound healing ends up in scar formation. The tensile strength of the skin gradually increases. (Kangal and Regan, 2020) The maximal tensile strength is reached about 11 to 14 weeks after the injury (Wallace *et al.*, 2019). The resulting scar will never have 100% of the original strength, it will stay around 80%, because the collagen in the scar will never be as organized as in the healthy skin (Kangal and Regan, 2020; Wallace *et al.*, 2019).

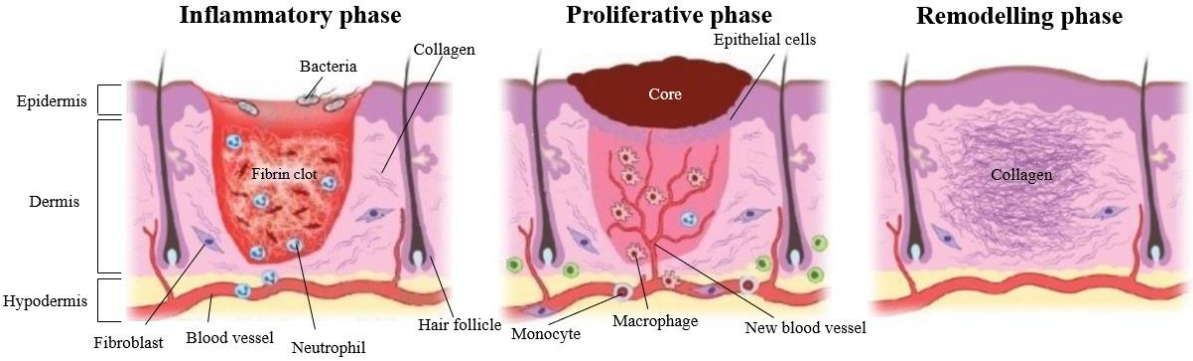

Figure 2. Phases of wound healing. Figure is changed according to Houschyar *et al.*, 2015.

1.3 Overview of TREM2

The protein Triggering receptor expressed on myeloid cells-2 (TREM2) is an extracellular immunomodulatory receptor that is part of the TREM family of receptors (Kober and Brett, 2017; Li and Zhang, 2018). These receptors are primarily expressed in myelogenous cells, such as dendritic cells, monocytes and microglia (Li and Zhang, 2018). TREM2 itself has a central role of myeloid cell activation and survival, but it also has several other functions, such as cell maturation, proliferation, phagocytosis and the regulation of inflammation (Kober and Brett, 2017). *TREM2* gene is located on human chromosome 6 and its total length is 4676 base pairs. TREM2 has 5 exons that can encode a protein with 230 amino acids (Figure 3). (Li and Zhang, 2018) TREM2 consists of an extracellular V-type immunoglobulin domain that is followed by short stalk and then by single transmembrane helix (Figure 3) which interacts with DNAX-activation protein 12 (DAP12). The receptor terminates with a short cytosolic tail (Figure 3), that does not have any known signalling or trafficking motifs. (Kober and Brett, 2017)

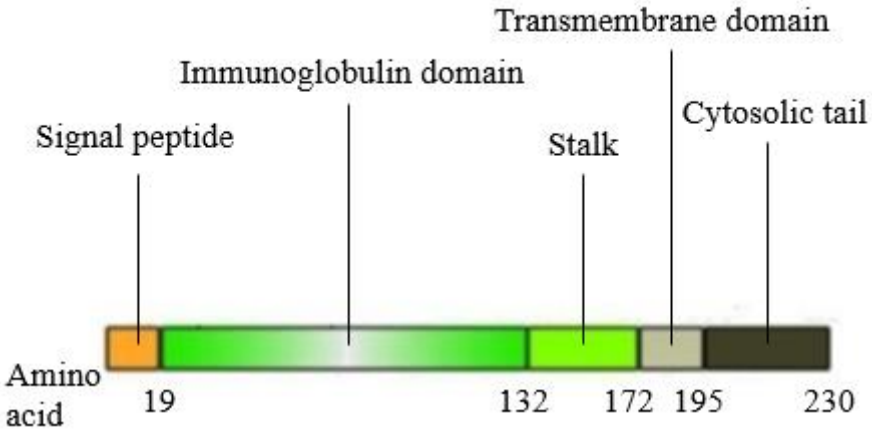

Figure 3. TREM2 domains. Figure changed according to Kober and Brett, 2017.

Soluble TREM2 (sTREM2) can be generated by proteolytic cleavage by ADAM proteases and it occurs within the protein stalk (Kober and Brett, 2017; Zhong *et al.*, 2017). Soluble TREM2 can also be generated by alternative splicing (Deczkowska *et al.*, 2020; Kober and Brett, 2017). After the cleavage or splicing the sTREM2 is secreted into the extracellular matrix (Walter, 2016). sTREM2 has been found in supernatants of mouse and human cell cultures and in the cerebrospinal fluid and peripheral blood (Li and Zhang, 2018). sTREM2 has been shown to promote myeloid cell survival under stress (Deczkowska *et al.*, 2020). In particular, sTREM2 enhances microglial proliferation, migration and clustering in the vicinity of amyloid plaques (Zhong *et al.*, 2019).

TREM2 activates signalling pathways that are involved in cell activation and differentiation, cell survival and in control of the actin cytoskeleton. The main TREM2-associated signalling pathway is the TREM2/DAP12 pathway. (Paradowska-Gorycka and Jurkowska, 2013) The cytoplasmic domain of DAP12 contain an immunoreceptor tyrosine-based activation motif (ITAM), that starts the recruitment and activation of downstream effector molecules. TREM2 and DAP12 molecular complex has significant cross talk with other signalling pathways as well, such as growth factor receptors and integrins. In addition, TREM2/DAP12 complex inhibits low-level Toll-like receptor ligand-induced cytokine responses in myeloid cells, that results in reduced pro-inflammatory cytokines and inflammation. (Xing *et al.*, 2015) TREM2 has also been associated with Wnt/β-catenin signalling pathway. Deficiency of TREM2 leads to dramatic downregulation of the signalling resulting in decreased microglial survival and enhanced cell death. This means that TREM2 regulates the survival and proliferation of microglia by activating this pathway. (Zheng *et al.*, 2017)

Variants of TREM2 (nonsense mutations, singe amino acid substitutions and frameshift) have been found to be associated with the risk of Alzheimer's disease and other neurodegenerative diseases (Jay *et al.*, 2017). It has been also found that genetic mutations that inactivate TREM2 or DAP12 lead to Nasu-Hakola disease with brain demyelination and cystic-like lesions of the bone that lead to presenile dementia and fractures (Zheng *et al.*, 2017). Elevated levels of sTREM2 have been found in the cerebrospinal fluid of patients with various neurological conditions, such as conditions described above. In addition, TREM2 has an important role in promoting an immune-suppressive tumour microenvironment in cancer. Studies have suggested that TREM2 has a role in tumour-associated macrophages as TREM2 levels in macrophages have a positive correlation with tumour progression. TREM2 mRNA and protein expression levels have also been shown to be higher in gastric tumour samples compared to normal gastric tissues. In addition, it has been reported that TREM2+ myeloid cells have a more potent inhibitory effect on the proliferation of T cells *in vitro*. (Deczkowska *et al.*, 2020) Additionally, it has been found that TREM2 enhances cell proliferation and invasion of glioma (X. Q. Wang *et al.*, 2016).

Even though TREM2 has associations with different diseases, researchers have found that efficient colonic mucosal wound repair requires TREM2 signalling (Seno *et al.*, 2009). Additionally, TREM2 has been found to be related to microglial migration and survival (McQuade *et al.*, 2020). Although the role of TREM2 in skin has not been researched much, it has been described that TREM2+ macrophages inhibit proliferation of hair follicle stem cells and therefore have an important role in controlling the hair growth and (E. C. E. Wang *et al.*, 2019).

2 THE AIMS OF THE THESIS

The main aim of present thesis was to examine the role of TREM2 protein in fibroblast and keratinocyte proliferation and migration. The stated hypothesis was that TREM2 has a role in skin fibroblast and keratinocyte proliferation and migration that could potentially promote wound healing. Therefore, to confirm the hypothesis, the following subaims were outlined:

- characterise the expression of TREM2 in human skin,
- investigate the role of soluble TREM2 protein in cell proliferation,
- investigate the role of soluble TREM2 protein in cell migration.

3 EXPERIMENTAL PART

3.1 Materials and Methods

3.1.1 Bacterial transformation and plasmid purification

To clone the DNA bacterial transformation was done by using *Escherichia coli* DH5α competent cells. Plasmid was purified from *E.coli* to isolate the plasmid DNA. Purification was performed by using NucleoBond Xtra Maxi Plus EF Kit (Macherey-Nagel).

On the first day

DH5α competent cells were melted on ice for 20-30 minutes and were then mixed with 100 ng replicative plasmid DNA containing KLC-TREM2 (kappa light chain signal peptide and TREM2 fusion protein) (Table 1). The suspension was incubated on ice for 15 minutes and then incubated at 42°C for 2 minutes. Bacterial cells were then cooled down on the ice again for 2 minutes. 1 ml of Luria-Bertani broth (LB) media was added to the cells and they were grown for at 37°C on a shaker for 1 hour for the ampicillin resistance gene to express. The cells were centrifuged 6000 rpm for 1 minute. Most of supernatant was discarded, approximately 100 µl was left in the tube. The bacteria were resuspended in the leftover supernatant and streaked on a previously warmed up Petri dish (Ø = 10 cm) of LB agar containing ampicillin (100 µg/ml) and incubated at 37°C for 14-16 hours.

Table 1. Original TREM2 sequence and KLC-TREM2 sequence. The sequence not used from the original TREM2 is shown with green. KLC sequence is shown with yellow. The start and stop codons in the KLC-TREM2 sequence are shown with red.

Original TREM2 sequence	KLC-TREM2 sequence
ACTCTGCTTCTGCCCTTGGCTGGGGA	cccAAGCTTatggacatgagggtccctgctcagctcct
AGGGTGGCATGGAGCCTCTCCGGCTG	ggggctcctgctgctctggctctcaggtgccagatgtGAG
CTCATCTTACTCTTTGTCACAGAGCTG	CCTCTCCGGCTGCTCATCTTACTCTTT
TCCGGAGCCCACAACACCACAGTGTT	GTCACAGAGCTGTCCGGAGCCCACAA
CCAGGGCGTGGCGGGCCAGTCCCTGC	CACCACAGTGTTCCAGGGCGTGGCGG
AGGTGTCTTGCCCCTATGACTCCATG	GCCAGTCCCTGCAGGTGTCTTGCCCC
AAGCACTGGGGGAGGCGCAAGGCCT	TATGACTCCATGAAGCACTGGGGGAG
GGTGCCGCCAGCTGGGAGAGAAGGG	GCGCAAGGCCTGGTGCCGCCAGCTGG
CCCATGCCAGCGTGTGGTCAGCACGC	GAGAGAAGGGCCCATGCCAGCGTGT
ACAACTTGTGGCTGCTGTCCTTCCTG	GGTCAGCACGCACAACTTGTGGCTGC
AGGAGGTGGAATGGGAGCACAGCCA	TGTCCTTCCTGAGGAGGTGGAATGGG
TCACAGACGATACCCTGGGTGGCACT	AGCACAGCCATCACAGACGATACCCT
CTCACCATTACGCTGCGGAATCTACA	GGGTGGCACTCTCACCATTACGCTGC
ACCCCATGATGCGGGTCTCTACCAGT	GGAATCTACAACCCCATGATGCGGGT
GCCAGAGCCTCCATGGCAGTGAGGCT	CTCTACCAGTGCCAGAGCCTCCATGG

GACACCCTCAGGAAGGTCCTGGTGGA GGTGCTGGCAGACCCCCTGGATCACC GGGATGCTGGAGATCTCTGGTTCCCC GGGGAGTCTGAGAGCTTCGAGGATG CCCATGTGGAGCACAGCATCTCCAGG AGCCTCTTGGAAGGAGAAATCCCCTT CCCACCCACTTCCATCCTTCTCCTCCT GGCCTGCATCTTTCTCATCAAGATTCT AGCAGCCAGCGCCCTCTGGGCTGCAG CCTGGCATGGACAGAAGCCAGGGAC ACATCCACCCAGTGAACTGGACTGTG GCCATGACCCAGGGTATCAGCTCCAA ACTCTGCCAGGGCTGAGAGACACGTG AAGGAAGATGATGGGAGGAAAAGCC CAGGAGAAGTCCCACCAGGGACCAG CCCAGCCTGCATACTTGCCACTTGGC CACCAGGACTCCTTGTTCTGCTCTGG CAAGAGACTACTCTGCCTGAACACTG CTTCTCCTGGACCCTGGAAGCAGGGA CTGGTTGAGGGAGTGGGGAGGTGGT AAGAACACCTGACAACTTCTGAATAT TGGACATTTTAAACACTTACAAATAA ATCCAAGACTGTCATATTTAGCTGGA	CAGTGAGGCTGACACCCTCAGGAAG GTCCTGGTGGAGGTGCTGGCAGACCC CCTGGATCACCGGGATGCTGGAGATC TCTGGTTCCCCGGGGAGTCTGAGAGC TTCGAGGATGCCCATGTGGAGCACAG CATCTCCAGGAGCCTCTTGGAAGGAG AAATCCCCTTCCCACCCACTTCtgaCTC GAGccg

On the second day

A colony was chosen from the Petri dish and it was suspended in 50 ml LB medium containing ampicillin (0,5 µl/50 ml). The suspension was incubated overnight at 37°C on a shaker.

On the third day

DNA was purified using NucleoBond Xtra Maxi Plus EF Kit. Suspension was centrifuged 5100 rpm for 5 minutes. Supernatant was discarded and pellet was resuspended in 4 ml Resuspension Buffer RES-EF containing RNase A. 4 ml of Lysis Buffer LYS-EF was added to the suspension and it was mixed by turning the tube slowly 5 times. The mixture was incubated at room temperature for 5 minutes. The suspension was neutralised by adding 4 ml of Neutralization Buffer NEU-EF, it was mixed by turning the tube a few times again until the suspension turned colourless. The suspension was incubated on ice for 5 minutes. Purification filter was moistened with 15 ml of Equilibration Buffer EQU-EF and after the buffer had flown through, suspension was poured into the filter. Filter was let to flow through again. The filter was washed with 5 ml of FilterWash Buffer FIL-EF and the filter and the tube around the filter was let to flow through again. After this step filter was discarded. The column inside the tube was washed with 35 ml of Wash Buffer ENDO-EF and the tube was let to flow through. The column was washed again

with Wash Buffer WASH-EF and was let to flow completely through once again. The column was moved to 15 ml centrifuge tube and 5 ml of Elution Buffer ELU-EF was added, to get the plasmid out of the small filter in the tube. 3,5 ml of isopropanol was added to precipitate the DNA from the outflow. Suspension was incubated at -20°C for 30 minutes and then centrifuged 5100 rpm for 15 minutes at 4°C. The supernatant was discarded and 2 ml of endotoxin free 70% ethanol was added to the DNA pellet. The tube was centrifuged again 5100 rpm for 5 minutes at 4°C. The supernatant was discarded and the DNA pellet was left to dry. The DNA pellet was suspended in 100 µl of Buffer TE-EF and the suspension was moved to 1,5 ml Eppendorf tube.

3.1.2 Polymerase chain reaction and gel electrophoresis

The concentration of DNA was measured with NanoDrop and DNA was diluted to 1000 ng/µl. Polymerase chain reaction (PCR) was used to control correct transformation. 10 µl per one sample PCR mixture contained Green Buffer (Thermo Fisher Scientific, Lithuania), pcDNA primers (forward primer 5´ CAACGGGACTTTCCAAA 3´; reverse primer 5´ GCAAACAACAGATGGCTG 3´) and mQ water. Two suspensions were mixed for both DNA samples (control (pcDNA) or TREM2). After mixing 1 µl of DNA (1000 ng/µl) was added tubes. The PCR was conducted with a thermal cycler (Applied Biosystems, No. 2720). The program was set up as following: 94°C for 30 s for initial denaturation, 94°C for 10 s for denaturation, 61°C for 30 s for annealing of the primers, 72°C for 1 minute for extension of primers. Program ended with final extension step at 72°C for 10 minutes. Middle steps of the program (denaturation, annealing and extension steps) were repeated for 26 times to amplify sufficient amount of DNA.

While the PCR was running, the agarose gel for the gel electrophoresis was done. The gel used contained 1% of agarose (Lonza, USA), so the final recipe contained 50 ml of 1x TAE (50 mM Tris-acetate, 1 mM EDTA, pH=8,2) buffer, 0,5 g of agarose and 2,5 µl of ethidium bromide.

When the PCR was done and the gel had set, the samples were mixed with loading dye (0,5 µl) and loaded into the gel. A GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Lithuania) was added to the first line of the gel to later determine the length of the DNA product. The electrophoresis was performed at 100 V for 25 minutes in 1x TAE buffer. After that, the gel was visualized under UV light to assess the presence of the DNA products and the size of them.

3.1.3 Cell lines, cultures and their cultivation

Keratinocytes have some limitations for using them in a scientific research. Keratinocytes require expensive supplementary growth factors for surviving and proliferating *in vitro* and once they start to differentiate, they tend to die rapidly. That is why HaCaT cells have been used as a replacement to keratinocytes. HaCaT cell line is an immortalised nontumorigenic monoclonal human keratinocyte line, that has largely similar properties to keratinocytes. For example, HaCaT cells express all the major surface markers and functional activities as keratinocytes. (Colombo *et al.*, 2017)

The following cell lines and cultures were used in this research project. HaCaT (immortalized human keratinocytes) cell line was a kind gift from Prof. Reet Kurg (Institute of Technology, University of Tartu). HEK293 (human embryonic kidney) cells were obtained from the American Type Culture Collection; human primary fibroblast culture was previously established in the laboratory by Dr. Klaas by explant culture method. Briefly, skin from healthy donors (collected from breast reduction surgeries, Tartu University Clinics; ethics permit 292/T-4) was cut into small pieces and adhered to Petri dishes. Skin pieces were covered in medium, that contained 89% DMEM with phenol red (Life Technologies Corporation, UK), 10% FBS (Life Technologies Corporation, Brazil) and 1% penicillin-streptomycin (Pen-Strep) solution (Life Technologies Corporation, USA). Cells were allowed to migrate from dermis for 10-14 days. Cells were passaged and stored as frozen stocks for further experiments. Before any experiments were done, cells were cultivated in a medium, that contained 89% DMEM with phenol red, 10% FBS and 1% Pen-Strep solution (medium A). Concentration of antibiotics in all the media used was penicillin 100 U/ml and streptomycin 100 ng/ml. The cells were cultivated in an incubator at the temperature 37°C and at the CO_2 level of 5%.

3.1.4 Cell transfection

<u>On the first day</u>

HEK293 cells were grown in the incubator at 5% CO_2 and at 37°C. Cells were washed with 5 ml PBS and then solution containing 0,05% trypsin, 0,02% EDTA (Life Technologies Corporation, Canada) was added. Cells were incubated at 37°C and 5% for 5 minutes and then suspended and mixed with 15 ml of medium A. Suspension was then centrifuged for 5 minutes at 300g at room temperature. Supernatant was discarded and cells were resuspended in 5 ml of medium A. The cells were then inoculated with the density of $2{,}2 \times 10^6$ cells per one 10 cm Petri dish in 10 ml of medium. Cells were grown overnight in the incubator at 37°C and 5% CO_2.

On the second day

Cells were transfected using TurboFect transfection reagent (Thermo Fisher Scientific, Lithuania). Two different media were used: one medium was the medium A, the other medium consisted of same ingredients as medium A, but DMEM was not coloured with phenol red (89% DMEM without phenol red, 10% FBS and 1% Pen-Strep solution). 1 ml of media was mixed with 10 ng of plasmid DNA (control (pcDNA) or recombinant soluble TREM2) and 16,6 µl of TurboFect transfection reagent. The suspension was incubated at room temperature for 15 minutes. Meanwhile medium on the cells was changed. There were two types of new media. One medium consisted of 98% DMEM with phenol red, 1% 100x insulin, transferrin and selenium (ITS) suspension (Life Technologies Corporation, USA) and 1% Pen-Strep suspension. The other medium consisted of same ingredients, but the DMEM was not coloured, so it consisted of 98% DMEM without phenol red (Life Technologies Corporation, USA), 1% 100x ITS suspension and 1% Pen-Strep suspension. After incubation, the transfection suspension was added to the cells and they were incubated for 24 hours at 37°C and 5% CO_2.

On the third day

Additional 100 µl of 100x ITS suspension was added to every dish and the cells were incubated for 24 hours at 37°C and 5% CO_2.

On the fourth day

Coloured conditioned medium (with phenol red) was collected to 15 ml tubes and was stored at 4°C until further usage. Uncoloured conditioned media (without phenol red) were also collected to 15 ml tubes and 100 µl of 100x protease inhibitor (Thermo Fisher Scientific, USA) and sodium aside (final concentration 0,04%) was added. The media were stored at 4°C until further usage.

3.1.5 Western blot

3.1.5.1 Preparation of cell samples for Western blot analysis

The transfected cells were washed with 5 ml PBS. After that the cells were typsinized with solution containing 0,05% of trypsin and 0,02% EDTA. Cells were suspended and transferred to 15 ml of medium A. The suspension was centrifuged at 1200 rpm for 5 minutes at 4°C. Supernatant was discarded, and the cells were suspended in 1 ml of PBS. Cells were centrifuged again at 5000 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet was

suspended in 120 µl of lysis buffer (150 mM NaCl, 50 mM TRIS pH=8,0, 1% NP-40), where 1% (1,2 µl) of 100x protease inhibitors (Thermo Fisher Scientific, USA) were added. The cells were lysed on the ice for 1 hour. After that the lysate was centrifuged at 12000 rpm for 15 minutes at 4°C. Supernatant was moved into a new tube. 12 µl of lysate was used to measure the concentration of protein using the Bradford method. For that 12 µl of lysate was mixed with 100 µl of WR solution made according to manufacturer's manual (Thermo Fisher Scientific, USA). The leftover lysates were frozen at -20°C until further usage.

3.1.5.2 Preparation of recombinant TREM2-conditioned media samples for Western blot analysis

To concentrate the medium for Western blot the uncoloured conditioned media was pipetted into dialysis bags (Spectrum Medical Industries, USA) and the bags were laid over polyethylene glycol 40000 (PEG40000) (SERVA, Germany) until the volume of the solution is approximately 300 µl. The bags were then cleaned of PEG40000 using mQ water and the suspension left in the bags were pipetted into Eppendorf tubes. The samples were diluted 5 times with acetone (StanChem, Poland) that had been cooled at -20°C. After that the samples were centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was suspended in 100 µl of mQ water. After that 30 µl of 4x Laemmli buffer (40% glyerol, 0,25 M Tris-HCl pH=6,8, 8% SDS, 0,008% bromophenol blue and 20% β-mercaptoethanol) was added and the samples were frozen at -20°C until further usage.

3.1.5.3 Electrophoresis and transferring proteins to PVDF membrane

The lysates were unfrozen on ice and mixed with 4x Laemmli buffer according to the protein concentration. The final volume of the samples was 37,5 µl. The samples were then heated at 100°C for 5 minutes. After that the proteins were first concentrated and then separated in the gel by electrophoresis. The concentration of the concentrative gel was – and the concentration of the separating gel was 12%. 5 µl of PageRuler ladder (Thermo Fisher Scientific, Lithuania) was added into one lane. The buffer solution used for the electrophoresis contained 25 mM of Tris-HCl (pH=8,3), 250 mM glycine and 0,5% SDS. The gel ran for approximately 2 hours at 100 V. When the gel was finished and all the proteins had separated, they were transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, USA). The transferring was done by using the wet electroblotting method. The transferring of the proteins lasted for approximately 1 hour at 100 V. The buffer solution used for transferring the proteins contained 50 mM Tris-HCl (pH=8,3), 40 mM glycine, 1,3 mM SDS and 20% methanol.

3.1.5.4 Binding of antibodies and treating with alkaline phosphatase

On the first day

After the transferring the proteins to the membrane, the membrane was first washed with a solution of PBS and 0,1% Tween-20 (BioTop Naxo, Estonia). The membrane was then blocked for 1 hour on a shaker with a blocking solution, that contained PBS that was mixed with 0,1% of Tween-20, 3% of bovine serum albumin (BSA) (AppliChem, Germany) and 3% of non-fat milk powder (AppliChem, Germany). After that the blocking solution was discarded and the membrane was incubated overnight at 4°C with the primary antibody (Table 2), that was added to the blocking solution.

On the second day

The blocking solution with primary antibodies was discarded and the membrane was washed 3 times 5 minutes using solution of PBS and 0,1% Tween-20 at room temperature on the shaker. After washing, the membrane was incubated with the secondary antibodies (Table 2), that had been mixed with the blocking solution. The incubation took place at room temperature on the shaker for 1 hour. After the incubation, the membrane was washed again 3 times 5 minutes with the solution of PBS and 0,1% Tween-20. After the washing, the membrane was incubated with blocking solution, where Streptavidin-AP conjugate (Dako, Denmark) was added (dilution 1:1000). The incubation was for 1 hour at room temperature on a shaker. The membrane was washed 2 times 5 minutes in the PBS, 0,1% Tween-20 solution and then 1 time 10 minutes with 1x TBS. Finally, the membrane was incubated in NBT/BCIP solution (Thermo Fisher Scientific, USA), to visualize the bands and after the bands could be seen, the reaction was stopped with washing the membrane in mQ water.

Table 2. Antibodies used in Western blot.

	Primary antibody	**Secondary antibody**
Target	Human TREM2	Goat IgG (biotin)
Host	Goat	Rabbit
Dilution	1:1000	1:10000
Manufacturer	R&D SYSTEMS	Dako
Catalog number	AF1828	E0466

3.1.6 *In vitro* wound healing assay

Fibroblasts and HaCaT cells were grown in the incubator at 37°C and 5% CO_2. The cells were then passaged with 0,05% trypsin, 0,02% EDTA to two 24-well plates, 10 wells for both cell lines. Fibroblasts were seeded at $1{,}5x10^4$ cells and HaCaT cells at $1x10^4$ cells per well. The cells were left to grow until nearly confluent.

24 hours before the start of the experiment, the medium was changed. The new media contained 75% medium A mixed with 25% conditioned medium (control (pcDNA) or recombinant soluble TREM2). The media of 5 wells of both plates was changed to the medium that contained pcDNA conditioned medium and the other 5 wells of both plates to medium that contained TREM2 conditioned medium.

The next day, scratches were made to each well using a pipette tip. At least 3 images were taken from each well starting from straight after the scratches were made and every 3 hours after that under light microscope with 10x objective lens, until each scratch had almost closed. This took about 12 hours for the fibroblasts and about 30 hours for HaCaT cells. For the HaCaT cells, after 12 hours a longer pause was left between the pictures.

The wound width was then measured from the pictures from 3 different places and wound closure percentage was calculated. Student's t-test was done with the help of Microsoft Office Excel 365. P-values below 0,05 were counted as statistically significant. This experiment was done twice to increase the statistical credibility.

3.1.7 Transwell migration assay

Fibroblasts were grown in the incubator at 37°C and 5% CO_2. The cells were then passaged with 0,05% trypsin, 0,02% EDTA to 2 wells of a 6-well plate.

<u>On the first day</u>

24 hours before the start of the experiment, the media was changed. The new media was mixed as following: control sample medium contained 75% of medium A mixed with 25% pcDNA conditioned medium; TREM2 sample medium contained 75% of medium A mixed with 25% TREM2 conditioned medium. The new medium for one well was with the pcDNA conditioned medium and for the other with TREM2 conditioned medium.

On the second day

Cells were trypsinized with 0,05% trypsin, 0,02% EDTA solution. After 5 minutes of waiting for the cells to lift, the cells were suspended and then added to the medium A. The suspension was the centrifuged for 5 minutes at 300 g at room temperature. Supernatant was discarded and cells were suspended in DMEM with phenol red without any additives. Cells were counted and a new suspension of cells was made. The suspension of included 75% DMEM with phenol red without any additives, 25% conditioned medium (pcDNA or TREM2 respectively according to media in the wells before trypsinizing) and $1{,}5x10^4$ cells per well. 400 µl of medium A was pipetted into the lower chamber. The upper chamber (6,5 mm Transwell® with 8,0 µm pore polycarbonate membrane insert, Corning Incorporated, USA) was loaded into the lower one and 200 µl of the cell suspension made before was loaded into the upper chamber, so that the cells could migrate through.

On the third day

24 hours later, cells are fixed with methanol for 3 minutes at room temperature. The cells are then dyed with 0,5% Coomassie Brilliant Blue G-250 dye for 20 minutes at room temperature. After that, the cells are washed with mQ water until the water was colourless. The cells that did not migrate through the membrane are removed with moist cotton swab.

Pictures are taken from 6 different places of each well under a light microscope with 10x objective lens. The cells that migrated through the membrane are then counted on the pictures. Student's t-test was done with the help of Microsoft Office Excel 365. P-values below 0,05 were counted as statistically significant. This experiment was done twice to increase the statistical credibility.

3.1.8 Proliferation assay

Fibroblasts cells were grown in the incubator at 37°C and 5% CO_2. The cells were then passaged with 0,05% trypsin, 0,02% EDTA solution into a 96-well plate as following: 3x5 wells for pcDNA conditioned medium and 3x5 wells for TREM2 conditioned medium (3 timepoints and 5 wells for each conditioned media in every timepoint). The final suspension in each well consisted of 75% of medium A, 25% of conditioned medium and the cells with the density of $5x10^3$ per well. In addition, 3x2 wells were filled with medium A as a negative control. The first timepoint, consisted of 5 wells of fibroblasts with medium A, because the measurements were done immediately. To do the measurements, the cells were incubated for 10 minutes at

room temperature with 100 µl of pre-mixed reagent from CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, USA). After the incubation, the cells were moved into another 96-well plate and the luminescence of metabolically active cells is measured by microplate reader (Infinite M200 Pro, Tecan). The measurements were taken every 24 hours.

Student's t-test was done with the help of Microsoft Office Excel 365. P-values below 0,05 were counted as statistically significant.

3.1.9 Immunofluorescence analysis

Tissue samples were collected and cryopreserved previously in the laboratory by Dr. Klaas. Briefly, skin samples from healthy donors (collected from breast reduction surgeries, Tartu University Clinics; ethics permit 292/T-4) were embedded in O.C.T compound (Tissue-Tek, Sakura) and stored at -80°C. 10 µm-thick frozen sections were cut for immunofluorescence analysis.

<u>On the first day</u>

Previously frozen and cut human tissue sections taken from the freezer were left on room temperature for 10 minutes to warm up. The tissue sections were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. After that, the sections were washed 3 times for 3 minutes in PBS and permeabilised for 15 minutes with 0,2% Triton X-100 in PBS at room temperature. The tissue sections were washed again 3 times for 3 minutes in PBS and then incubated in blocking solution, which consisted of 5% normal donkey serum (Sigma-Aldrich, Germany) in PBS for 1 hour at room temperature in a humidified chamber. After that, the cells were incubated with primary antibodies diluted in blocking solution overnight at 4°C in a humidified chamber. Used antibodies are described in Table 3.

<u>On the second day</u>

The sections were washed 3 times for 5 minutes with PBS at room temperature. The tissue sections were then incubated with secondary antibodies diluted in blocking solution for 1 hour at room temperature in a humidified chamber. After the incubation, the cells were washed 3 times for 5 minutes in PBS at room temperature. To colour the nucleus, the cells were incubated in DAPI solution (final concentration 0,5 µg/ml) in PBS for 2 minutes at room temperature. The tissue sections were washed again 3 times for 3 minutes in PBS at room temperature. The sections were then mounted by putting a drop of Dako mounting medium (Agilent Technologies, USA) on the tissue section and putting a cover glass on top of that. The sections

were analysed with a confocal microscope Olympus IX81 CellR microscope (Olympus Corporation, Hamburg, Germany) equipped with Hamamatsu Orca ER (Hamamatsu Photonics, Herrsching am Ammersee, Germany) camera. The pictures were then layered using Hokawo software (Hamamatsu Photonics).

Table 3. Antibodies used in the immunofluorescence analysis. There were 3 patients and 3 tissue sections from each patient. The antibodies were placed as following: every tissue section was incubated with TREM2 and every tissue section was additionally incubated with 1 of these other 3 antibodies.

Primary antibodies				
Target	**Host**	**Dilution**	**Manufacturer**	**Catalog number**
TREM2	Goat	1:100	R&D SYSTEMS	AF1828
ITGB4	Mouse	1:200	R&D SYSTEMS	FAB4060R-100UG
CD68	Mouse	1:100	eBioscience	12-0681
Vimentin	Rabbit	1:300	abcam	EPR3776
Secondary antibodies				
Target	**Host**	**Dilution**	**Manufacturer**	**Catalog number**
Goat IgG (Alexa Fluor 488)	Donkey	1:1000	Life Technologies	A11055
Mouse IgG (Alexa Fluor 647)	Donkey	1:1000	Life Technologies	A31571
Rabbit IgG (Alexa Fluor 568)	Donkey	1:1000	Life Technologies	A10042

3.2 Results

3.2.1 Expression of TREM2 in human skin

To get a better knowledge about the expression of TREM2 in human skin an immunofluorescence analysis was done. ITGB4, CD68 and vimentin were used as markers for different cell types. ITGB4 is a receptor that is expressed in the keratinocytes of the basal layer of epidermis (Lipscomb and Mercurio, 2005; Watt and Jones, 1993). In addition, it can be expressed by hematopoietic cells such as macrophages (Evans *et al.*, 2019). CD68 is a glycoprotein that is exclusively expressed by macrophages and that is why it is most widely used marker for this cell type (Chistiakov *et al.*, 2017). Vimentin is fibroblast intermediate

filament that is found in addition to fibroblasts, in macrophages and melanocytes located in dermis as well (Robinson-Bennett and Han, 2006).

Based on Figure 4, there seemed to be some coexpression of ITGB4 and TREM2, but the coexpression seemed to be in the dermis, which means that TREM2 is probably expressed in the macrophages. In addition, there was some coexpression between CD68 and TREM2 (Figure 5), meaning that TREM2 is expressed in dermis, in the macrophages. This was supported by Figure 6, where some coexpression of vimentin and TREM2 be seen. TREM2 was not only expressed together with the markers, but it was also found in the extracellular matrix of dermis.

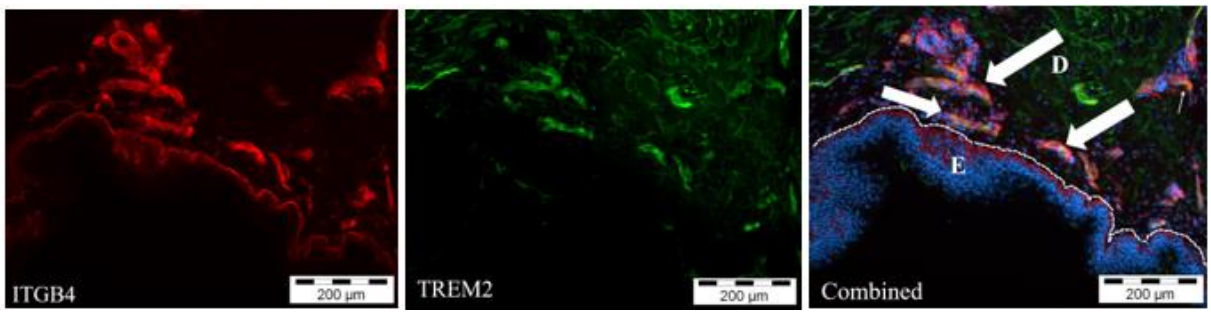

Figure 4. Expression of ITGB4 (red) and TREM2 (green) in human skin. The last picture is ITGB (red) and TREM2 (green) combined with DNA dye DAPI (blue). In the last picture epidermis (E) and dermis (D) are separated with a line. Places of coexpression are shown with arrows.

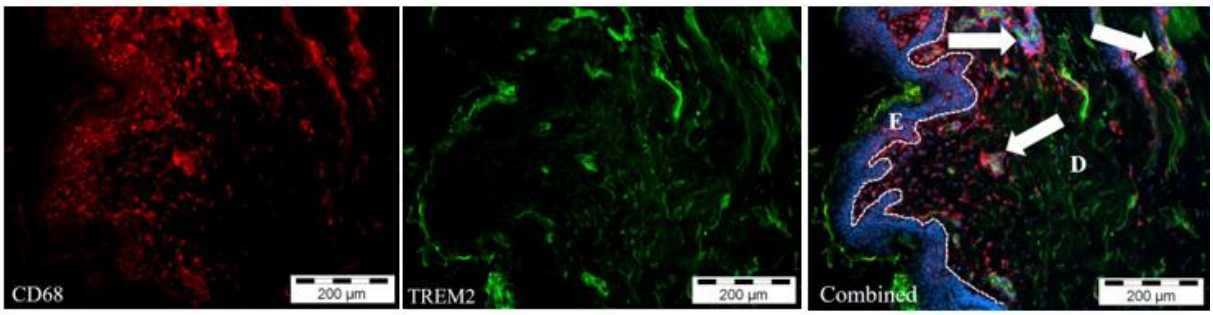

Figure 5. Expression of CD68 (red) and TREM2 (green) in human skin. The last picture is CD68 (red) and TREM2 (green) combined with DNA dye DAPI (blue). In the last picture epidermis (E) and dermis (D) are separated with a line. Places of coexpression are shown with arrows.

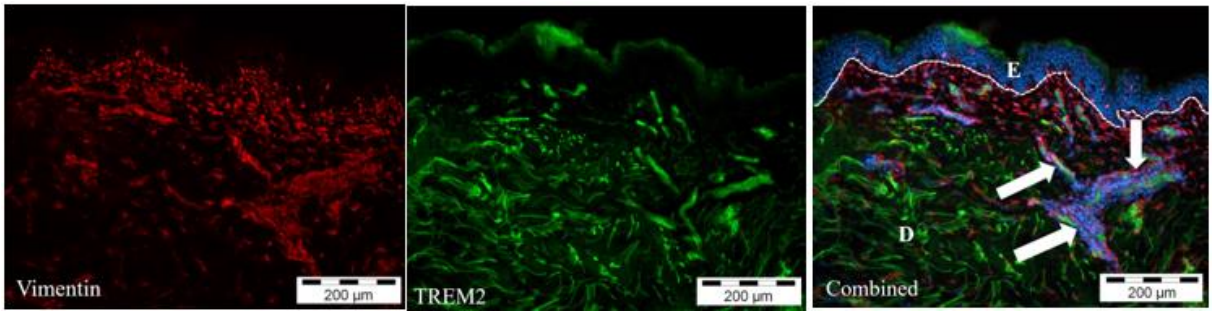

Figure 6. Expression of Vimentin (red) and TREM2 (green) in human skin. The last picture is vimentin (red) and TREM2 (green) combined with DNA dye DAPI (blue). In the last picture epidermis (E) and dermis (D) are separated with a line. Places of coexpression are shown with arrows.

3.2.2 Results of Western blot

Before any experiments with recombinant TREM2 protein were conducted, it was checked if the cell transfection worked. The TREM2 conditioned medium and pcDNA (mock-transfected) conditioned medium were analysed by Western blot to detect TREM2 expression (Figure 7). TREM2 protein in the HEK293 cell lysates appeared as double bands of about 25-35 kDa in size. These bands could not be seen in the HEK293 cells containing pcDNA. The size of the band for TREM2 in the medium from HEK293 cells was different than the size of the HEK293 cells containing TREM2. It was about 40 kDa. This could be due to glycosylation. The medium from HEK293 cells containing pcDNA did not show any significant bands, meaning that the control medium did not contain TREM2 protein.

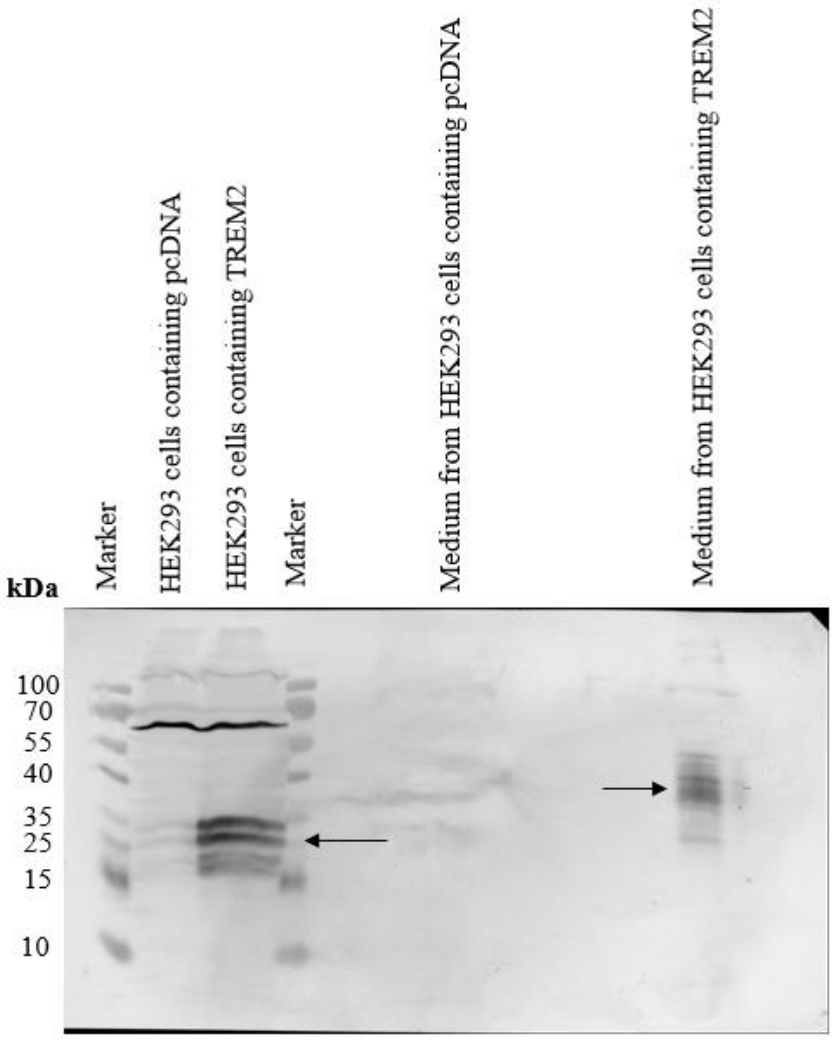

Figure 7. Western blot showing that transfection resulted in TREM2 production and secretion by HEK293 cells. TREM2 was missing from cells transfected with pcDNA. TREM2 is synthesised only in the cells that were transfected with the KLC-TREM2 plasmid vector.

3.2.3 The effect of TREM2 on the proliferation of fibroblasts

To see if treating cells with TREM2 conditioned medium makes affects cell proliferation, the amount of ATP available was measured with CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, USA). This is in correlation with the number of metabolically active (alive) cells. This means that the more luminescence is generated, the more cells are metabolically active. In the beginning, it seemed that the TREM2 inhibited the growth of the cells, but after some time the growth of the cells treated with TREM2 conditioned medium reached the same level as the cells treated with pcDNA conditioned medium (Figure 8). The p-value was below the 0,05 threshold at one timepoint (day 2). Although the p-value for the final timepoint (day 3) was above 0,05, the overall result of this experiment was inconclusive.

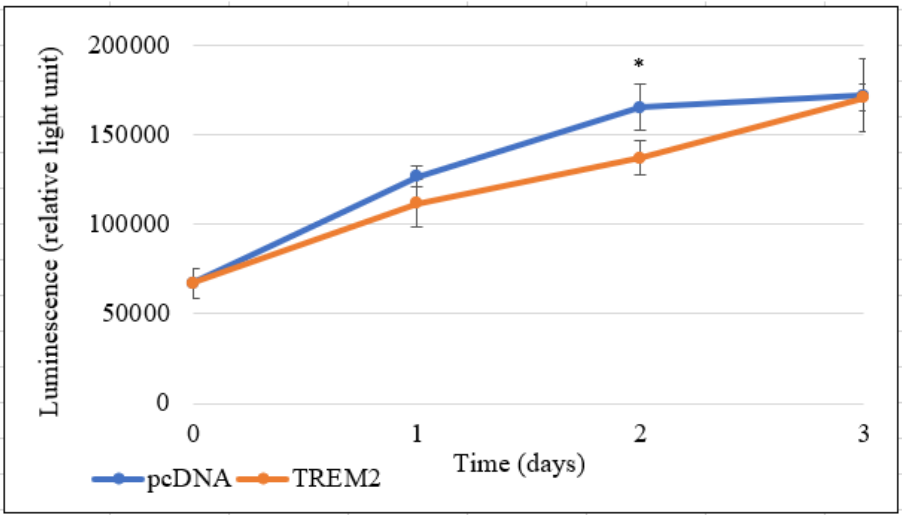

Figure 8. Results of proliferation assay. The measurements were done every 24 hours. 5 repetitions were done with both conditioned media and the average of the results from each well was calculated. The statistical significance was determined with the Student's t-test, where the value below 0,05 was counted as statistically significant. * means that the p-value was less than 0,05. Error bars show standard deviation.

3.2.4 The effect of TREM2 protein on the migration of cells

The effect of TREM2 on the migration of cells was measured by 2 different experiments. Scratch wound healing assay showed that TREM2 does not affect the migration of fibroblasts and HaCaT cells. As this experiment was done twice, the results differed a little, but not significantly. The graphs of the first experiment showed the cells that were treated with TREM2 conditioned medium seemed to migrate faster as the wound closure percentage at the last 12h timepoint was 40,5% for fibroblasts that were treated with TREM2 and 29,2% for fibroblasts that were treated with pcDNA (Figure 9A). The p-value was still above 0,05, which means that even though there was a trend, the difference was statistically insignificant. Similar results could be seen with the HaCaT cells (Figure 9B). The difference between cells treated with pcDNA and TREM2 was almost the same, as it was in the experiment made with fibroblasts. The wound closure percentage at the final 12h timepoint for the HaCaT cells treated with pcDNA was 22,6% and for HaCaT cells treated with TREM2 33,6%. The p-value was still above 0,05.

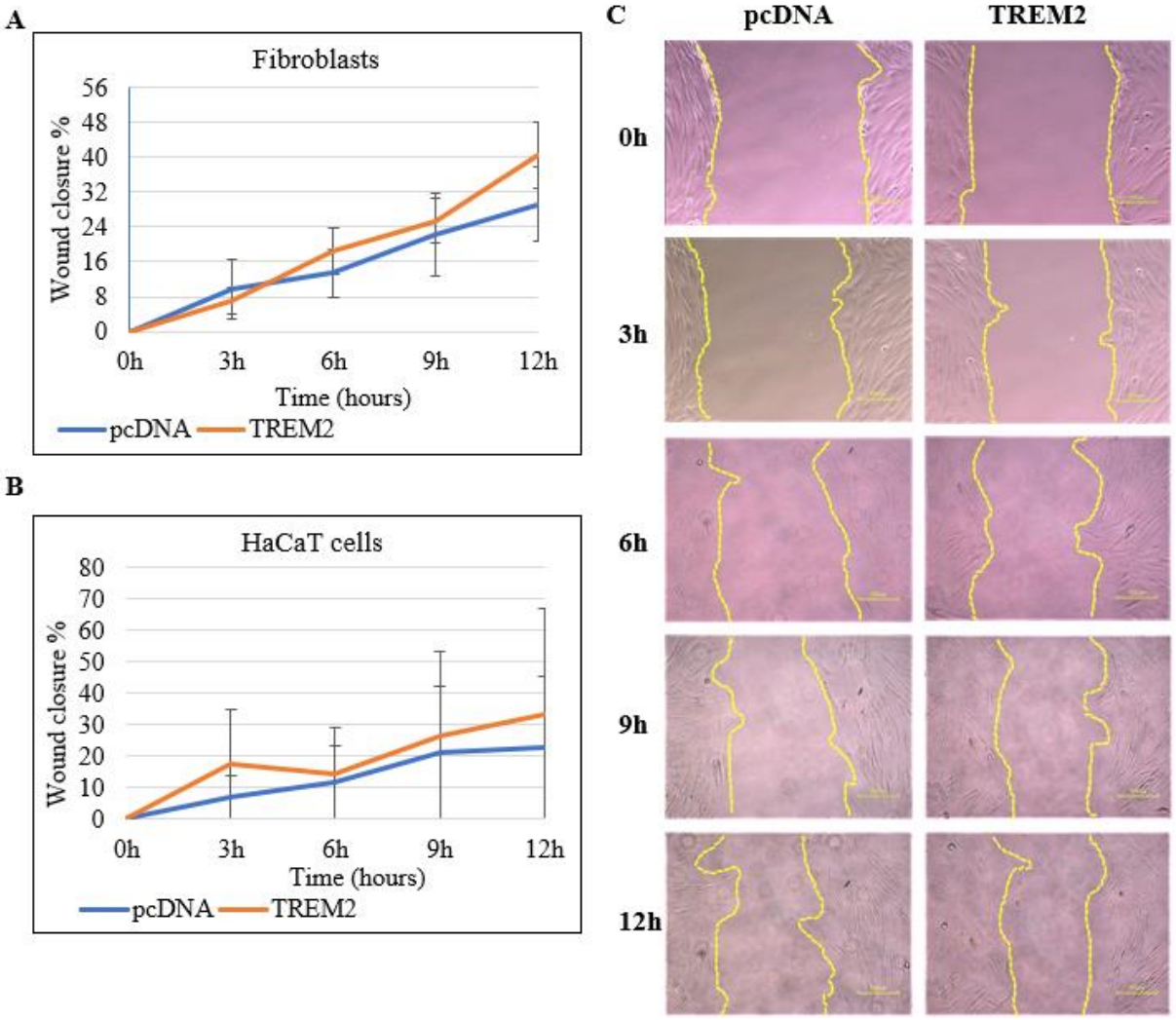

Figure 9. Graph A shows the results of wound healing assay done with fibroblasts, Graph B shows wound healing assay done with HaCaT cells and Graph C representative images from wound healing assay from the first experiment done with fibroblasts. Student's t-test was used to determine whether the result is statistically significant or not. The threshold of significance was 0,05. If the p-value was under 0,05, it was considered statistically significant. Error bars show standard deviation.

The results of the second repetition of this experiment differed, but the difference was not big. Similarly to the first experiment, the second experiment's graph of fibroblasts showed, that the cells treated with TREM2 migrated more. Although, the difference between the results of cells treated with TREM2 and the cells treated with pcDNA was statistically insignificant (Figure 10A). The percentages were 23,6% for the fibroblasts treated with TREM2 conditioned medium and 19,2% for the fibroblasts treated with pcDNA conditioned medium in the final 12h timepoint. The results of the experiment done with HaCaT cells differed the most from results of the other experiments. While in other experiments there was a difference between the cells treated with TREM2 and the cells treated with pcDNA, the difference could not be seen in this

experiment. The wound closure percentage of different treatments of cells was almost identical (Figure 10B). The percentage in the final 30h timepoint was 25,7% for cells treated with pcDNA and 26,6% for cells treated with TREM2. This means that the p-value was also above the 0,05 threshold, so the difference was statistically insignificant.

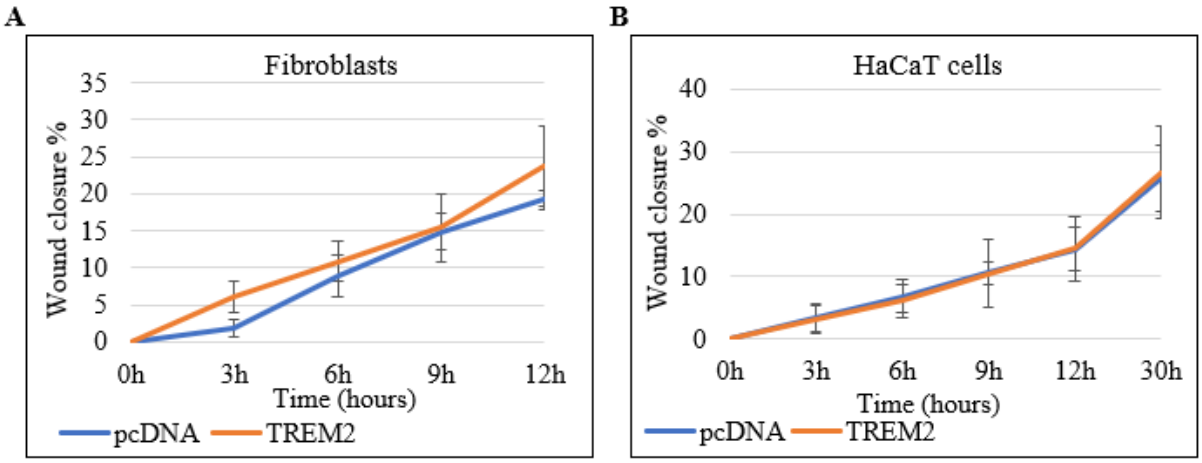

Figure 10. Graph A shows the results of the second wound healing assay done with fibroblasts and graph B shows the results of the second wound healing assay done with HaCaT cells. The error bars show standard deviation.

The second method used to measure the migration of cells was the transwell migration assay. The experiment was conducted twice and the results of both experiments were similar. The only difference between two experiments was the magnification of the objective lens used to record the imaged from the transwells. This is the reason why the number of cells counted in the first experiment is higher than the number of cells counted in the second experiment. TREM2 seemed to inhibit the migration of fibroblasts in both cases (Figure 11). The p-value was less than 0,05 in both cases, so the difference between pcDNA and TREM2 treated cells seemed to be statistically significant.

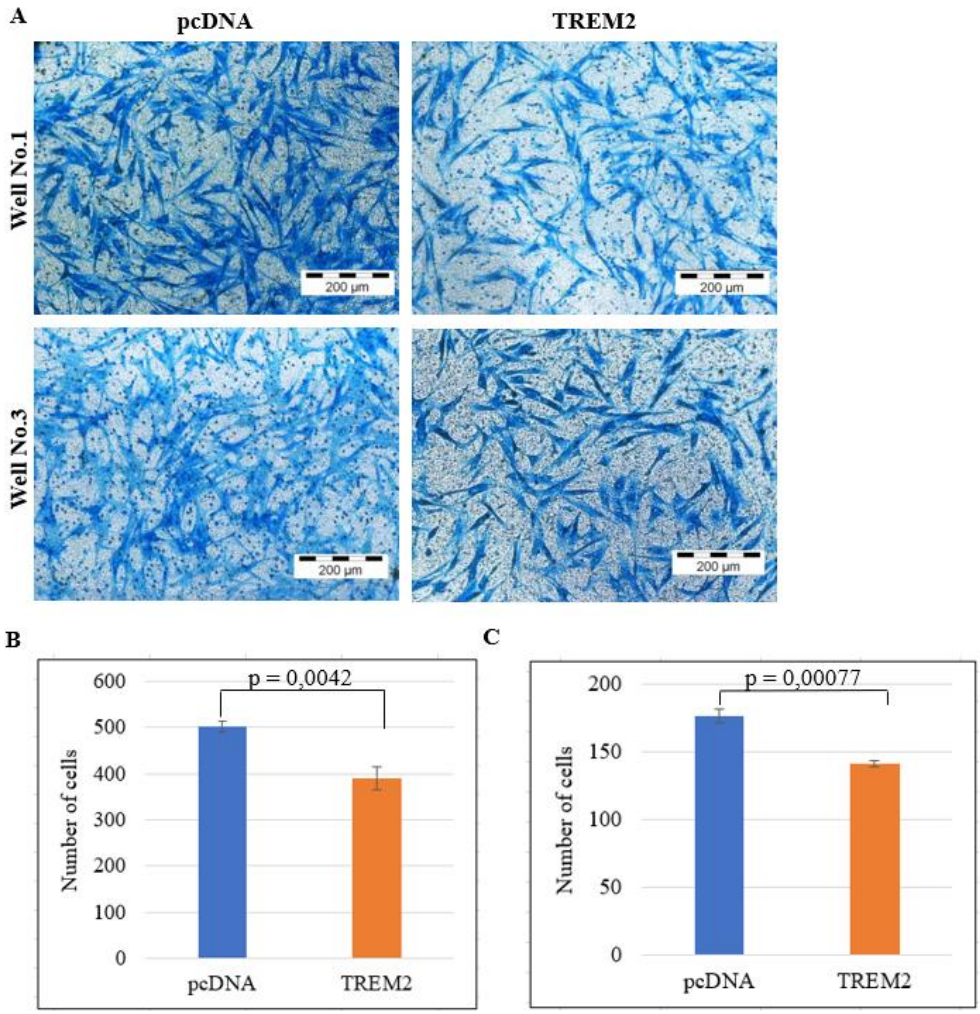

Figure 11. Graph A shows representative images from the second transwell migration assay from different wells, Graph B shows results of the first transwell migration assay and graph C shows results from the second transwell migration assay. The representative pictures are from the same experiment but from different wells. Error bars show standard deviation.

3.3 Discussion

The aim of this thesis was to investigate whether TREM2 could contribute to wound healing and skin regeneration. At first, the expression of TREM2 in human skin was investigated by immunofluorescence analysis. TREM2 was shown to be expressed in the dermis, since there was some coexpression between TREM2 and ITGB4 (expressed in macrophages), TREM2 and CD68 (expressed in macrophages) and TREM2 and vimentin (expressed in fibroblasts and macrophages). The coexpression between TREM2 and vimentin was little, which can mean that TREM2 is only expressed in macrophages and not in fibroblasts, since vimentin is primarily expressed in fibroblasts. TREM2 was also expressed in the extracellular matrix. Soluble TREM2 is secreted by proteolytic cleavage by ADAM proteases within the protein stalk, which results in releasing the TREM2 into extracellular matrix (Kober and Brett, 2017; Walter, 2016).

Proliferation assay was conducted to see if TREM2 affects cell proliferation. The results showed that TREM2 tends to inhibit cell proliferation in the first timepoints (day 1 and day 2), but in the last timepoint (day 3) the cells treated with TREM2 reached the same level of proliferation as cells treated with pcDNA. To author's knowledge the effect of TREM2 on the proliferation of fibroblasts has not been previously researched, although E. C. E. Wang *et al.*, 2019 showed that TREM2+ macrophages inhibit proliferation of hair follicle stem cells, X. Q. Wang *et al.*, 2016 showed that TREM2 enhances cell proliferation of glioma and Deczkowska *et al.*, 2020 described TREM2 to have inhibitory effect on the proliferation of T cells. Even though X. Q. Wang *et al.*, 2016 also measured the proliferation of cells, glioma cells are tumorous cells and the properties of them differ a lot from the fibroblasts. The TREM2 might still inhibit fibroblast proliferation, even though the level of proliferation was the same on the final day. The reason might be that too many cells were initially plated. When looking at the graph (Figure 6), it can be seen that the level of cells treated with pcDNA conditioned medium reached equilibrium already at day 2. This means that if fewer cells would have been plated, the cells might have continued proliferating, since contact inhibition may have stopped the cells from continuing to proliferate. If this was the case, the results would be similar to the results described by Deczkowska *et al.*, 2020, E. C. E. Wang *et al.*, 2019 and TREM2 could inhibit the proliferation different cells, such as T cells, hair follicle stem cells and fibroblasts.

Wound healing assay was conducted to measure the effect of TREM2 on the migration of the cells. The results showed that even if TREM2 has some effect on the migration of the cells, it is not significant (Figures 7-11). The results of wound healing assay did not overlap with the results of the transwell migration assay, where TREM2 seemed to inhibit cell migration (Figures 12-14). Overall conclusion would be that TREM2 does not have an effect on the migration of fibroblasts and keratinocytes. To author's knowledge the effect of TREM2 on the migration of fibroblasts and HaCaT cells has not been previously researched, but McQuade *et al.*, 2020 found that TREM2 is important for microglial migration and Seno *et al.*, 2009 described that TREM2 signalling is needed in efficient colonic mucosal wound repair. The results of previous researches contradict with the results of this research. This might be due to different cell types compared to other researches or lack of repetitions of the experiments done for this research.

The results of this research indicate that TREM2 is expressed in healthy human skin but it does not have a positive effect on fibroblast and keratinocyte migration and proliferation. TREM2 seemed to inhibit the proliferation of fibroblasts and have very little or inhibiting effect on the migration of the cells.

SUMMARY

Wound healing and skin regeneration are very important since skin acts as a protective shield from the external environment and is constantly exposed to possible injuries (Takeo *et al.*, 2015). The main aim of this present thesis was to examine the role of TREM2 protein in fibroblast and keratinocyte proliferation and migration. The main aim was fulfilled by fulfilling all the subaims.

The first subaim was to characterise the expression of TREM2 in human skin. Immunofluorescence analysis was done with the help of 3 markers – ITGB4, CD68 and vimentin – to see where TREM2 is expressed in the skin. TREM2 seemed to be expressed mainly by the macrophages in dermis, but it was also expressed in the extracellular matrix of dermis.

The second subaim was to investigate the role of soluble TREM2 protein in cell proliferation. The subaim was fulfilled by doing the proliferation assay. Even though the result seemed to be inconclusive, TREM2 still might inhibit fibroblast proliferation.

The third subaim was to investigate the role of soluble TREM2 protein in cell migration. Two different *in vitro* assays - wound healing assay and the transwell migration assay were performed. The results of these experiments contradicted with each other. Even though the result was not statistically significant, wound healing assay seemed to show a trend of some beneficial role of TREM2 in cell migration, while transwell migration assay showed that TREM2 inhibits migration. That is why the overall conclusion would be that TREM2 does not affect the migration of fibroblasts and keratinocytes.

The stated hypothesis was that TREM2 has a role in skin fibroblast and keratinocyte proliferation and migration that could potentially promote wound healing. The hypothesis was disproved as TREM2 seemed to inhibit the skin fibroblast and keratinocyte proliferation and not to affect the migration. The get a more accurate result, experiments should be done with more repetitions.

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