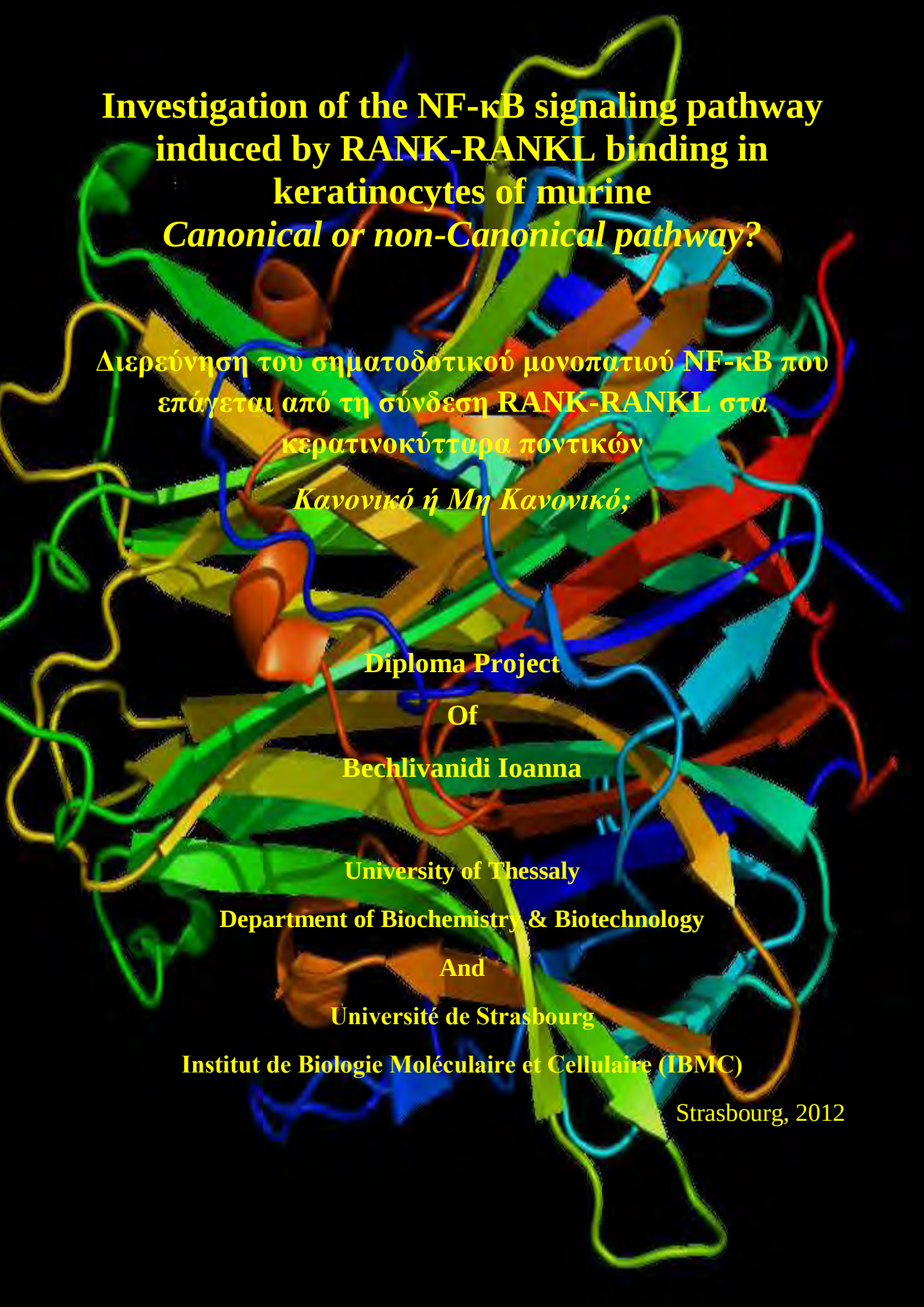

Investigation of the NF-κB signaling pathway induced by RANK-RANKL binding in keratinocytes of murine

Canonical or non-Canonical pathway?

Διερεύνηση του σηματοδοτικού μονοπατιού NF-κB που επάγεται από τη σύνδεση RANK-RANKL στα κερατινοκύτταρα ποντικών

Κανονικό ή Μη Κανονικό;

Diploma Project

Of

Bechlivanidi Ioanna

University of Thessaly

Department of Biochemistry & Biotechnology

And

Université de Strasbourg

Institut de Biologie Moléculaire et Cellulaire (IBMC)

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny..."
Isaac Asimov (1920-1992)

Contents

Abstract

Receptor activator on NF-κB (RANK) is known as a key molecule in bone metabolism and bone disorders. Moreover, the recptor is shown to act during the development of a functional lactating mammary gland, by triggering the proliferation of mammary epithelial cells. Taking under consideration the fact that, hair follicles and mammary glands are both skin appendages, and in addition that bone and epidermo-pilosebaceous unit -which consists of hair follicle, hair shaft and sebaceous gland- have all a renewal activity conducted by the same operating molecules, enable us to use the epidermis and hair follicles as models for RANK's activity. In this project, we used transgenic (Tg) mice overexpressing RANK in the hair follicle in comparison with wild type (WT) mice to determine whether or not RANK activates hair cycle and epidermal growth. For that reason, measurements of concentration of the total protein content of epidermis were performed indicating that Tg mice have thicker epidermis (epithelial hyperplasia) and augmented protein concentration. RANK was found to induce both canonical and non canonical NF-κB signaling pathway depending on the circumstances. Hereto, to identify which one is induced by the binding of RANK-RANKL, on epithelial cells of epidermis in Tg an WT animals, isolation of the epidermis' protein content had taken place.

Περίληψη

Ο υποδοχέας που ενεργοποιεί το NF-κΒ σηματοδοτικό μονοπάτι (RANK), είναι γνωστό ότι διαδραματίζει ένα ρόλο κλειδί στο μεταβολισμό των οστών και στις παθήσεις που σχετίζονται με αυτόν. Επιπλέον, έχει βρεθεί ότι δρα στην ανάπτυξη λειτουργικών μαστικών αδένων, ενεργοποιώντας τον πολλαπλασιασμό των μαστικών επιθηλιακών κυττάρων. Έχοντας υπόψη ότι τα τριχοθυλάκια και οι μαστικοί αδένες είναι δερματικές αποφύσεις, και επιπλέον ότι η μονάδα του δέρματος- που συνίσταται από το τριχοθυλάκιο, το σμηγματογόνο αδένα και τον ορθωτήρα μυ- και τα οστά έχουν αναγεννητική δυνατότητα που καθορίζεται από τα ίδια μόρια, μας επιτρέπει τη χρήση της επιδερμίδας και του τριχοθυλακίου ως μοντέλο για τη μελέτη της λειτουργίας του RANK. Στην παρούσα εργασία χρησιμοποιήθηκαν διαγονιδιακά ποντίκια που υπερεκφράζουν τον υποδοχέα RANK στο τριχοθυλάκιο σε σύγκριση με άγριου τύπου για να καθοριστεί αν ο RANK ενεργοποιεί τον κύκλο της τρίχας και την επιδερμική αύξηση. Για αυτό το λόγο πραγματοποιήθηκαν μετρήσεις της συνολικής συγκέντρωσης των πρωτεϊνών στην επιδερμίδα, υποδεικνύοντας ότι τα διαγονιδιακά ποντίκια όντως έχουν παχύτερη επιδερμίδα και αυξημένη πρωτεϊνική συγκέντρωση. Έχει βρεθεί ότι ο RANK ενεργοποιεί τόσο το κανονικό όσο και το εναλλακτικό NF-κΒ σηματοδοτικό μονοπάτι ανάλογα με τις συνθήκες. Για αυτό το λόγο, για να διευκρινιστεί ποιο μονοπάτι τελικά ενεργοποιείται από την πρόσδεση RANK-RANKL στα επιθηλιακά κύτταρα της επιδερμίδας διαγονιδιακών και άγριου τύπου ζώων πραγματοποιήθηκε απομόνωση του επιδερμικού πρωτεϊνικού περιεχομένου.

1. Introduction

1.1 RANK/RANKL

Tumor necrosis factor (TNF) is identified as the first member of a large family, now called the TNF superfamily (TNFSF) *(Wong 1997a, Anderson 1997)*. Not surprisingly, the receptors for these proteins also constitute a superfamily with sequence homology, named TNF Receptor superfamily (TNFRSF) *(Locksley G., et al. 2001)*.

RANK (Receptor Activator of NF-κB) is a transmembrane protein, member of TNFRSF. It is also known as TRANCE Receptor or TNFRSF11A. RANK is the receptor for RANK-Ligand (RANKL). RANK is a 616 amino acid (aa) (90kDa), type I transmembrane protein. Its extracellular domain consists of 184 amino acids, its transmembrane domain has 21 amino acids, and its cytoplastic domain consists of 383 amino acids. Like other members of the TNFR family, it has four extracellular cysteine-rich pseudo-repeat domains *(Anderson et al., 1997)*.

RANKL is a member TNFSF, which are type II transmembrane proteins with their carboxy terminus outside of the cell. It is also known as TNFSF11, TNF activation-induced cytokine (TRANCE), Osteoprotegerin Ligand (OPGL) (35kDa), and Osteoclast differentiation factor (ODF). Murine RANKL is composed of 316 aa, with a cytoplasmic domain and an extracellular domain. RANKL's extracellular domains are similar to other TNF family members in regard to the structural homology. These proteins are able to cleave from the cell surface as a soluble form *(Ikeda et al., 2001, Hikita et al., 2006)*.

RANKL is the ligand of two receptors, RANK and OPG (TNFRSF11b). OPG (osteoprotegerin) was the first of this protein triad to be discovered in a search for an inhibitor of osteoclastogenesis *(Simonet et al., 1997)*. OPG-ligand was then isolated and cloned using OPG as bait *(Lacey et al., 1998; Yasuda et al., 1998)*. OPG-ligand turned out to be identical to TRANCE, cloned during a search for apoptosis-regulatory genes in T cells *(Wong et al., 1997b)*, and RANKL identified as the ligand for RANK that had attracted attention for its homology to CD40 *(Anderson et al., 1997)*. The affinity of RANKL for OPG is 1000-fold higher than for RANK *(Nakagawa et al., 1998)*, which is dependent on the ability of OPG to homodimerize *(Schneeweis et al., 2005)*. OPG is also a ligand for TNF-related apoptosis-inducing ligand (TRAIL), however, its affinity for TRAIL is 10,000 times less, compared to RANKL *(Body et al., 2006)*. Generally, one trimeric ligand engages three monomeric receptors, a key event for the activation of intracellular signaling pathways.

RANK is known for controlling bone mass but also has an important role in epithelial cell growth and differentiation *(Walsh MC, et al. 2003, Tanaka S., et al 2005)*. The RANK-RANKL complex regulates diverse physiological functions and organ development in the body such as lactating mammary glands *(Fata JE, et al. 2000; Beleut M, et al. 2010)*, proliferation of epithelial cells, lymph nodes, teeth *(Kong YY, et al. 1999)* and immune responses. It is known that is acting on the early development of the lymphoid tissue and on its homeostasis. In its absence bone mass control is deregulated and lymph nodes fail to develop *(Mueller C.G. and Hess E. 2012)* so as the teeth.

After RANKL binds on RANK on the cell surface, trimerization of the reception is induced and intracytoplasmic signalization pathways are activated to transfer the signal to the cell nucleus. Signaling is mediated by adaptor proteins that interact with the cytoplasmic domain of the receptor. The adaptor proteins that RANK can bind are TRAF (1,2,3,5,6) *(Galibert et al., 1998)*. Downstream of RANK several signaling pathways are activated such as JNK, NF-κB, ERK and Akt/PKB *(Wong 1999, Walsh 2003)* (Figure 1). RANK mediates activation of both canonical and non-canonical NF-κB signaling pathways. TRAF6 seems particularly important for RANK signaling, because $TRAF6^{-/-}$ mice present similar phenotypes as $Rank^{-/-}$ mice *(Naito et al., 1999; Mueller C.G. and Hess E. 2012)*.

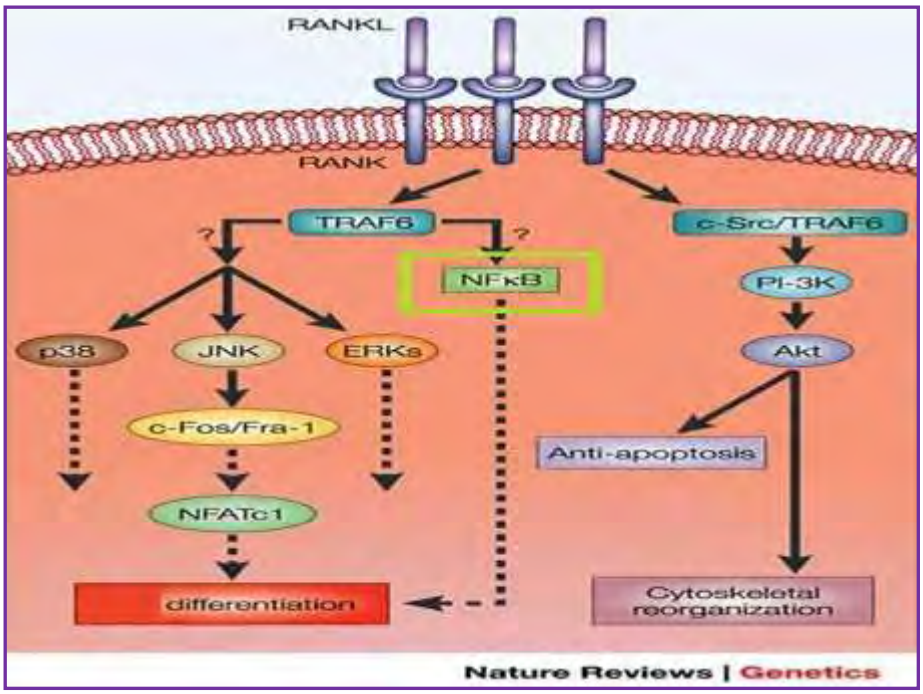


Figure 1 **Intracytoplasmic signalization pathways activated by RANK-RANKL binding** *(Nature Reviews, Genetics)*

1.2 NF-κB signaling pathway

The nuclear factor-κB (NF-κB)/REL, family of transcription factors has a central role in coordinating the expression of a wide variety of genes that control immune responses. There has been intense scientific activity in the NF-κB field owing to the involvement of those factors in the activation and regulation of key molecules that are associated with diseases ranging from inflammation to cancer. The constitutive activation of NF-κB pathways is often associated with inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and asthma.

Although NF-κB target genes have been most intensely studied for their involvement in immunity and inflammation *(Imler, J. L. & Hoffmann, J. A. 2000*), this transcription factor also regulates cell proliferation, apoptosis and cell migration. Therefore, it is not surprising that NF-κB has been shown to be constitutively activated in several types of cancer cells.

NF-κB proteins are present in the cytoplasm in association with inhibitory proteins that are known as inhibitors of NF-κB (IκBs). After activation by a large number of inducers, the IκB proteins become phosphorylated, ubiquitylated and, subsequently, degraded by the proteasome.

The NF-κB transcription factor family in mammals, is sectioned in five related members: p50/p105 (NF-κB1), p52/p100(NF-κB2), RelA (p65), c-Rel and RelB. This distinction is based on the presence of a Rel homology domain (RHD), which is responsible for dimerization and DNA binding. The NF-κB family can be divided in two subgroups, the Rel and NF-κB groups. Only Rel proteins contain a trans-activating domain through which they activate transcription. Moreover, they are synthesized in their mature forms. On the other hand, NF-κB group is first synthesized as large precursor molecules (p100 and p105) before their proteolysis into p52 and p50 respectively *(Silverman et al. 2001)*, and they are not able to promote transcription unless they form a heterodimer with one of the proteins of Rel group (Figure 2). The NF-κB group can also create homodimers which can move to the nucleus

and act as repressors of transcription. Its transcriptional activity is silenced, as mentioned above, by interactions with inhibitory Inhibitor κB (IκB) proteins present in the cytoplasm. There are currently seven identified IκB family members - IκBα, IκBβ, IκBγ, IκBε and Bcl-3. These regulatory proteins are identified by the presence of many ankyrin repeats. p100 and p105 NF-κB proteins contain similar ankyrin repeats and can function as IκB-like proteins *(Verma I., et al. 1995)*. Bcl-3 was found to interact with the NF-κB group and act like a co-activator or as inhibitor when interacts with p50.

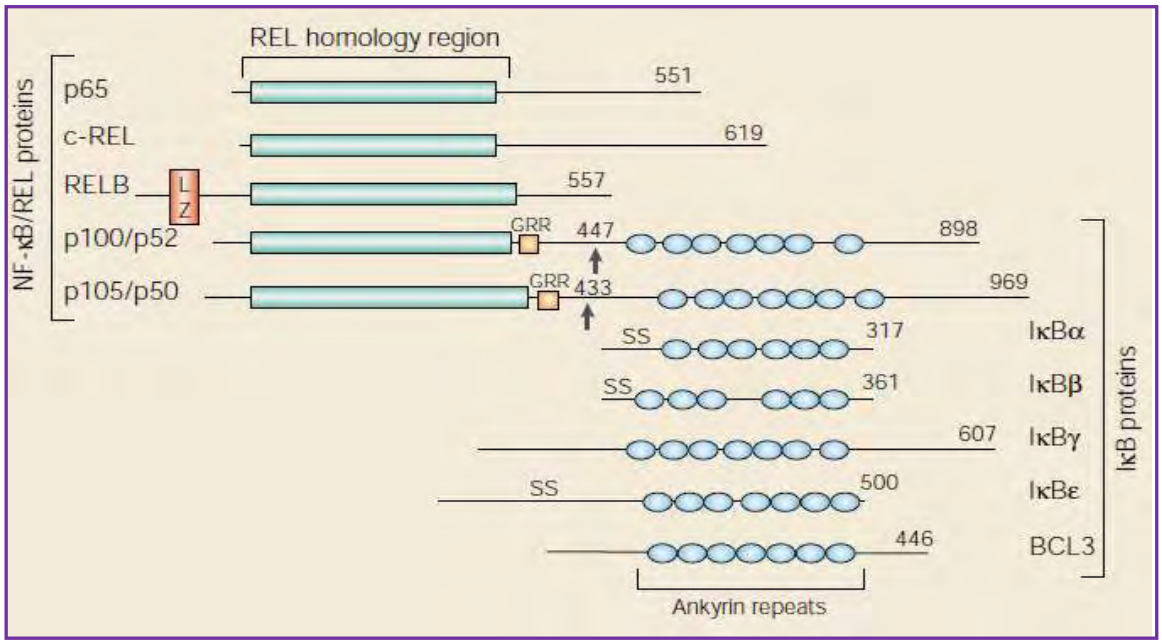


Figure 2 NF-κB and IκB proteins domains (*Michael K, et al. (2002) NF-κB in cancer: from innocent bystander to major culprit. NATURE REVIEWS CANCER 2, 301)*

There are two signaling pathways including the **NF-κB** family, known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway). A crucial regulatory step in both processes is the signal-induced phosphorylation of IκB or IκB-similar regions (p100 and p105) at specific amino-terminal serine residues, which is mediated by the IκB kinase (IKK) complex *(Glosh S., et al 1998, Glosh S., et al 2002)*.

Both pathways regulate cell survival and death; the classical pathway is responsible for inhibition of programmed cell death under most conditions. The alternative pathway is important for survival of premature B cells and development of secondary lymphoid organs.

The **classical pathway** involves the p50-RelA dimer, which is rendered inactive in the cytoplasm by its bounding with IκB. The canonical pathway is normally triggered in response to microbial and viral infections and exposure to proinflammatory cytokines, all of which activate the IKK complex. The classical pathway starts with the activation of the IKK, which is consisting of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit IKKγ/NEMO. The activation of IKK results in the phosphorylation of IκBs followed by their ubiquitanation and proteosomal degradation. The p50-RelA dimer translocates to the nucleus and induces gene transcription (Figure 3) *(Glosh S., et al 1998, Glosh S., et al 2002)*.

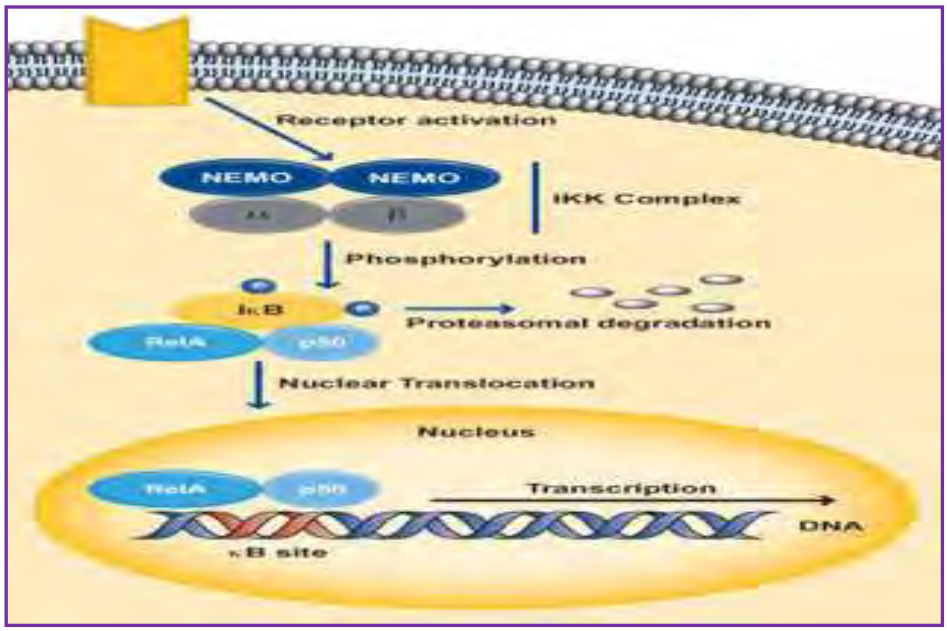


Figure 3 NF-κB Classical pathway *(Prajabati B., et al (2010) Review Article Role of NFkB in Various Immunological & Inflammatory Disorders. International Journal of Toxicological and Pharmacological Research 2, 35-39.)*

The **alternative pathway**, on the other hand, involves the p52-RelB dimer, which is settled inactive in the cytoplasm by an IκB-homologous domain in the C terminus of the precursor p100. This processing dependent pathway is triggered by certain members of the TNF cytokine family that selectively activate the catalytic subunit IKKα, along with another protein kinase called NF-κB inducible (NIK). The first step is the activation of kinase NIK. Secondly, NIK phosphorylates and activates the IKKα homodimers which consequently leads to the phosphorylation, ubiquitination and cleavage of the p100 to p52. Finally the p52-RelB dimer is transferred to the nucleus (Figure 4) *(Glosh S., et al 1998, Glosh S., et al 2002)*.

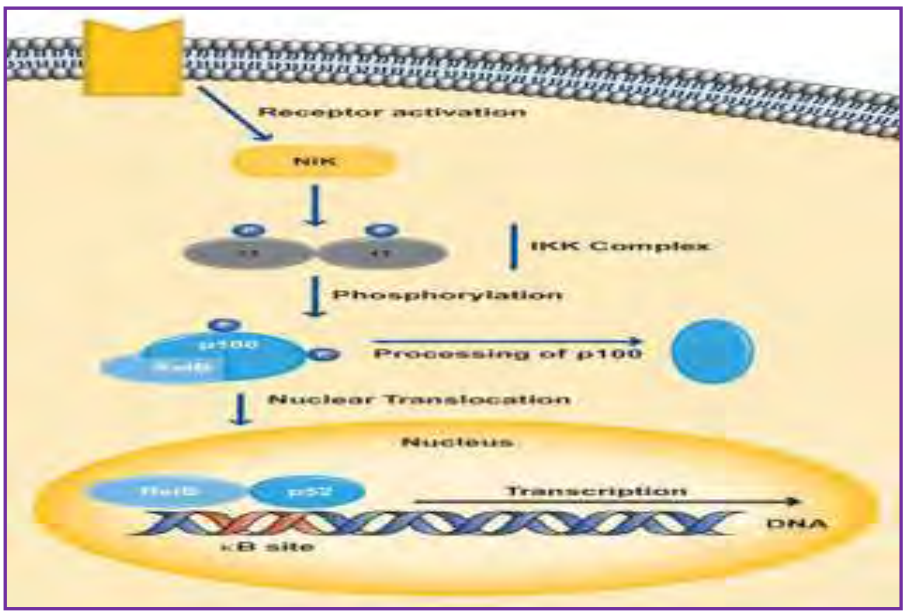


Figure 4 NF-κB Alternative pathway *(Prajabati B., et al (2010) Review Article Role of NFkB in Various Immunological & Inflammatory Disorders. International Journal of Toxicological and Pharmacological Research 2, 35-39.)*

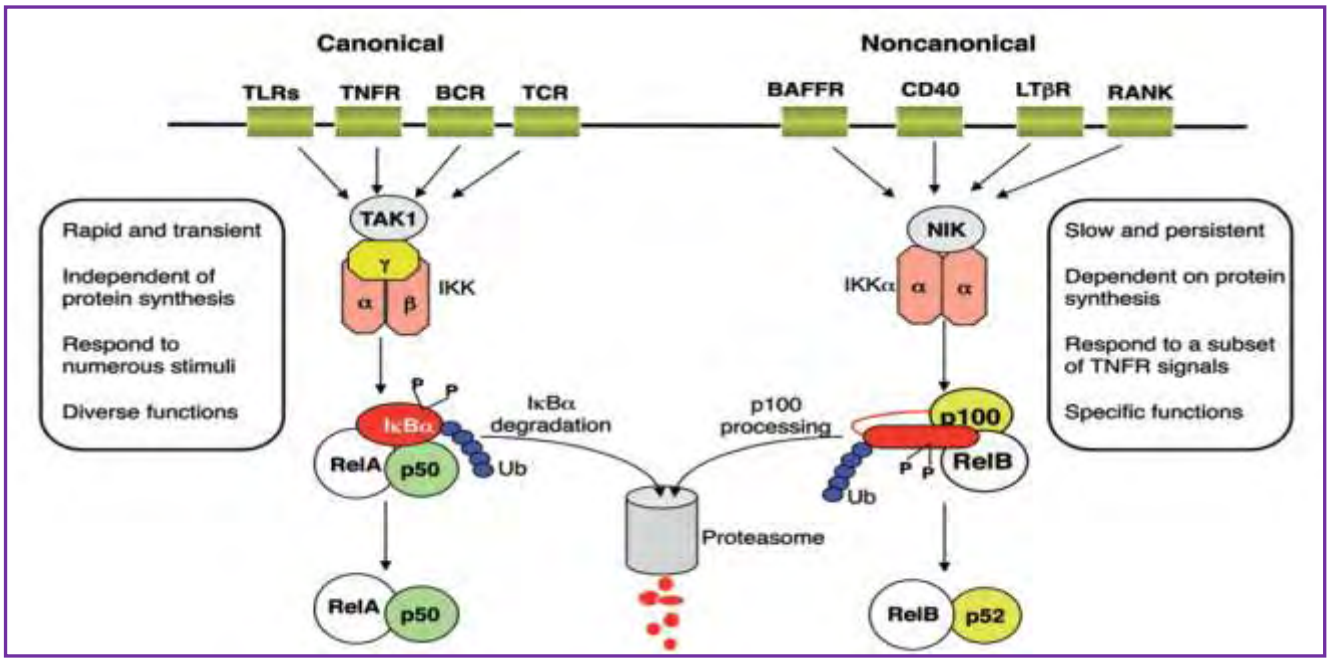


Figure 5 **Canonical VS Non Canonical pathway** *(Shao-Cong Sun (2011) Review Non-canonical NF-κB signaling pathway. Cell Research 21, 71-85.)*

1.3 Physiology of the skin

The skin is simultaneously the largest epithelial surface and a complex organ. It serves multiple functions for the organism such as protection from external threats, temperature homeostasis, perception of the environmental circumstances and metabolism.

The skin consists of three layers, the epidermis, the dermis and the hypodermis and contains blood and lymph vessels, nerve fibers, glands and the errector pili muscle (Figure 6). Specialized structures like pilosebaceous unit and sweat glands are found mainly in the dermis but connected with epidermal surface.

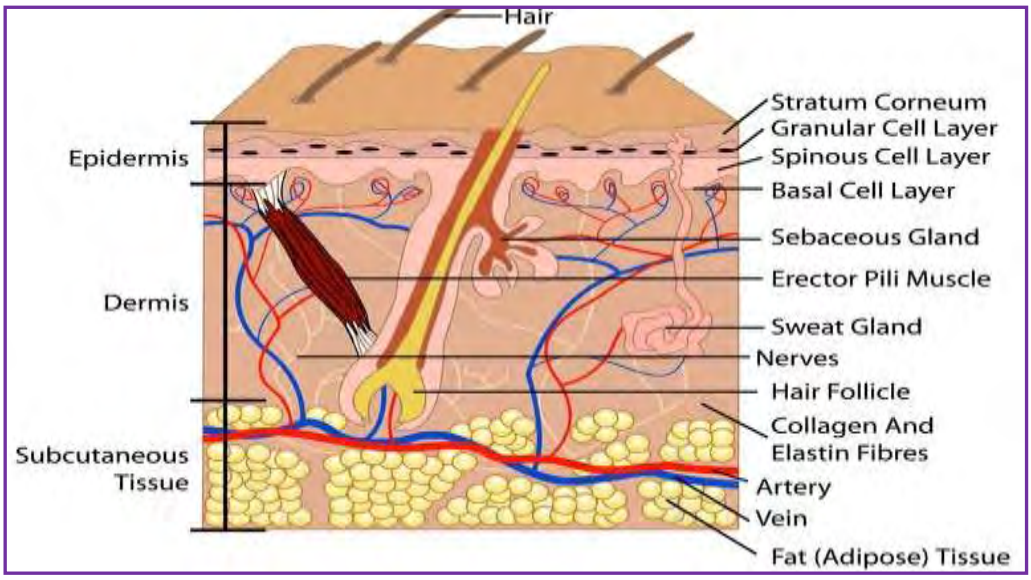


Figure 6 **Representation of skin organization** ***(****Dr. Rudyard Health pictures* *http://www.rudyard.org/human-skin-diagram/)*

The epidermis is composed by stratified epithelium, layers of cells that are continually renewing. The epidermis is avascular, nourished almost exclusively by diffused oxygen from the surrounding air. It is mostly populated by keratinocytes and more specifically by proliferating basal and differentiated suprabasal keratinocytes. The layers are structured based on their differentiation stage. The epidermis is composed of 4 or 5 layers depending on the region of skin. Those layers in descending order are:

1. cornified layer (*stratum corneum*)
2. clear/translucent layer (*stratum lucidum*, only in palms and soles)
3. granular layer (*stratum granulosum*)
4. spinous layer (*stratum spinosum*)
5. basal/`germinal layer (*stratum basale/germinativum*) (Figure 7).

Keratinocytes of basal layer is the most undifferentiated cells and they maintain the capacity of proliferating in an asymmetrical division (*Blanpain C., et al 2009)*. By the time they leave the basal layer, keratinocytes start differentiating simultaneously as they move to the surface. This movement of keratinocytes is linked to various stages of morphological changes. Even though the signals responsible for keratinocytes' differentiation are not fully understood, NF-κB appears to have a role in this process.

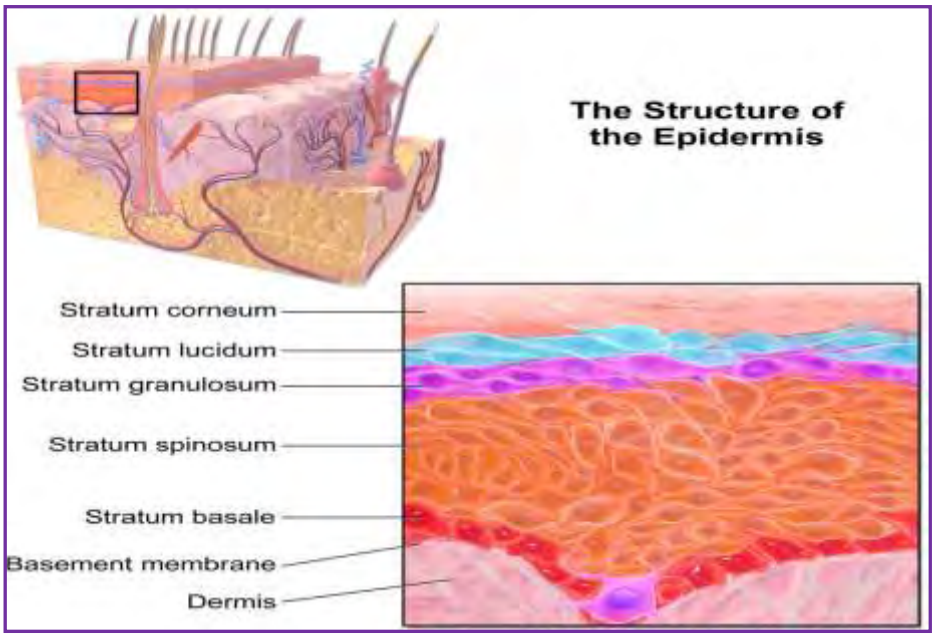


Figure7 Organization of the epidermis
(http://upload.wikimedia.org/wikipedia/commons/6/68/Blausen_0353_Epidermis.png)

Throughout our whole lifetime bone is constantly being remodeled and approximately every ten years our skeleton is being completely renewed *(Leibbrandt A., et al. 2008)* with the triad of RANK/RANKL/OPG having an operator role. Bones, mammary gland, teeth and hair follicle (HF) are all skin appendages where epidermal renewal and differentiation integrates the NF-κB pathway. It was not long ago noticed, that in a similar way RANK/RANKL controls murine hair follicle growth and epidermal homeostasis. This was contributed to the fact that the key molecules controlling both activities are the same. Because bone and the epidermo-pilosebaceous unit of the skin share a lifelong renewal activity where similar molecular players operate, and because mammary glands and hair follicles are both skin appendages, and undergoing cycles of growth, regression and relative

quiescence, the function of RANK has been addressed in the hair follicle and the epidermis *(Cotsarelis G 2006; Fuchs E 2007; Blanpain C 2007; Watt FM 2000)*. RANK stimulation of HF stem cells is required for entry into anagen. RANK was found to be expressed in basal keratinocytes and RANKL in superbasal keratinocytes *(Duheron V., et al. 2011)*. More specifically, keratinocytes of the basal layer divide and terminally differentiate to replace upper-lying cells. RANKL is expressed by activated IFE and the epidermal Langerhans dendritic cells carry RANKL *(Loser K, et al. 2006; Barbaroux J, et al. 2008)*. These findings supporting the idea that RANK is a regulator of epidermal keratinocytes' homeostasis.

1.4 The epidermo-pilosebaceous unit

The hair coat, which keeps most mammals warm, dry and protected from harmful elements, requires a constant supply of new hairs throughout the lifetime of the animal. Skin which is the largest epithelial surface and its epidermo-pilosebaceous unit, which provides the hair coat, includes the interfollicular epidermis (IFE), the hair follicle (HF), and the sebaceous gland *(Alonso L. & Fuchs E, 2006)*. HF is divided in two parts, a permanent upper part and a continually remodeled lower part (Figure 8). The hair growth cycle describes the changing morphology of the shaft, grossly, and the follicle, histologically, over time *(Dry FW, 1926*).

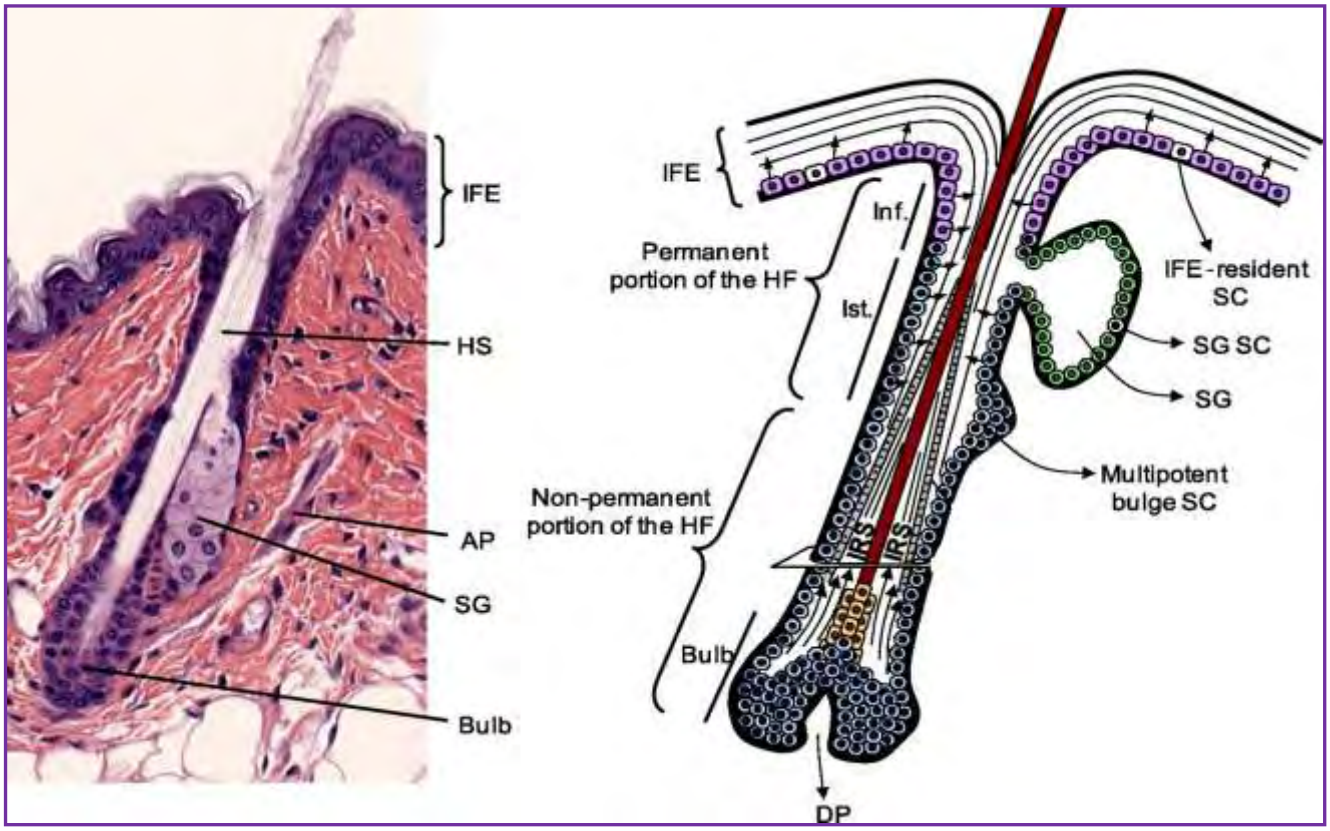


Figure 8 Epidermo-pilosebaceous unit *(Margadant C., et al (2010) Unique and redundant functions of integrins in the epidermis. The FASEB Journal 24, 4133-4152)*

Although, the number of HFs is fixed and determined during the embryonic life, existing follicles undergo cycles of growth (anagen), regression (catagen) and rest (telogen) in order to produce new hair on the existing follicles. In this cycle, different skin stem cell pools are contributing including IFE, HF and sebaceous glands *(Blanpainand C., 2009)*. During each anagen phase, follicles produce an entire hair shaft from tip to root; during catagen and telogen, follicles reset and prepare their stem

cells so that they can receive the signal to start the next growth phase and make the new hair shaft (Figure 9). The hair cycle represents a remarkable model for studies regarding the regulation of stem cell quiescence and activation, as well as transit-amplifying cell proliferation, cell-fate choice, differentiation and apoptosis in a regenerative adult epithelial tissue *(Alonso L. & Fuchs E, 2006)*.

The time points when the hair follicle enters the next step of the cycle are clearly defined in mice, thus making it a convenient model for studying factors controlling hair production.

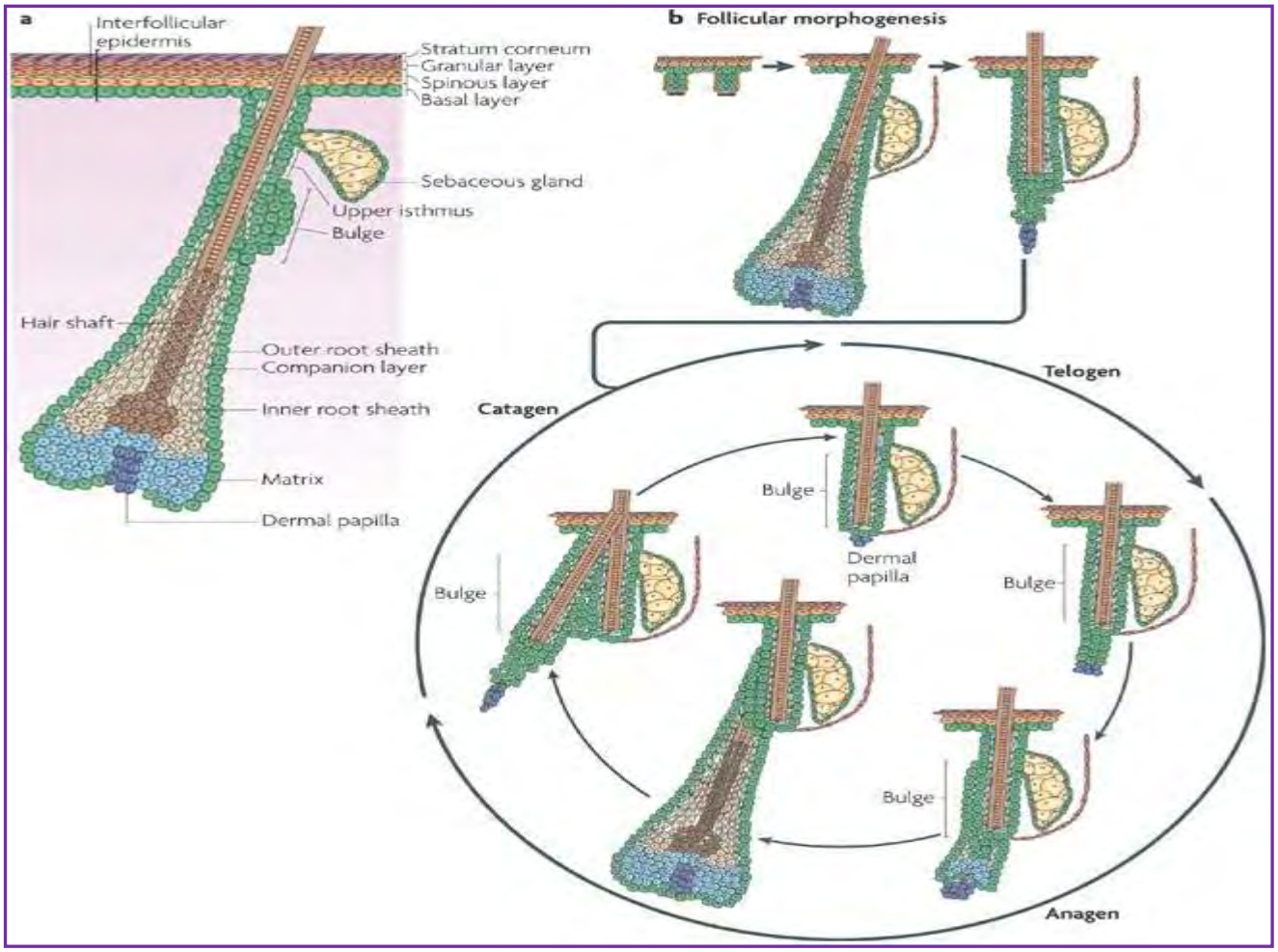


Figure 9 The hair cycle *(Blanpain C., et al (2009) Epidermal Homeostasis: a balancing act of stem cells in the skin. Nat. Rev. Mol. Cell. Biol. 10, 1209-1219.)*

1.5 Mice as model organisms

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its striking genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

Mice are small, easy to maintain in the laboratory and (compared to most mammals) have a short breeding cycle (about 2 months). They can produce 10-15 offspring per litter and approximately one litter every month. These feautures make them suitable for analysis. Laboratory mice are usually of

the species *Mus musculus* or also known as the house mouse. Mice belong to the Euarchontoglires clade, which includes humans. This close evolutionary relationship to humans, make mice particularly suitable models for human-oriented research.

Mouse models are nowadays available for genetic research and include thousands of unique inbred strains and genetically engineered mutants. There are mice prone to different cancers, diabetes, obesity, blindness, Lou Gehrig's disease, Huntington's disease, anxiety, aggressive behavior, alcoholism and even drug addiction.

The laboratory mouse has been a favorite subject for hair studies, and the pigmented C57BL/6 *(Chase HB. 1954; Paus R., et al. 1989)* and C3H *(Hattori M. 1983)* mice are the most commonly used strains**.** The rationale for choosing these mice is that their truncal pigmentation is entirely dependent on their follicular melanocytes; their truncal epidermis lacks melanin-producting melanocytes. Because pigment production is active only during the follicle growth (anagen) phase, the only time skin is dark is when the hair is growing. Therefore, by assessing the skin color one can also assesses the follicle growth phase.

Mouse hair develops in two consecutive waves. In mice 28 days old, the second generation of follicles was well advanced, comparable to 3 or 4 days postnatal growth of the first generation. In most rodents, however, large collections of follicles cycle together; in this situation, synchronous follicle growth occurs in waves that sweep posteriorly and dorsally *(Ahmed W., et al. 1998).* As the mouse ages, the waves become less frequent so that in the mature and senile mouse synchronous hair growth occurs only in relatively small patches.

Heretofore, we have discussed how the body and skin environment influence hair growth; the converse also is true, namely, the hair cycle influences the character of the skin. It has long been appreciated that the whole skin organ is influenced by the hair cycle. In fact, the entire skin architecture changes whenever thousands of hair follicles more or less simultaneously pass from one hair cycle stage to the next. With anagen the epidermis and dermis thicken, dermal vascularity and stromal content increase, and the subcutis layer increases in depth *(Hansen LS, et al. 1984)*.

Figure 10 Mouse as model organism *(http://www.nc3rs.org.uk/category.asp?catID=78)*

2. Aim of the project

The main objectives that were settled in order to be determined in this series of experiments concern

- the role of RANK in epidermal thickness and
- its affect on signal transduction in keratinocytes

Firstly, in order to certify, that Tg mice, which are overexpressing RANK, presenting elevated protein content on the epidermis during the 28^{th} post-natal day, when the second hair cycle is in anagen, BCA quantification protocol and statistic analysis was performed. This aimed to verify the notion that the proliferation of keratinocytes is linked to the RANK /RANKL function.

Secondly, we tried to identify which NF-κB signaling pathway- canonical or alternative- was induced by the overexpression of RANK in Tg mice in comparison with the WT mice. This was attempted by Westerrn Blot analysis protocols in different conditions and with various antibodies. As a consequence, this would be useful to define the genes that RANK's overexpression is inducing and how this overexpression determines the fate of the cell (differentiation//survival) through this pathway.

3. Materials & Methods

3.1 Animal breeding and maintenance

Mice were kept in a pathogen-free barrier facility following faculty guidelines and within its animal bioethics accord *(Duheron V., et al. 2011)*. Because mice generally breed at night, breeding performance is best when a consistent and uninterrupted light-dark cycle is maintained. Laboratory mice breed best when the temperature is between 16-26^{o}C (64-79^{o}F) and the humidity is between 40-60%.

Maintaining a colony of transgenic mice can be challenging. The mice were fed, watered and kept in individually ventilated cages.

Figure 11 Example of individually ventilated cages (Fawcett A., (2012) Guidelines for the Housing of Mice in Scientific Institutions *Animal Research Review)*

3.2 Transgenic mice

Transgenic mice overexpressing a protein have extra gene in their genome. Rank overexpression was under the control of the human macrophage-related protein (*mrp*) 8 promoter, which is specific for expression in epidermal keratinocytes.

In the Tg mice, epidermal thickness reached a maximum at 4 weeks (28 days), as it was noticed in previous experiments, and this is why 27-28 days old mice were chosen to be sacrificed.

The Rank-Tg mice were genotyped by PCR.

3.3 Tissue Lysis for PCR

For the preparation of samples for genotyping, cells from the tail were used, from both Tg and WT mice. Firstly, cell lysis and DNA extraction were conducted with use of Genomic DNA Purification Kit (from Thermo Scientific). This kit can be used for extraction and purification of high-quality genomic DNA from: whole blood or serum, cell culture, plant tissues, mammalian tissues, epithelium samples and bacteria.

Procedure	
	1) Cut the tissue into small pieces or disrupt it using a homogenizer. 2) Collect the material into a microcentrifuge tube and resuspend it in Digestion Solution. Add Proteinase K Solution and tissue Lysis Solution and mix thoroughly by vortexing to obtain a uniform suspension. 3) Add RNaseA Solution, mix by vortexing then incubate for 10 min at room temperature. 4) Incubate the sample at room temperature for 10 min, until the tissue is completely lysed and no particles remain. During incubation vortex the vial occasionally. 5) Incubate in 95 for 3 min. Add of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 × g. 6) Keep supernatant for PCR.

3.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical method in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

Figure 12 Thermo cycler *(http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html)*

In this part of identification of RANK Tg mice we enhanced the RANK gene using specific primers. The mix prepared for PCR assay contained:

PCR Mix
1. H_2O 2. Green Buffer (KCl and $(NH_4)_2SO_4$) 3. dNTP 4. Primer 5. Taq 6. DNA 7. $MgCl_2$

The Green Buffer contains an additional density reagent and two tracking dyes. The density reagent allows direct loading of PCR products in a gel. The blue dye (migrates with 3 to 5 kb DNA fragments in 1% agarose gel) and the yellow dye (migrates faster than 10 bp DNA fragments in 1% agarose gel) are included for monitoring electrophoresis progress.

3.5Agarose gel Electrophoresis

Gel electrophoresis is an easy and quick method to separate a mixed population of DNA, to measure nucleic acid quantity in a matrix of agarose. Biomolecules can pass through agarose gel pores and channels and separate, based on their length and charge. An electric field is applied to the DNA and RNA samples, as they are negatively charged molecules and consequently migrate to the positively-charged anode.

The PCR products were prepared for the electrophoresis in PCR mix which is compatible for the gel electrophoresis.

The preparation of agarose gel is summed up below:

For a 1.5% gel the required quantity of agarose is weighed; 1,5g per 100 ml. The first step is to place the solid agarose into an Erlenmeyer flask and dilute it up to the desired volume of concentrated TAE buffer stock solution. Next, we the mix placed in the microwave just until the appearance of boiling signs. After that the flask was removed and carefully swirled the agarose mixture, and the flask was

returned in the microwave to boil again. This step was repeated until there was no sign of any solid bits of agarose remaining. After that, the mix was stained with ethidium bromide in concentration 1/1000. This intercalating factor is used to visualize DNA/RNA as it fluoresces under UV light. When the mix temperature decreased it was placed in an electrophoresis casting tray, to solidify, and then in the electrophoresis tank. The tank was filled with Tris/Acetate/EDTA (TAE) DNA buffer, the same that was used to prepare the gel. After the agarose gel has been solidified, sample loading and electrophoresis can begin. Firstly, 1kb plus Invitrogen DNA ladder was loaded on the first well, the ladder provides bands with standard length to compare with the samples. After that the DNA samples were also loaded in the wells of the gel. The electrophoresis was performed over night (O/N) at 25V. In the end the electrophoresised gel was viewed and photographed using an ultraviolet (UV) transilluminator.

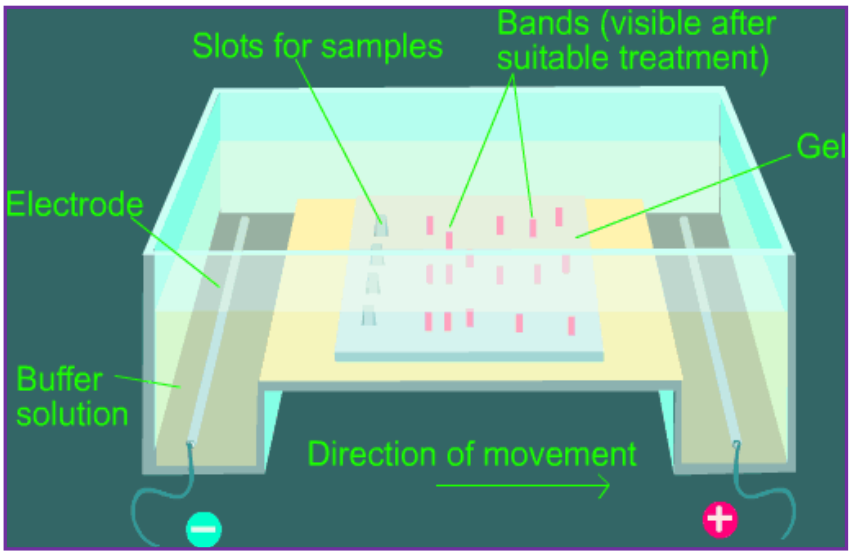


Figure 13 Diagram of an electrophoresis device
(http://animalsciences.missouri.edu/biotech/low/tech/findit/gel/diagram/index.htm)

3.6 Segregation of Epidermis

For the second part of this project the epidermis of WT and Tg mice will be needed. In this part we demonstrate a protocol for the isolation of the epidermis.

Different types of mice were sacrificed at the age of 27-28 days old where the second hair cycle is in the anagen phase. An advantage of mice in this age is that the epidermis is more easily detached. The dorsal skin was removed from the mice and weighed in order to have the same weight for the quantification protocol and used to continue.

Firtsly, the hypodermis was removed by razor, as it has a slightly darker color and thus is easily detected.

Secondly, a disassociation solution for tissue dissociation was prepared containing RPMI medium, FCS 2% and DISPASE II in 1/1000 dilution. RPMI is a general purpose media with a broad range of applications for mammalian cells and is used for cell cultures and cell deriving from living organisms. It contains aa, vitamins and all the necessary components for cell survival. FCS (Fetal Calf Serum) is

the blood fraction remaining after the natural coagulation of blood, followed by centrifugation to remove any remaining red blood cells. The rich variety of proteins in FCS, maintains cultured cells and isolated cells in a medium in which they can survive, grow, and divide. DISPASE II, is a neutral protease, fibronectinase and Type IV collagenase that can be characterized as a gentle agent for separating intact epidermis from dermis *(Kurt S., et al 1989)*. Every sample was placed in a plate with disassociation solution and the epidermis on the upper part, not in direct contact with the solution. The samples were incubated at 37^{o}C for 3 hours.

Thirdly, the epidermis was separated (peeled off) from the dermis with forceps. The epidermis has a distinct colloidal texture.

The samples were kept in ice with addition of TBS to avoid further protein degradation and keep them intact.

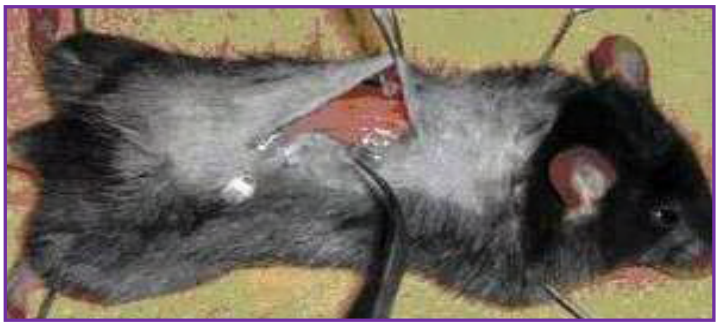


Figure 14 Removal of dorsal skin as an intact sample

3.7 Keratinocytes' Lysis and Potein Extraction

To prepare samples for gel electrophoresis, cells and tissues need to be lysed to release the proteins of interest. This solubilizes the proteins so they can migrate individually through a separating gel. There are many recipes for lysis buffers but a few will serve for most western blotting experiments. In brief, they differ in their ability to solubilize proteins, with those containing sodium dodecyl sulfate and other ionic detergents considered to be the harshest and therefore most likely to give the highest yield.

For the purpose of the experiment we had to resolve the keratinocytes and extract the proteins. We used two different protocols for this action. The first one would allow us to perform protein quantification by BCA assay in next steps, while the second one is known to have a well recognized action on lysis and extraction.

1. For this first assay, that would enable us quantification, we begun with the preparation of the lysis buffer containing:
    - 10ml Lysis-M buffer EDTA-free (ROCHE)
    - 1tablet Protease inhibitors
    - 1tablet Phosphatase inhibitors

The lysis kit full name was: copmplete Lysis-M kit (by Applied Science Roche). This kit contains a mild detergent in 25 mM bicine buffer (pH7.6). This simple extraction method allows very efficient and gentle extraction of proteins from both the cytoplasm and the nucleus of mammalian cells. The copmplete, Mini tablets provided with this kit allow the inhibition of a broad spectrum of serine,

cysteine and metalloproteases as well as calpains. Due to the optimized composition of the tablets, they show excellent protease-inhibiting effects and are therefore very well suited for the protection of proteins isolated from mammalian cells.

After the buffer's preparation we continued as following:
The epidermis was grinded with the addition of liquid Nitrogen, until a fine powder was formed. After that, immediately the powder was transferd to a microcentrifuge tube and 500μl of Lysis-M buffer was added. The samples were vortexed and placed on ice for 10min. The samples were sonicated for 4times/1min, taking some time for pausing between and put tubes in ice to avoid overheating. Then, the samples were incubated in room temperature for 30min and were vortexed ocassionaly. Finally, centrifugation was performed at 15000rpm/30min and the supernatant was kept and stored in -20^{o}C for further usage.

2. For the second essay, which combines lysis with preparation gel electrophoresis (Western blotting), cells might be lysed directly in 1x Laemmli buffer (obtained from Bio-Rad contained Tris-HCl, SDS, glycerol and bromophenol blue).

Laemmli buffer is especially formulated for protein sample preparation to be used in the Laemmli SDS-PAGE system. The SDS detergent denatures the proteins and subunits and gives each an overall negative charge so that each will separate based on size. The bromophenol blue serves as a dye front that runs ahead of the proteins and also serves to make it easier to see the sample during loading. The glycerol increases the density of the sample so that it will layer in the sample well. We prepared a lysis buffer containing Laemmli and β- mercapteptoethanol (9:1), which reduces the intra and inter-molecular disulfide bonds.

After the buffer's preparation we continued as following:
Part of the buffer added on the epidermis samples and they were grinded with mortar and pestle. When two faces appeared clearly (solid and fluid), we removed the liquid solution and transferred it in tubes. Centrifugation was performed in max speed for 5 min. The supernatant was kept and stored in -20^{o}C for further usage.

3.8 Bicinchoninic Acid Assay (BCA) for protein quantification

After having isolated the protein content of dorsal skin, coming from Tg and WT mice, we performed quantification in order to demonstrate that Tg mice are having more proteins on their epidermis in comparison with WT and moreover, to establish the difference between those two types and complete a statistic analysis.

On this protocol only samples that were treated with complete Lysis-M buffer were used as the protocol described above is compatible with the quantification assay. The quantification is performed automatically by a computer software and the preparation of the samples is described below.

This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid.1The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000μg/mL). The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA. Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known

concentration are prepared from the protein and assayed alongside the unknown(s) samples before the concentration of the unknown samples is determined based on the standard curve.

Pierce BCA Protein Assay Kit was used .This kit provides:

- BCA Reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide
- BCA Reagent B, 25mL, containing 4% cupric sulfate
- Albumin Standard Ampules, 2mgcontaining bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide

The procedure is summarized: A BCA buffer mix from both reagents is fixed with a ratio 25:1 and amount of this mix was placed in first well of plate and BSA solution protein of known concentration is added too. The BSA is diluted and in the same way the samples were diluted too with the addition of the buffer mix. The samples were incubated for 20min in 37^{o}C. The samples' optical density (OD) was measured at 570nm.

Figure 15 Example of color degradation appeared with samples' dilutions
(http://t3.gstatic.com/images?q=tbn:ANd9GcTnn9HaGAcGgiI7wsX1k8z_LMWsVGWoPSh-wTBDpFNKN36jPW0Xlw)

3.9 Denaturating Polyacrylamide gel electrophoresis (SDS-PAGE)

As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called SDS-PAGE. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

Acrylamide gels achieve separation in a size-selective way when electricity is applied to them. This happens as proteins migrate through the gel, in response to the electric field. The smaller sized proteins move rapidly as opposed to the larger proteins which are found closely to the start of the gel. The gel has a vertical orientation and is mounted between two buffer chambers, which make the gel itself the only way for the electricity to pass.

Laemmli buffer and PBS was added to the samples that where treated with Lysis- M, as well as for the samples treated with Laemmli for protein extraction. Both Laemmli and PBS were added, so as to achieve the protein concentration needed, in the appropriate volume for the gel. The samples were

heated in 90^{o}C for 15 min and at the same time Pre-mixed running buffer (NOVEX Tris-Glycine SDS 1x) was prepared. Precast gradient gels were used (Bio- Rad, Mini-Protean TGX) for the electrophoresis. The gel cassette was placed in the electrophoresis device and the samples were loaded to the wells. On the first well marker "Presicion plus protein standards dual color" was loaded. For positive control for the canonical pathway we used protein extraction from 293T cultured cells which were stimulated by TNF-α and for negative control RANK $^{-/-}$ mice cells. Then the buffer was added and the electrophoresis began. It was performed for 30 min at 200V.

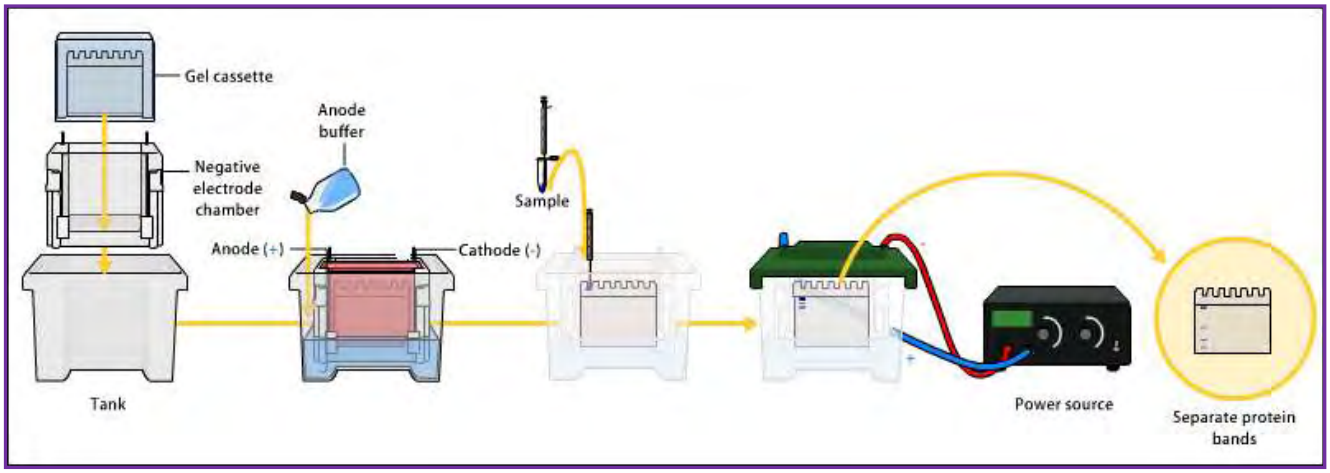


Figure 16 Schematic representation of SDS- PAGE
(http://en.wikipedia.org/wiki/Polyacrylamide_gel_electrophoresis#mediaviewer/File:SDS-PAGE_Electrophoresis.png)

3.10 Coomassie Brilliant Blue staining Assay

Coomassie Brilliant Blue is the name of two similar triphenylmethane dyes that is commonly used for staining proteins in analytical biochemistry, to stain proteins in sodium dodecyl sulfate and blue native polyacrylamide gel electrophoresis (SDS-PAGE and BN-PAGE, respectively) gels. The gels are soaked in dye and excess stain is then eluted with a solvent ("destaining"). This treatment allows the visualization of protein bands. The gel usually contains a set of molecular weight can be estimated in an unknown solution during the visualization.

The gel that was prepared as described above, could be used for Coomassie Brilliant Blue G250 staining assay. This assay took place to identify if the extraction assays, combined to the electrophoresis conditions were befitted and allowed the proteins to run properly to the gel.

The procedure followed to produce the Coomassie Blue G-250 staining regeant was:

- 0.2g dye in 100 ml H_2O at 50^{o}C was dissolved.
- The solution had been let to cool and 100 ml 2N H_2SO_4 were added.
- Incubation at room temperature O/N had followed.
- Filtration of the solution and addition of 22.2 ml 10N KOH and 28.7g TCA.
- The solution was allowed to stand for 3h, and then was filtered again.

The gel was immersed in the solution above. Bands would begin to appear within 15 min. Intensity and sensitivity would continue to improve for several hours. These gels were left over night (O/N) in the solution.

3.11 Western Blotting Assay

The western blot (sometimes called the protein immunoblot) is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein *(Towbin H., et al. 1979; Renart J., et al. 1979)*. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis.

Following the separation of the protein mix the polypeptide bands are transferred to a membrane carrier. For this purpose the membrane is attached to the gel and this so-called sandwich is transferred to an electrophoresis chamber. The applied electric charge causes the proteins to travel out of the gel vertically to the direction they traveled in on the gel, onto the membrane. The protein bands are thereby bound to the membrane. The "blotted" bands are now available to be treated further (e.g. for detection of specific proteins with specific antibodies). The membrane is then incubated with labels antibodies specific to the protein of interest. Specific antibodies bind to "their" band of proteins. Unspecifically binding antibodies are removed by washing with detergent-containing buffers. Additionally, unspecific binding pockets can be blocked before the addition of specific antibodies. Primary antibodies are usually applied first, which are then recognized by a secondary antibody. The secondary antibody is conjugated with colour, radioactivity or an enzyme for detection. Biotin-conjugated antibodies are also used for this purpose. Analysis of the western blot is then carried out using a variety of different imaging systems (e.g. autoradiography).

Gels that were electrophoresised as described above have been used for this protocol of Western Blot. The proteins were transferred in a PVDF membrane from Trans Blot Turbo blotting system by Bio-Rad. This is a semi dry Western blotting technique that reduces the transfer time (7 min).

Figure 17 The Trans Blot Turbo blotting system by Bio- Rad *(http://www.bio-rad.com/en-us/product/semi-dry-rapid-blotting-systems/trans-blot-turbo-transfer-system)*

After the transfer the following steps are needed to detect the protein.

First, the blocking of the membrane had to be performed**.** Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, steps must be taken to prevent the interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS) or Phospate Buffered Saline (PBS) , with a minute percentage (0.1%) of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the western blot, leading to clearer results, and eliminates false positives.

Second, the detection took place**.** During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when exposed to an appropriate substrate this enzyme drives a colorimetric reaction and produces a colored product. This process traditionally takes place in two steps for various reasons.

- Primary antibody

After blocking, the membrane is incubated with a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) under gentle stirring. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight.

- Secondary antibody

After rinsing the membrane to remove excess or unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary antibody will bind to almost any mouse-sourced primary antibody, which allows some cost saving by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly, a horseradish peroxidase-linked secondary antibody is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed on top of the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blotted membrane. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark purple stain that can be photographed without using specialized photographic film.

As a first step on this project we used an antibody for β-actin to detect the existence of protein on the membrane. Actins are an essential component of the cytoskeleton, with critical roles in a wide range of cellular processes, including cell migration, cell division, and the regulation of gene expression *(Bunnell T. M. et al. 2011)*. Actins are also highly conserved proteins that are involved in cell motility, structure and integrity. Beta actin is usually used as a loading control, and among others, to detect protein degradation, and also in PCR and Western blotting. Its molecular weight is approximately 42 kDa.

For this antibody we worked as it follows:

A blocking solution of PBST milk 5% on PBS 0,2%, was prepared and in which the membrane was incubated for 90min. The primary β-actin mouse antibody (Ab) from mouse was added on the membrane. The solution for the Primary Ab was 1:5000 on PBS T milk 1%. The membrane was incubated with the Primary Ab O/N at 4°C on a shaker. Following, five, 5min washes under gentle stirring took place, with PBST 0,1% and the Secondary Ab was added. The secondary Ab was Goat anti Mouse linked to the reporter enzyme horseradish peroxidase (GAM-PO) (1:10000) in PBST milk 1%. The membrane was incubated for 1h with the Secondary Ab. Then the membrane was washed 5 times for 5 min each time, under gentle stirring in PBST 0,1%. Consequently, the membrane was gently dried and placed with the blotted proteins facing up and the mix for the substrate was prepared (Immobilon Western Chemiluminescent HRP Substrate), mixing equal volumes of Luminol Reagent and Peroxide Solution. The blot was incubated for 5 minutes at room temperature and then the excess substrate was drained and the blotted membrane was covered with a clean plastic wrap and any air bubbles were removed. A photographic film was exposed to the membrane for 15 sec.

After having certified that proteins can be detected on the membrane we had to decide which particular Ab we should use in order to identify which NF-κB signaling pathway is induced by the RANK/RANKL interaction on the surface of keratinocytes, and if the signaling pathway which is induced is different between Tg and WT mice. Aiming in the detection of this distinction we decided to use rabbit NF-κB p105 / p50 Ab in order to detect the classical pathway and rabbit NF-κB p52/p100 to detect the alternative pathway. The methodology followed was the same for both Ab and as disccribed above for the β-actin. In detail:
A blocking solution of PBST BSA 5% on PBS 0,2%, was prepared and in which the membrane was incubated for 90min. A Primary rabbit Antibody (Ab) was added to the membrane. The solution for the Primary Ab was 1:5000 on PBST BSA 1%. The membrane was incubated with the Primary Ab O/N at 4°C on a shaker. Then, the membrane was washed 5 times for 5 min each time under gentle agitation, with PBST 0,1% and then, the Secondary Ab was added. The secondary Ab was Goat anti Rabbit linked to the reporter enzyme horseradish peroxidase (GAR-PO) (1:10000) in PBST BSA 1%. The membrane was incubated for 1h with the Secondary Ab. Then, the membrane was washed 5 times for 5 min every time, with gentle agitation with PBST 0,1%. Consequently, the membrane was gently dried and placed with the blotted proteins side up and the mix for the substrate was prepared (Immobilon Western Chemiluminescent HRP Substrate), mixing equal volumes of Luminol Reagent and Peroxide Solution. The membrane was incubated for 5 minutes at room temperature and then the excess substrate was drained and the blot was covered with a clean plastic wrap and any air bubbles were removed. Finally, a photographic film was exposed to the membrane for 15 sec.

4. Results

4.1 Agarose gel Electrophoresis

The mice were constantly genotyped so as to avoid mixed breeding and the Tg mice were identified by the presence of the transgene band. PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. Specific primers were designed so as to be complementary to this specific region of the transgene promoter. The results are shown on the picture below.

Figure 18 Genotyping of WT and Tg mice (positive).

4.2 Bicinchoninic Acid Assay (BCA) for Protein quantification (BCA)

The quantification of protein content was calculated based on the known concentration of BSA solution and using the standard method of BCA quantification automated by computer software. In order to determine the concentration of the samples and the difference between Tg and WT mice we used standard curve designed based on BSA concentration. More specifically:

1. We diluted the solution of BSA of known concentration (2mg/ml) with the mix which was prepared as described in: "Methods". A series of successive dilutions were performed so as to plot the standard curve. Firstly, 20 μl of the solution of BSA 2mg/ml was used and brought up to a 200μl of final volume after the addition of 180μl of the mix. Every other dilution was made using 100 μl from the previous dilution and adding 100 μl of the mix (total volume in every well was 100 μl). The standard curve was plotted using the dilutions against the absorption of each dilution.
2. Moreover, we diluted the unknown concentration samples. The samples were diluted in 10/100 and 1/100, adding in the first well 10 μl of the sample and 90 μl of the mix and in the second well 1 μl of the first well and 99μl of the mix.
3. The plates were incubated for 30min at 37^{o}C.
4. Spectrophotometric analysis of the samples in the well plates at 570nm.
5. The absorption of BSA minus the absorption of the blank estimated the standard curve.

6. The average of two 10/100 dilution absorptions and of two 1/100 dilution absorptions were used to define the average protein concentration in the primary protein extraction.
7. The protein concentration from every sample of each mouse type was used to determine the average protein concentration of WT and Tg keratinocytes.

Abs (570nm)	Abs (570nm)	Average Abs	[C] BSA (µg/µl)
0,999	0,943	0,971	0,2
0,588	0,584	0,586	0,1
0,393	0,382	0,387	0,05
0,269	0,270	0,270	0,025
0,203	0,193	0,198	0,0125
0,164	0,159	0,162	0,00625
0,144	0,139	0,141	0,003125
0,128	0,128	0,128	0,001563
0,121	0,116	0,119	0,000781
0,118	0,113	0,115	0,000391
0,110	0,113	0,112	0,000195
0,112	0,111	0,111	

Table 1 Absorption and correspondent concentrations of BSA

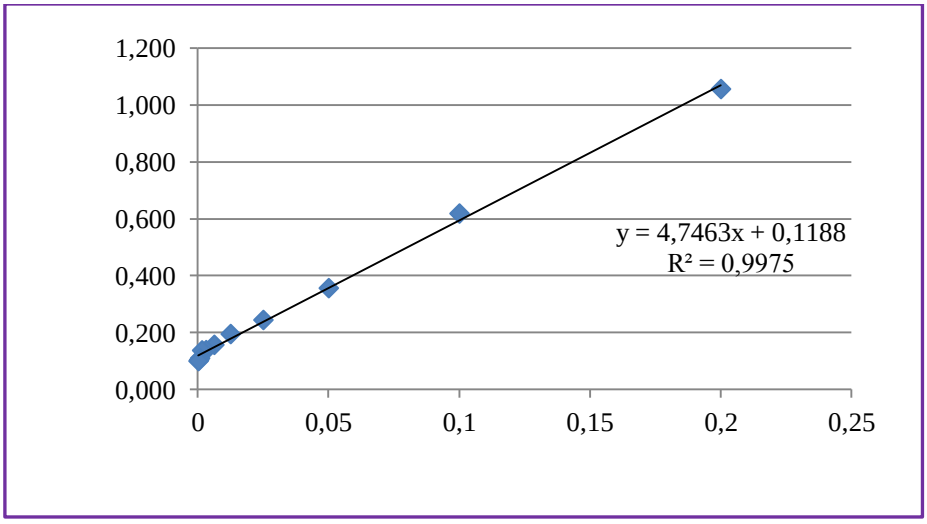


Figure 19 Standard curve designed based on the absorption of known concentration of BSA solution

	10/100 Abs	1/100 Abs	Average Abs		[C] (µg/µg)		Average [C] (µg/µl)
29.TG	1,688	1,151	1,727	1,131	4,003	260,415	**132,209**
29.TG	1,766	1,111					

Table 2 Example of calculation of average concentration of one sample Tg mice

The data below demonstrates the fact that keratinocytes from WT mice have lower protein concentration in comparison with Tg mice.

	WT (µg/µl)	TG (µg/µl)
1.	32,299	40,140
2.	41,853	54,724
3.	48,370	77,979
4.	72,481	80,962
5.	80,520	121,610
6.	104,930	122,409
7.	105,861	132,209
8.	108,840	135,984
9.	109,460	138,110
10.	128,738	138,938
11.		156,181
12.		168,655
13.		179,570
Average	**78,290**	**113,992**

Table 3 The average protein cocetration of every sample used

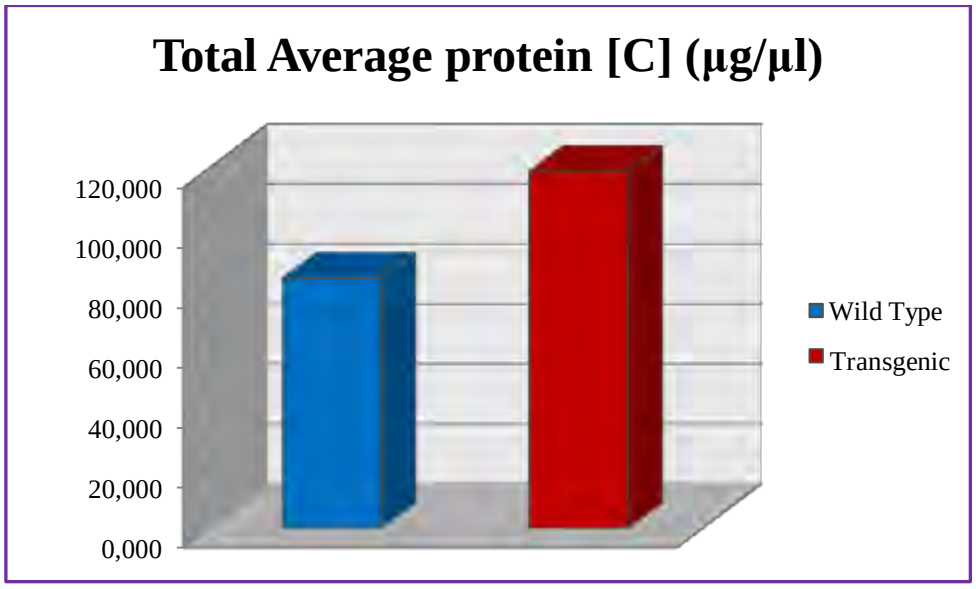
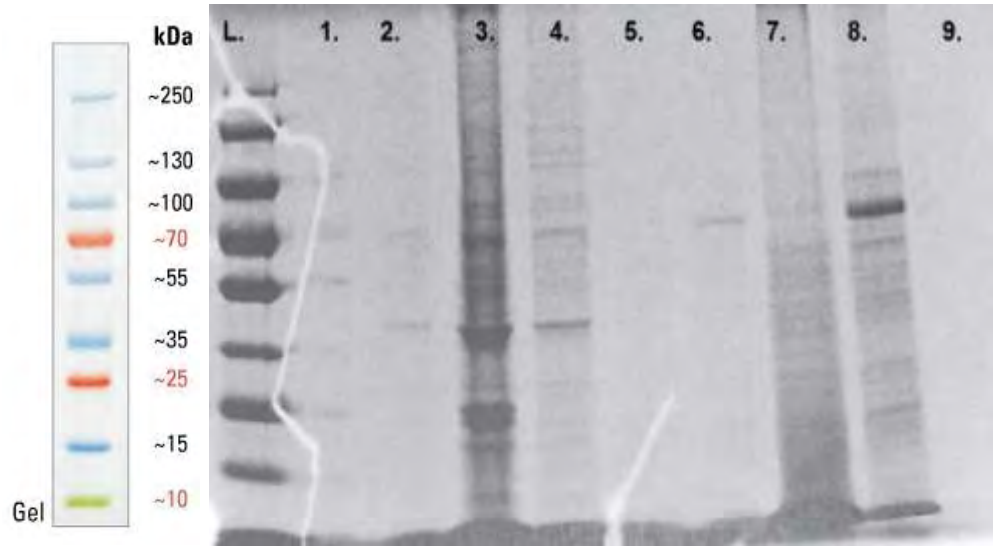


Figure 20 Comparison of WT and Tg protein concentration in keratinocytes of the epidermis

Based on the absorption of each sample protein concentration was calcaluted for each sample and for each type an average value was calculated to exhibit the antithesis between those two types. For this statistic measurement we used 8 WT samples and 13 Tg samples, as shown on Table 3.

4.3 Coomassie Brilliant Blue Assay

The primitive gel used for this assay was used to determine the optimal running conditions of the proteins under denaturating conditions (SDS-PAGE) on precast gradient minigels and so as to visualize the electrophoresised proteins, as shown in Figure 21.



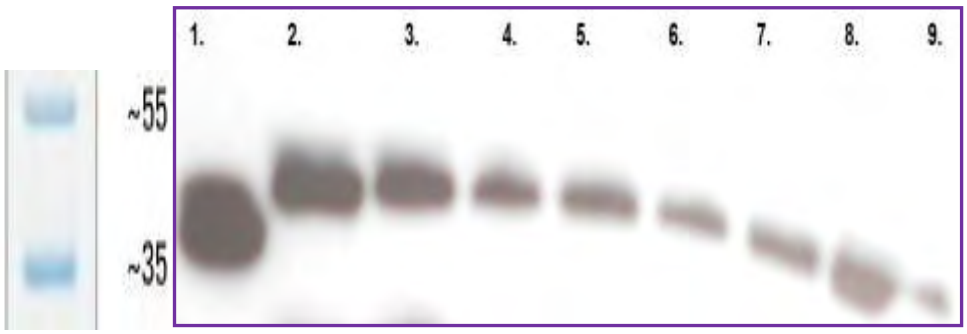
Ladder	L
Empty	1. 9.
WT	2. 5. 6.
Tg	3. 4. 7. 8.

Figure 21 Gel stained with Coomassie Brilliant Blue G-250

We continued using the same conditions of protein extraction and SDS-PAGE assays.

4.4 Western Blotting

On this gel samples of both Tg and WT mice were probed with β- actin. The results are shown in the picture and the protein is detected around 42 kD as expected, with higher signal on the control cells and Tg mice.



Positive control cells	**1.**
Tg	**2. 3. 4. 5. 8.**
WT	**6. 7. 9.**

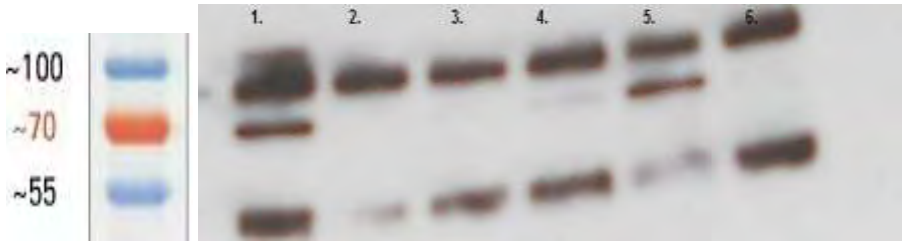
Figure 22 β-actin is detected in all samples at 42kD.

We faced some difficulties with the antibodies for both NF-κB signaling pathways as the Ab seemed to bind with no specificity. Beside this, after many experiments under different conditions, different concentrations and different saturation media we finally came to a result.

For the canonical pathway we used p52/p100 in different dilutions for the primary Ab and secondary Ab, in different saturation media (milk, BSA) and with different buffers (TBS, PBS).

We also used NIK Ab which gave us a disappointing image of non specific binding.

We determined that in both Tg and WT mice the alternative pathway is induced. As the picture shows p52/p100 was detectable in all samples.



WT	**1. 4. 5.**
Tg	**2. 3. 6.**

Figure 23 p52/p100 (52/100 kDa) detection proves induction on the alternative signaling NF-κB pathway

The antibodies used for the canonical pathway gave no image, indicating that this pathway was not induced. For the canonical pathway we used p50/p105 Ab. To exclude the induction of the canonical signal pathway we also used p65 (RelA) Ab which gave no signal as well.

5. Discussion - Conclusion

RANK and RANKL are mainly known for their activity in bone development and homeostasis. Epidermal homeostasis is critical to maintain the capacity of the skin to protect the organism against external aggressions. A number of molecular pathways have been implicated in regulating epidermal renewal, proliferation and differentiation. NF-κB pathway is one of those implicated. RANK-deficient mice as shown in previous experiments demonstrate a thinner epidermis and reduced epidermal renewal indicating tha RANK may be implicated in skin homeostasis, as an important regulator.

We ought to prove that Tg mice for RANK, with tissue specific promoter, display thicker epidermis and protein content as well as high basal cell proliferation rate. The overexpression of RANK provokes an increased expression of soluble RANKL, too. The high protein content of Tg mice was proven by quantification assays and by the comparison with WT mice. The difference between those types of mice is given by statistic analysis of protein concentration.

On the second and more complex part of the experiment, we had to decide which Ab should be used for both signaling pathways. This took a lot of effort and experimentation. Additionally, the chosen Ab should also be used in optimal conditions (saturation media, dilution, dilution of secondary Ab, probing time, exposure time on the film). The optimization of these conditions for Western Blot was the most challenging part.

This assessment was the result of many experiments which had already failed in:

a. Isolation of the target protein
b. Estimation of the right concentration of the protein on the gel
c. Transfer of proteins from the gel to the membrane
d. Estimation of concentration of the Blocking Reagent (too diluted)
e. Right dilution for the Ab (too diluted, too concentrated)
f. Incubation time (too short)
g. Wash time (too long, too short)
h. Non specific binding of the Secondary Ab
i. Signal development time (too long, too short)

Based on the final and repetitive results we agreed on the fact that, both types of mice used seemed to induce the alternative pathway in keratinocytes as was proven with Western Blot assays, detecting p52/p100 which participates in the aforementioned pathway.

The value of this finding may lay on the fact that the alternative pathway induces the transcription of specific families of genes. Even though the finding that p52/p100 are present in both types of cells (WT and Tg) should enforce the investigation of which genes are induced into transcription, we should also have in mind that p52 formulates a complex with Bcl-3 that can act as an inhibitor of the transcription of certain genes. This identification could be conducted with in situ hybridization, using a specific Ab for the protein p50 and p52 or RelA and RelB we could clarify by their localization (in the nucleus) if they act as transcription (or inhibitors) factors. Furthermore, to identify if the role of p52 is to inhibit the transcription of certain genes we could try to detect or co-localize Bcl-3 as well, because it is known that when they form a complex that acts as an inhibitor.

In conclusion, the fact that RANK is an important regulator of epidermal homeostasis and keratinocytes proliferation can be supported by the obtained data. It activates keratinocytes' proliferation increasing their protein content. Moreover, we showed that the alternative signaling pathway is induced by the RANK/RANKL binding. These results, can be the start of using RANK

antagonistic molecules in the treatment of skin disorders where the epidermal homeostasis is dysfunctional.

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