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Molecular biology

The role of the uPAR in tumor progression

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Abstract

The urokinase receptor (uPAR) is well known for its role in cell migration, invasion and tumor progression. Using uPAR knock out (ko) mice, we tested the hypothesis that uPAR may participate in tumor development by regulating expansion of the stem cell compartment, migration of these same skin stem cells within the tissue and inhibition of differentiation. After skin activation by an injury or simply by shaving the mice, so inducing the anagen phase, uPAR ko epidermis showed a reduced capacity to heal the wounds and a reduced hyperproliferation of the hair germ which is moving downwards into the dermis, compared to the wild type mice.

Emigration of the upper follicular stem cells (progeny population) into the epidermis is clearly decreased in uPAR ko skin, demonstrating that uPAR contributes for populations of follicular keratinocytes to epidermis regeneration during a time of tissue expansion, which could be after an injury or during early stages of tumor progression.

Besides, other experiments demonstrated that uPAR deficiency, not only reduces keratinocytes proliferation and delays epidermis and hair follicle regeneration, but accelerates also the terminal differentiation. Involucrin is in fact up-regulated by uPAR ko mice both in anagen and telogen phase.

The fact that during the anagen phase, Rac1 activation is increased and that Myc expression is down-regulated in uPAR wt mice compared to the ko, suggests the possible mechanism by which uPAR regulates activated epidermis regeneration. These findings offer an opportunity for gaining insight into the earliest stages of neoplastic development.

Samenvatting

De urokinase receptor (uPAR) is bekend voor zijn rol in cel migratie, ivasie, en tumor progressie. Door uPAR knock out muizen te gebruiken, hebben we de hypothese getest dat uPAR een rol zou hebben in de ontwikkeling van tumoren doordat het een regulerende functie heeft op; de expansie van het stam cel compartiment, de migratie van deze stam cellen door het weefsel en de remming van de differentiatie van deze cellen.

Wanneer de huid geactiveerd wordt door een wond of door het scheren van de huid, gaat de huid in de anagen fase. De uPAR ko epidermis liet in deze een verminderde capaciteit zien om wonden te genezen, en daarnaast een verminderde hyperproliferatie bij de neergaande beweging van de haar beginselen in de dermis, vergeleken met de wild type muizen.

De migratie van de bovenste folliculaire stam cellen (ook wel voorloper cellen) in de epidermis is duidelijk vertraagd in de uPAR ko muizen. Dit betekent dat uPAR een bijdrage levert aan folliculaire keratinocyten die de epidermis genereren tijdens uitzetting van het huidweefsel. Dit zou kunnen na een wond, of tijdens de vroege fases van tumor ontwikkeling.

Daarnaast lieten eerdere experimenten zien dat de afwezigheid van uPAR niet alleen de proliferatie van keratinocyten en de hernieuwde groei van de epidermis en de haarfollikel reduceert, maar dat het ook de differentiatie van de huid versnelt. De differentiatie marker involucrine is daadwerkelijk verhoogd in uPAR ko muizen in zowel de anagen als de telogen fase van de haar cyclus.

Het feit dat tijdens de anagen fase van de haar cyclus de activering van RAC1 verhoogd, en de expressie van myc juist verlaagd is in wt muizen vergeleken met de ko muizen, suggereert de mogelijke mechanismen waarmee uPAR de heropbouw van de geactiveerde epidermis reguleert. Deze bevindingen geven een mogelijkheid om een beter zicht te krijgen op het begin van neoplastische groei.

List of Abbreviations

BrdU	Bromodeoxyuridine
C	Celsius
DMBA	Dimethylbenzanthracene
DNA	Deoxyribonucleic acid
DP	Dermal papilla
ECM	Extracellular matrix
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine Serum
FFPE	Formalin fixed paraffin embedded
GPI	Glycosylphosphatidylinositol
H	Hour
H&E	Haematoxylin & Eosin
IRS	Inner root sheath
kDa	Kilo Dalton
KO	Knock Out
LRC	Label retaining cell
MMP	Metalloproteinase
NK	Natural killer
ORS	Outer root sheath
PA	Plasminogen activator
PAI	Plasminogen activator inhibitors
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pro	Pro-enzyme
RNA	Ribonucleic acid
RPM	rounds per minute
RT	Room temperature
SC	Stem cell
suPAR	Soluble uPAR
TGF	Transcription growth factor
TPA	Tetradecanoylphorbolacetate
TA	Transient amplifying
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
Vn	Vitronectin
WT	Wild Type

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Introduction

uPAR

The specific cell surface urokinase plasminogen activator receptor uPAR/CD87 is produced as a 313 amino acid polypeptide. This peptide will be processed post-translationally at the carboxyl terminus into a protein which contains 283 amino acids. [1, 2] UPAR was first described as the cellular binding site for uPA in 1985 [3], and is connected to the cell membrane at Gly_{283}, which binds to a glycosylphosphatidylinositol (GPI) anchor [4]. Therefore it is obvious that uPAR is a member of the Ly-6 superfamily of GPI anchored proteins and it doesn't contain a transmembrane or a cytoplasmic domain [5].The 55-60 kDa protein uPAR is heavily glycosylated and consists of three cysteine rich homologous domains (D1, D2 and D3) which are connected by short linker regions. The primary binding site for uPA and vitronectin is the amino-terminus of domain D1. The binding affinity increases with the presence of D2 and D3.

Cell surface uPAR has been shown to undergo two major types of covalent modifications which alter the function of the receptor. The first type of uPAR cleavage is either a proteolytic cleavage close to the GPI anchor or a hydrolysis of the GPI-anchor by a phospholipase [6, 7]. This cleavage releases the entire receptor from the cell surface (suPAR), with concomitant functional changes, but does apparently not notably alter the ligand binding properties of the receptor. This process is called uPAR *shedding*. The second type is a proteolytic cleavage in the linker region connecting D1 and D2 and results in the release of the D1 fragment from the rest of the receptor. This cleavage changes the biochemical properties of uPAR completely. This phenomenon is called uPAR *cleavage*.

Recent advances in the study of uPAR shedding and cleavage supports the possibility that these processes are important in the malignant process of tumor invasion and metastasis. The GPI-anchoring of uPAR renders the protein prone to release from the cell surface and soluble forms of uPAR can indeed be found both *in vitro* and *in vivo.*

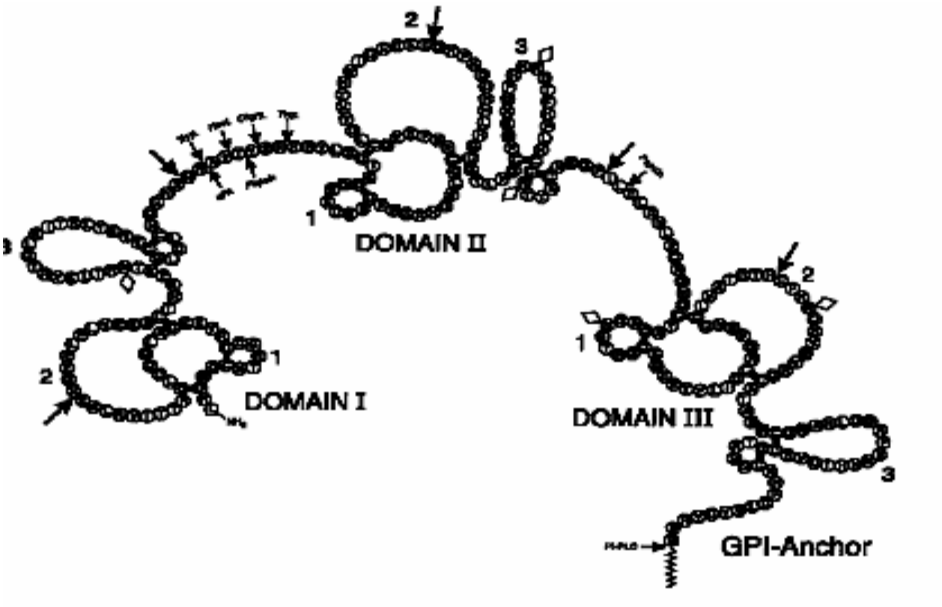


Fig 1 The amino acid chain of uPAR showing its 3 domains.[8]

In most of the human tissues uPAR cannot be detected, but there are a few exceptions. The receptor is detected in some haematopoietic cells, such as monocytes, eosinophils neutrophilic granulocytes, skin mast cells, dendritic cells, activated T-lymphocytes and natural killer cells, but it is also expressed in non haematopoietic cells, such as endothelial cells, hepatocytes, fibroblasts, keratinocytes, smooth muscle cells and placental throphoblasts.[9]
Besides its ability to bind uPA to the cell surface, uPAR is capable of localizing itself to specific sites where plasminogen activation is required. Usually uPAR is located on specific parts of the cell surface. It's mainly found at cell-ECM contacts, cell-cell contacts and in particular to the leading edge of migrating cells. The mechanism that regulates the localisation of uPAR on the cell surface appears to depend on the association of uPAR with uPA, integrins, and the ECM protein vitronectin. [10]

Plasminogen activation

The zymogen plasminogen is produced mainly by the liver, and is present in the plasma as well as extravascularly in the interstitial fluids. When plasminogen is cleaved at the Arg_{561} – Val_{562} bond, it is converted to the active serine protease plasmin. The two poly peptide chains of plasmin are held together by a disulfide bond. There are several proteases which have the ability to activate plasminogen, but its specific physiological activators are uPA and tissue type PA (tPA) [4, 11, 12].

Plasmin degrades extracellular matrix components, such as fibrinogen, fibronectin en vitronectin. Besides that, plasmin activates zymogens of different metalloproteinases (MMP's), which also degrade the extracellular matrix [1, 4, 13]. Together with these functions in proteolysis, plasmin can also activate a latent growth factor, being transforming growth factor β1 (TGF- β1). The active form TGF- β1 is involved in ECM repair [14].

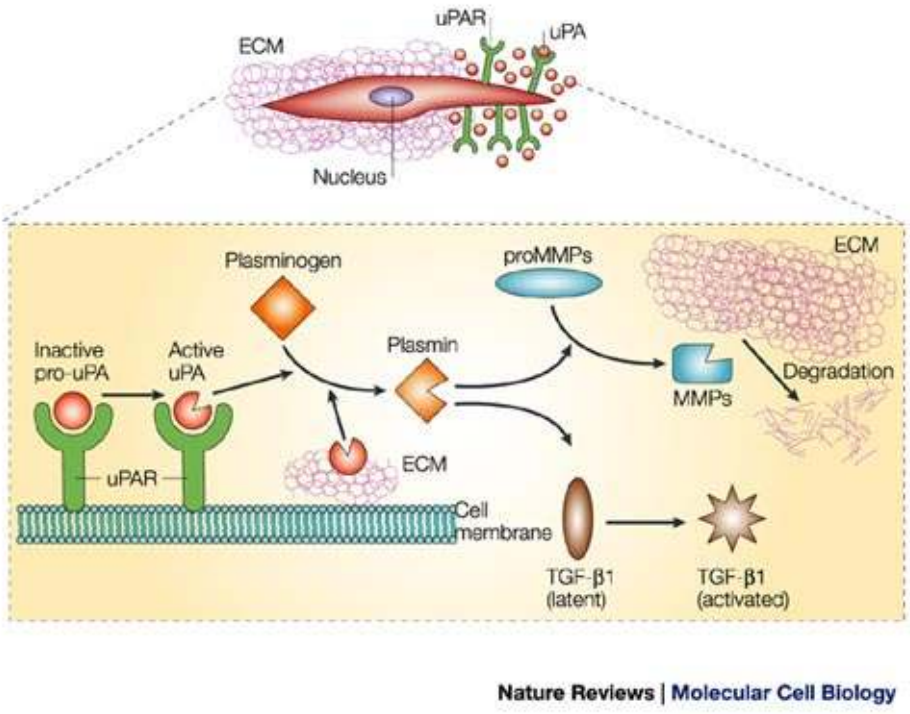


Fig 2 A schematic overview of the PA system and its main functions. [14].

Pro-uPA and uPA

Pro-uPA is the single chain proenzyme form of uPA, is synthesized and secreted as a single glycoprotein chain of 52 kDa. The enzyme undergoes several post-translational modifications before it binds to its receptor uPAR. After secretion, pro-uPA is bound to uPAR and can be cleaved by several proteases, but the main protease which cleaves pro-uPA at k158-k159 into uPA is membrane bound plasmin. Once pro-uPA is activated, the molecule contains three major functional domains: the N-terminal growth factor domain which is responsible for the binding to uPAR, the C-terminal protease domain which cleaves plasminogen, and finally the kringle domain [5, 14].

Many different cell types secrete pro-uPA and elevated levels of production are in particular associated with the transformed phenotypes of cells. In adherent cells uPA is almost exclusively localized at cell-cell contacts and at focal adhesion contacts with the ECM [15, 16].

Over the last years evidence has accumulated for an important role of uPAR/uPA in regulating cell adhesion. Firstly, uPA/uPAR promotes cellular adhesion to the ECM protein vitronectin (VN) by a direct high affinity interaction between uPAR and VN. Secondly, uPAR modulates cell adhesion to a variety of ECM proteins by lateral interactions with adhesion receptors from the integrin family. This is reasonable, considering the fact that in order to spread, cells must bind to vitronectin, and induce the integrin dependent signalling pathways that lead to reorganisation of the cytoskeleton.

The interaction between uPAR and Vn causes a potent induction of actin cytoskeleton rearrangement and cell motility. This mechanism also requires rac activation [10].

High levels of uPAR also activated the ERK/MAPK1/2 signalling, which in turn are key factors in cell proliferation, differentiation and cell survival [14].

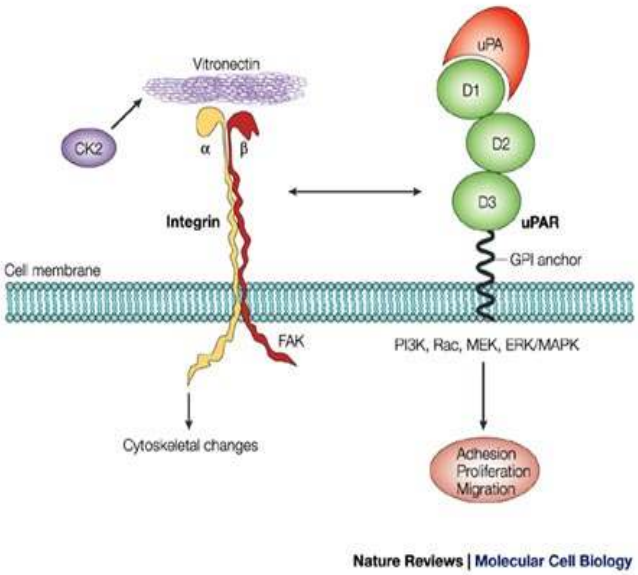


Fig 3 Shows the attached uPAR molecule, its components and its involvement in functional processes.[14]

Plasminogen activators inhibitors

Inhibition of the plasminogen activator complex is done at the level of the plasminogen activators uPA and tPA, by PA inhibitors (PAIs).

PAI-1, in particular, forms 1:1 complexes specifically with the active (two chain) forms of the plasminogen activators [17] . PAI-1 is a 52 kDa glycoprotein secreted by a wide range of cell types including vascular endothelium, platelets, hepatocytes, fibroblasts, and many different tumor cell lines. This protein interacts with circulating and ECM forms of Vn which stabilizes PAI-1 in its active configuration [18-21]. It is considered to be the principal regulator of fibrinolysis in the vasculature and recent findings demonstrate that it also plays important roles in processes as cell migration, adhesion and tumor angiogenesis [22].

It was demonstrated that the binding sites of PAI1 and uPAR on Vn are overlapping. Therefore the binding of these proteins is competitive and mutually exclusive. An excess of active PAI1 over uPA will inhibit cell adhesion mediated by uPAR, whereas an excess of uPA will stimulate this process [1, 10].

uPAR and cancer

Breakdown of the extracellular matrix by cell surface proteolysis has an important role in fibrinolysis, artherosclerotic plaque formation, inflammation, angiogenesis, mammary gland involution, and matrix remodelling during wound healing [8, 23].

However, degradation of the extracellular matrix by controlled proteolysis is also crucial for cancer invasion and metastasis. Indeed, both uPA and uPAR were found to be overexpressed not only in several human tumors including leukemias, tumor of the breast, lung, bladder colon, liver, pleura, pancreas and brain [24-34]. but also in various tumor-associated cells like macrophages, mast cells, endothelial cells, NK cells and fibroblasts [8, 11, 12, 35]. Several molecular components that cause matrix degradation, in fact, have clinical value as strong prognostic markers in different types of cancer. For example, elevated uPA, PAI-1 and uPAR protein levels in tumor tissue and blood are associated with a poor relapse-free and overall survival [10, 11].

Tumor angiogenesis, a necessary event in tumor progression to sustain tumor growth and metastasis dissemination, is also modulated by the uPA/uPAR system [9, 13, 36-39].

Since uPAR is involved in cell adhesion and migration and since cell migration is a process that includes tightly regulated steps of adhesion and de-adhesion, the counteracting uPAR-dependent effects of proteolysis and adhesion play a key role in migration, tumor cell invasion and metastasis [40].

uPAR as target for anti-cancer therapy

The important role in cell adhesion, proliferation, migration and invasion, makes uPAR an attractive potential drug target in cancer treatment. [41]. However, since these proteases have also functions in healthy humans, it‘s inevitable that this inhibition might have toxic side effects. Besides that, due to an overlap in different protease systems more inhibitors should be used to get an effective therapy. Therefore, for every type of cancer, a therapy with the best effect and the lowest toxicity has to be found. [11].

Most experimental strategies have been focused on reducing uPA-mediated plasminogen activation, a goal which may be obtained by a variety of approaches. Firstly by reducing the expression of uPAR or uPA, for example by using a vector expressing an antisense transcript against uPAR. An example, in this case, is the reduced invasive potential of highly malignant human squamous carcinoma cells. In fact, the lower level of uPAR achieved by this method can lead to a permanent state of tumor dormancy, and also it induces apoptosis in vitro [10, 40, 42, 43].

Secondly by using anti-uPAR drugs which block the cell surface uPA/uPAR interaction. These agents range form soluble forms of the receptor and catalytically inactive fragments of uPA, to synthetic peptides constructed on the basis of the uPAR binding region of uPA.

Thirdly by direct inhibition of uPA because inhibiting the uPA/uPAR interaction may not be sufficient to block the uPA mediated plasminogen activation [10].

Elevated tumor-tissue levels of proteins of the uPA/uPAR system are indicative of poor prognosis in most cancers and decision-making based on these markers is currently being implemented in the clinic [12].

The epidermal skin components

The epidermis is the multilayered epithelium that covers the skin. In mammals, it comprises the interfollicular epidermis and the adnexal structures, including the sebaceous glands and the hair follicles. It protects animals from water loss, temperature change, radiation, trauma and infections. Therefore it is necessary for animal survival.

The epidermis is a stratified keratinized epithelium that contains several cell types, including the keratinocytes, which are specialized epithelial cells responsible for epidermal renewal, cohesion and barrier function. A complex basement membrane separates the epidermis from the underlying dermis, the connective tissue responsible for the mechanical properties of the skin. The dermis contains several types of mesenchymal cells (fibroblasts), adipocytes (fat cells) and the so-called epidermal appendages, being the hair follicles, the sebaceous glands and sweat glands.

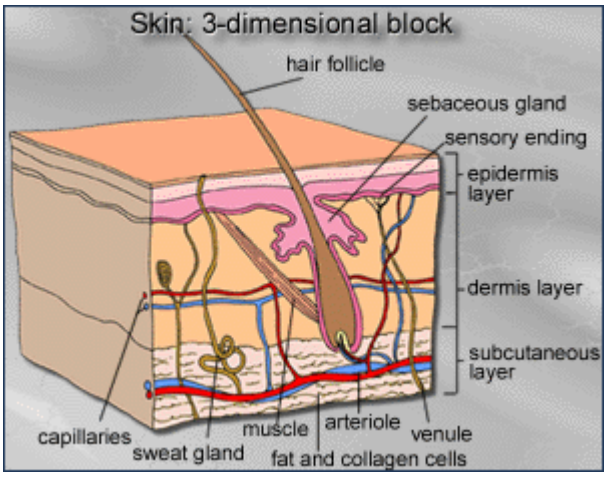


Fig 4 An overview of the skin and its layers.

Mature epidermis is a stratified squamous epithelium whose outermost layer is the skin surface. Only the innermost (basal) layer is mitotically active. The basal layer produces, secretes, and assembles an extracellular matrix (ECM), which constitutes much of the underlying basement membrane that separates the epidermis from the dermis. The most prominent basal ECM component is laminin5, which utilizes α3β1-integrin for its assembly. As cells leave the basal layer and move outward toward the skin surface, they withdraw from the cell cycle, switch off integrin and laminin

expression, and execute a terminal differentiation program. In the early stages of producing spinous and granular layers, the program remains transcriptionally active. However, it culminates in the production of dead flattened cells of the cornified layer (squames) that are sloughed from the skin surface, continually being replaced by inner cells moving outward.

The terminal differentiation program is constituted by the expression of several differentiation markers such as keratins. Keratins are the major structural proteins in the skin and the epithelial cells (keratinocytes) express different keratins in different layers of the epidermis. There are 4 main layers in the epidermal skin, being the basal layer, the suprabasal (spinous) layer, the granular layer and the cornified layer. The terminal differentiation step of cornification involves the destruction of the nucleus and the other cellular organelles, while the cell lipid bilayers will be extruded onto the scaffold of the cornified envelope, packaged in lamellar granules. [44-47]

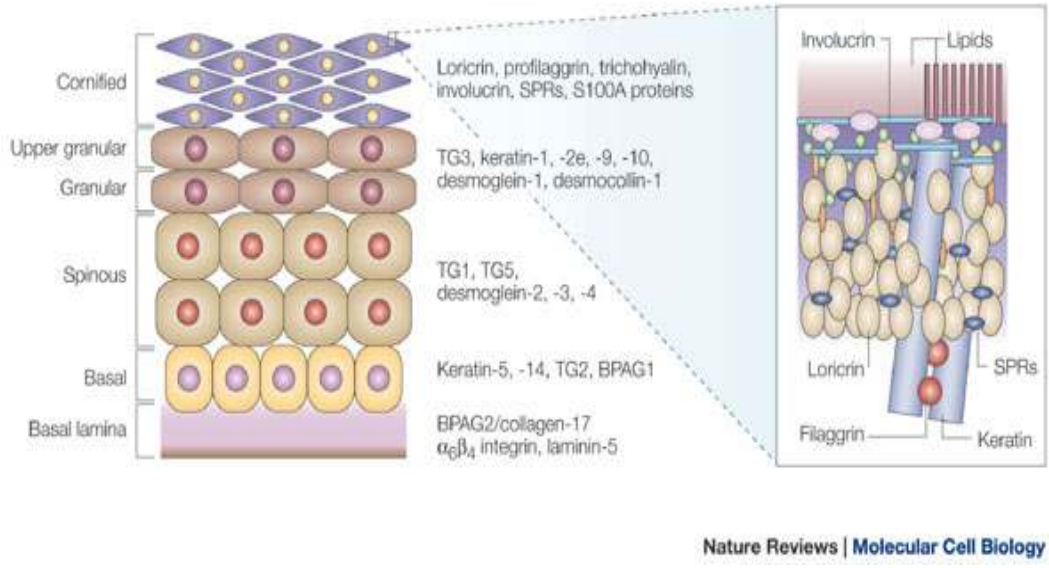


Fig 5 Provides an overview of the layers of the epidermis and its way to cornification .[45].

Skin stem cells

The terminally differentiated cells in all regions of the epidermis are continually shed from the skin and must be replaced throughout adult life. Given that the terminally differentiated cells of the epidermis cannot divide, their replacement depends on less highly differentiated cells, stem cells. These are cells with an extensive self-renewal capacity and which produce progeny that undergo terminal differentiation along the

different epidermal lineages. The best characterised stem cell population lies in a region of the hair follicle known as the bulge, but there are also stem cells in the interfollicular epidermis and, potentially, the sebaceous gland.

The skin is constantly renewed and remodelled with a strict balance between keratinocytes proliferation and differentiation. A rupture of this equilibrium could lead to a variety of conditions including improper scaring and cancers that are known to develop more frequently on chronic cutaneous wounds [48, 49]. The multipotent stem cells have the ability to differentiate into any of the other epidermal cells. When they are activated, epithelial stem cells can generate highly proliferating progeny that are often called transient amplifying cells (TAs) [50]. Despite the fact that stem cells are surrounded by many high proliferating cells, they rarely undergo mitosis [51]. The TA cells, instead, will actively divide for several times, so creating the source that later differentiates along a particular cell lineage to (re)generate tissue (interfollicular epidermis, hair follicle growth, sebaceous gland)[50].
The stem cells have to be able to sense and refill the vacancies in the basal layer.
Besides, in case of interfollicular epidermal wounding, they will change their biochemistry, exit from the bulge region, migrate and proliferate in order to repair the damaged skin [50, 52-54]. Also when the sebaceous gland loses some functional capacity, the skin stem cells will be activated to proliferate into sebocyte progenitors [55] .

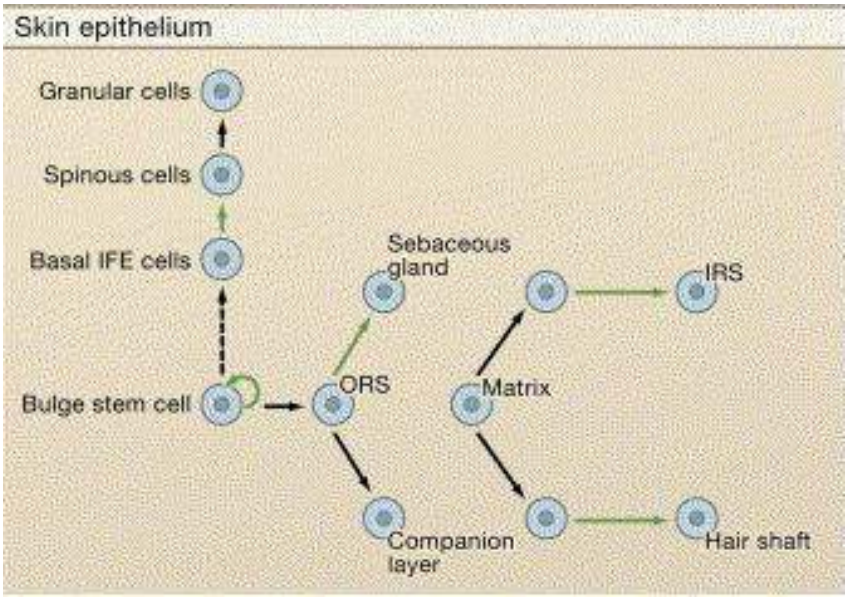


Fig 6 shows the proliferation and the differentiation possibilities of bulge skin stem cells [50].

Many skin disorders, such as cancer, chronic wounds, skin atrophy and fragility, hirsutism, and alopecia, can be viewed as disorders of the skin stem cells. Defining gene expression patterns that control the quiescent, non-cycling nature of skin stem cells is a major goal in epithelial stem cell biology, since it could lead to a better understanding of the abnormal proliferation that these cells undergo in the development of skin cancer [56]. In addition, elucidation of genes involved in skin stem cell proliferation and self-renewal may provide insights into alopecia and abnormal wound healing, since bulge cells are important for hair growth and normal wound healing [57].

Since epithelial stem cells are thought to have a lifespan as long as that of the organism, they are thought to be susceptible to multiple genetic "hits", which cumulatively may result in tumor formation [49]. A great deal of evidence in the mouse system points to hair follicles and stem cells as the origin of many skin tumors [58] [59]

Stem cell markers

The effort to identify epidermal stem cells has recently gained momentum. Stem cells and transit-amplifying cells were first identified according to their different proliferative characteristics [48, 60, 61]. In the normal epidermis, stem cells are quiescent and tend not to divide, but in response to tissue damage or to being placed in culture they are capable of sustained self-renewal. By contrast, transit-amplifying cells are actively dividing *in vivo*, but in culture they undergo terminal differentiation within a few rounds of division. These characteristics have led to the use of retention of a DNA label (such as 5-bromo-2-deoxyuridine [BrdU]) as an *in vivo* stem cell marker, and clonal growth as an *in vitro* marker.

Molecular markers of epidermal stem cells have been identified through a candidate approach and, more recently, by global gene expression profiling. Mouse bulge markers identified by the candidate approach include CD34, keratin 15 (K15) and the α6 and β1 integrin subunits [51, 54, 60, 62]. Bulge genes identified by microarray analysis include genes (such as Wnt inhibitors) that maintain stem cells in quiescent

and undifferentiated states, genes that regulate cell adhesion, and genes that mediate communication with other cell types, including melanocytes [54, 60, 62].

Stem cell maintenance and quiescence

Although integrin-mediated adhesion to the extracellular matrix negatively regulates terminal differentiation of cultured keratinocytes, epidermal deletion of integrins *in vivo* does not lead to a stem cell depletion phenotype [63]. This has led to a search for pleiotropic effectors of integrins and other cell surface receptors that might be required to maintain the stem cell compartment *in vivo*. It turns out that deletion of the Rho family GTPase Rac1, which regulates cell adhesion and growth factor responsiveness, results in stem cell depletion and a failure to maintain the interfollicular epidermis, hair follicles and sebaceous glands [64]. Deletion of Rac1 enables the temporal sequence of events following Rac1 deletion in adult epidermis to be followed. There is an initial, transient increase in proliferation and c-Myc expression, followed by loss of proliferating cells and by the onset of terminal differentiation [64].

One way, indeed, in which Rac1 maintains the stem cell compartment is by negative regulation of Myc. This occurs through PAK2 phosphorylation of Myc, which decreases Myc binding to Max and DNA [64]. Activation of Myc causes cells to exit from the epidermal stem cell compartment and stimulates differentiation into interfollicular epidermis and sebaceous glands.

Other factors that are important in triggering stem cell proliferation are Tert [65], which promotes stem cell mobilisation in the absence of changes in telomere length, and β-catenin stabilisation, which promotes the transition from quiescent stem cell to proliferating transit-amplifying cell in the bulge [66]. The changes in Rac1, Myc, Tert and β-catenin activity that are found in epithelial tumours [60, 64, 65] would be consistent with the hypothesis that increased stem cell proliferation predisposes the epidermis to cancer.

The hair follicle cycle

The hair follicle is a sensitive mini-organ whose cyclic transformations are divided in phases. The anagen is a phase of rapid growth in which progenitor cells of the bulb area generate the hair and its surrounding inner root sheath. When the hair is finished, the cells stop proliferating and the epithelial cells below the bulge either differentiate or die by an apoptotic process during the catagen phase. After that the hair follicle reaches its relative quiescence telogen phase [54, 67]). The hair that was produced is now dead and will be removed during the exogen phase [47, 51]. In a mouse, in the first hair cycle, telogen lasts approximately one day, but in subsequent cycles, this phase becomes increasingly extended, suggesting the need to reach a biochemical threshold before the next hair cycle can be activated. The new cycle of hair regeneration (anagen) begins with the emergence of a proliferating hair germ, and the progression to form the mature follicle bears a significant resemblance to embryonic folliculogenesis [68] (figure 1.7). The periodic cycling of hair growth and degeneration persists throughout the life of the animal and implicates the existence of SCs to fuel the regenerative process. All these phases are profoundly influenced by numerous growth factors, cytokines, hormones, neuropeptides and pharmaceutical products [68]

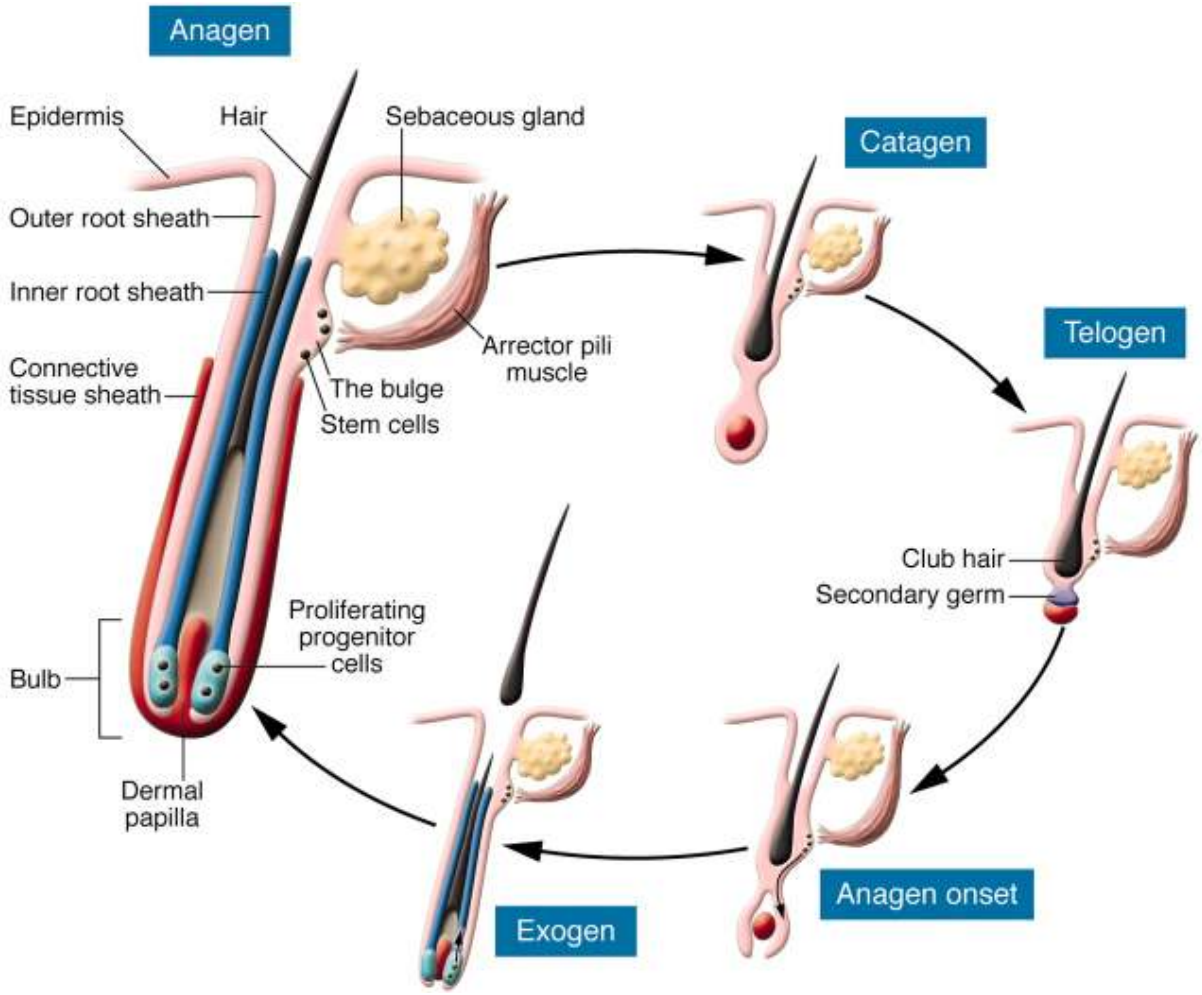


Fig 7 Gives a clear view of the hair cycle phases anagen, catagen, telogen and exogen [51].

UPAR and wound healing

Malignant tumors do not show a common “cancer-specific” pattern of gene expression, but each type of cancer has a unique distribution of roles between the different cells. Such patterns of gene expression reflect the expression during non-neoplastic tissue remodelling programs in the tissue from which the cancer originates [69].

The simplest example comes from skin biology. Here the epithelial cell is responsible for the synthesis of most proteolytic components. This is true for both the non-neoplastic process of skin wound healing and for the invasive growth of squamous cell skin carcinoma. In normal skin keratinocytes, uPAR and uPA are undetectable by in situ hybridization. When the skin is wounded, well-coordinated tissue repair reactions start healing the wound. This process includes proliferation of epidermal keratinocytes at the wound edge and their migration beneath the wound scab to

regenerate the new epidermal layer. The migration of keratinocytes through the fibrin-rich matrix appears to require extracellular proteolysis. Therefore the expression of uPA and uPAR in keratinocytes is elevated to break down the fibrin rich matrix.
During this process uPAR and uPA were detected by in situ hybridization, so both molecules required for plasminogen activation were found on the surface of the migrating keratinocytes [70], but even if the role of uPA, tPA and plasminogen are clear during wound healing [70] ( ), the role of uPAR is still not well understood.

Aim of the project.

The invasive growth of squamous cell carcinoma needs protease activity to break down the extracellular matrix so that cancer cells can migrate through the tissue. uPAR and all the components of the fibrinolytic system, which play an important role in plasminogen activation, were found to be overexpressed in many type of cancers. They are in fact considered as markers of poor prognosis. The same happens with several non-neoplastic tissue remodelling processes, such us wound healing.

Before my arrival in this laboratory, Blasi's group has demonstrated that the absence of the urokinase plasminogen activator receptor delays keratinocytes migration, proliferation and laminin-5 production *in vitro*, in particular under permissive conditions.

Besides, they found that, after chemically-induced carcinogenesis through an initiation (DMBA)/promotion (tPA) protocol , uPAR knock-out mice show a reduced number of papillomas, while in terms of melanocytes hyperproliferation only 4 out of 40 uPAR wild-type mice develop malignant tumors, such as melanomas, compared with 0 out of 40 uPAR knock-out mice (unpublished data).

These results show that uPAR plays an important role in activated skin *in vivo* or for stimulated keratinocytes behaviour *in vitro*.

Since stem cells could be the origin of many skin tumors and are on the basis of many non-neoplastic tissue remodelling processes, during my internship at San Raffaele, we have tried to better understand the role of uPAR, first in the skin stem cell niche and then during wound healing, comparing uPAR wild-type and uPAR knock-out mice.

To investigate this, we have used several markers to label, count and visualize different populations within the skin stem cell compartment and we have tried to understand a possible mechanism by which skin stem cells move from their niche toward the upper follicle and the interfollicular epidermis.

Since it turned out that deletion of the Rho family GTPase Rac1, which regulates cell adhesion and growth factor responsiveness, results in stem cell depletion and a failure to maintain the interfollicular epidermis, hair follicles and sebaceous glands [64] and since uPAR has found to directly activate Rac1, we have tried to study a

possible correlation between these two proteins, during activation of the skin stem cell compartment.

Defining gene expression patterns that control the quiescent, non-cycling nature of skin stem cells is a major goal in epithelial stem cell biology, since it could lead to a better understanding of the abnormal proliferation that these cells undergo in the development of skin cancer

Material and Methods

UPAR wild type (WT) and knock out (KO) mice.

For my experiments I used uPAR-wild type and uPAR-homozygous deficient mice, which have been generated over 10 years ago by Dewerchin. In her experiments, the uPAR gene was isolated from a D3 embryonic stem cell library. The gene encoding for uPAR is organised into 7 exons. The first exon encodes the signal peptide, and the three homologous domains are each encoded by 2 exons. The targeting vector pPNT contains neomycin resistance and herpes simplex virus thymidine kinase genes. The genomic sequences of exon 2 through 5 of the uPAR gene were replaced by the neomycin resistance gene.

Targeted clones containing a disrupted uPAR gene were injected into C57BL/6 host blastocytes. Injected embryos were transferred into pseudo pregnant B6D2F1 foster mothers. The inactivated uPAR mice display normal viability, growth, and fertility. And so the uPAR -/- was transmitted by Mendelian inheritance[71].

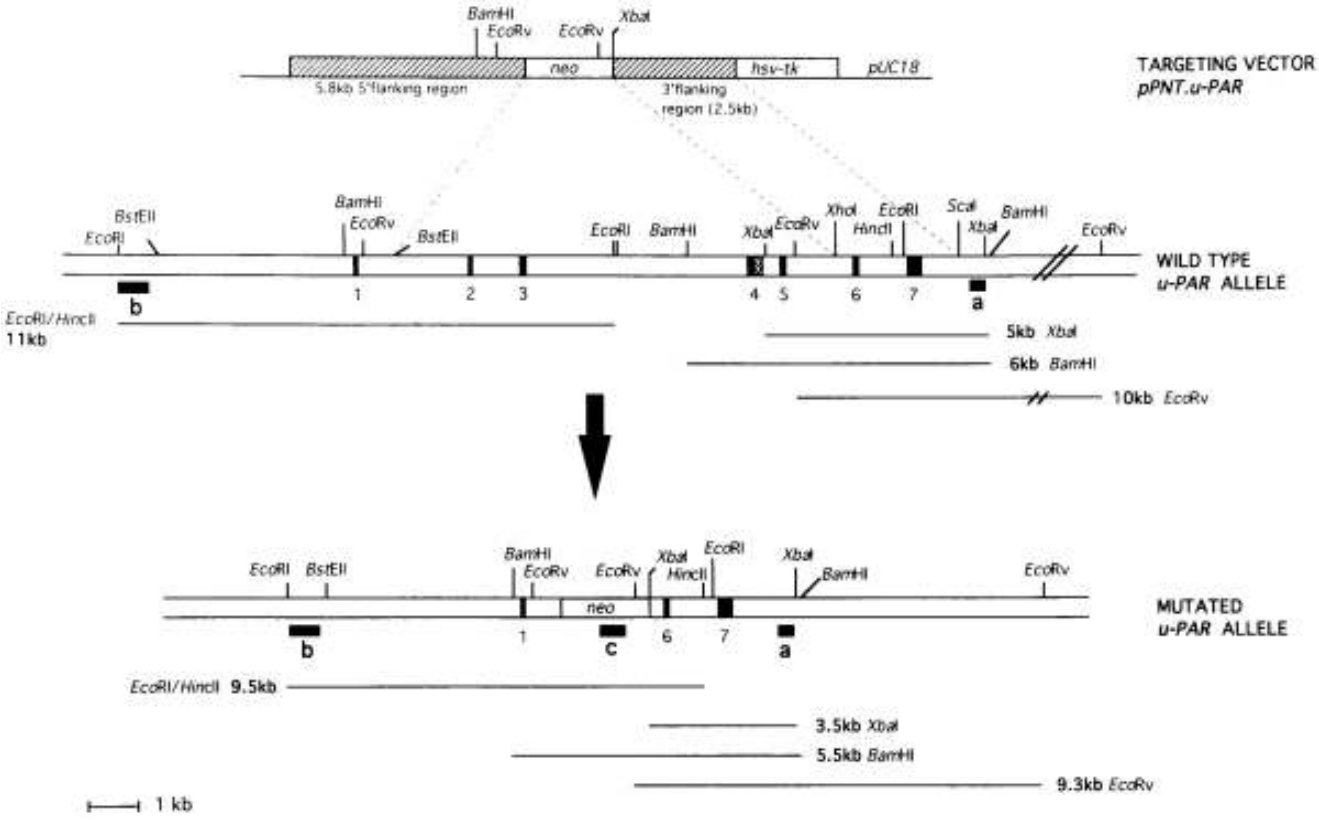


Fig 1 shows the insert that is placed in the mouse DNA to substitute the uPAR gene. This method was used to develop the uPAR knock out mice.[71]

DNA extraction from fresh mouse tissue

In order to extract the DNA and genotype the mice, In order to extract the DNA and genotype the mice, the tissue has to be digested. Therefore we used the serine protease proteinase K to cleave the peptide bonds. So the cells will break down and the DNA is ready to be used for PCR.

Materials

- Gitschier Buffer
    - 16,6mMammonium sulphate
    - 67mM Tris PH 8.8
    - 0,5mM β-mercaptoethanol
    - 6,7mM EDTA
- Proteinase K
    - 500µg/ml proteinase K
    - 0,5% triton X100 Sigma
- Heating block

Methods:

To check the genotype of the mice, fingers were cut off and in case of number 0 I took a piece of the tail. These tissue samples were incubated for digestion overnight at 56°C in a mix of proteinase K and gitschier buffer. The next day the proteinase K was deactivated by heating the samples at 95°C for 5 minutes and the genotype for uPAR was controlled by PCR.

PCR

PCR is an often used method for DNA amplification. By doing a denaturation, annealing and elongation step every cycle, the DNA of each sample will be amplified. A normal PCR program consists of 30/35 cycles. In this case 2 primers are designed to amplify the uPAR gene to identify wild-type mice, and two others to amplify the insert located in the uPAR knock out construct. Since these primers amplify a different gene with a different amount of nucleotides, later in the gel electrophoresis the genotype can be determined by the weight of each band.

Materials:

- Primers
    - WT forward - CAC ACC TGG AAC TCT ATT ACT AGG
    - WT reverse - ACG CCC GAC TCA CCG GGT CTG GGC CTG TTG CAG
    - KO forward - CGA CAG GGA ACG AAG ATG AGC AC
    - KO reverse - CGC AGC GCA TCG CCT TCT ATC GCC
    - primer working stock
        - 10µl WT forward
        - 10µl WT reverse
        - 7µl KO forward
        - 3,5µl KO reverse
        - 69,5µl H_2O
- PCR tubes
- Taq polymerase easy
- DNTP's
- Magnesium
- DNA samples
- Taq PCR buffer

Methods:

The PCR reaction mix was made and each tube contained:

- 1µl DNA sample
- 35,8µl H_2O
- 5µl Taq PCR buffer
- 3µl Magnesium
- 1µl DNTP's
- 4µl primer working stock
- 0,2µl Taq polymerase easy
-

The PCR program used is the following:

Cycles	Process		Time
1	Denaturation	95°C	2 min
35	Denaturation	95°C	30 sec
	Annealing	60°C	1 min
	Elongation	72 °C	3 min
1	Elongation	72°C	5 min

At the end of the PCR a 2% Agarose gel in Tris Acetate EDTA buffer containing Ethidium bromide were run to separate PCR products, at 145 Volt for 30 minutes. The length of the DNA determines the velocity of migration and the ethidium bromide, which is in the gel, will make the DNA samples visible under Ultra Violet light. The weight of uPAR wild-type band is 514 base pairs; while the uPAR knock out is 780 base pairs.

Materials:

- PCR product
- Loading buffer
- Tris Acetate EDTA (TAE) buffer
- Agarose
- Ethidium bromide
- Electrophoresis chamber and power supply

BrdU injections

Mice were labelled using BrdU to visualize slow-cycling Label retaining cells. These cells will incorporate BrdU in the DNA during the S phase of the cell cycle.

Materials:

- BD Pharmingen^{TM} flow BrdU kit BrdU 10mg/ml in PBS
- Syringes
- Mice
- Razor blades

Methods:

The back skins of the mice were shaved using a razor blade. The mice were held firm and the BrdU was injected subcutaneously.

Skin shaving of mice

The hair shafts of mice were removed by using a wax/rosin mixture. At the time of shaving all the dorsal skin hair follicles of the mice are in telogen, evidenced by the pink colour of the skin. The hair removal induces homogenous anagen development over the entire depilated back skin of the mouse. After the anagen phase, the catagen and the telogen are entered spontaneously. Compared to spontaneous anagen development, there are two major differences. First, depilation induced anagen is fully synchronized over the entire area of depilation, whereas spontaneous anagen develops in a wave like pattern. Second, a slight inflammatory effect of plucking has been demonstrated. Nine days after depilation, the induced anagen hair follicle reach their maximal length and are morphologically indistinguishable from spontaneously developing anagen follicles. Even though the plucking trauma induces a short wound healing response immediately after the depilation [68].

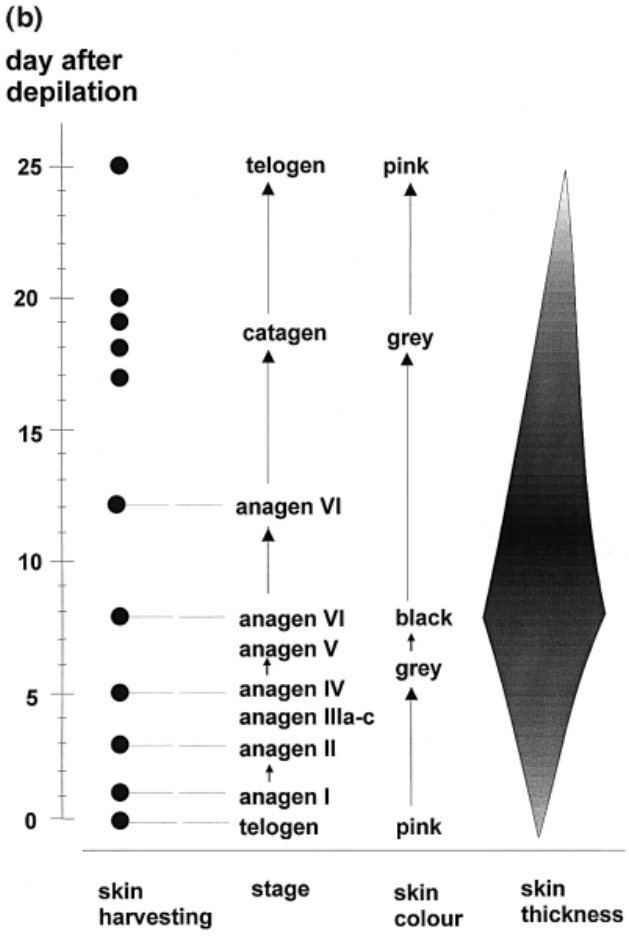


Fig 2 The period of hair development and skin colours after depilation of the mice[68].

Materials:

- Mice in telogen phase
- Veet depilation
    - Cream
    - Hair removing tool

Methods:

-The depilation cream was rubbed on the back of the mice, we waited for couple of minutes and the skin was removed using the tool from the depilation kit.

Back skin isolation and inclusion in paraffin

Skins isolated from the back of the mice were embedded in paraffin, after fixation in formalin. Since fixation is very strong, the paraffin blocks are easily stored at room temperature. Besides the standard immunohistological analysis, paraffin-embedded sections could be used to isolate DNA, RNA and proteins

Materials:

- Mice
- Dry Ice
- pipettes
- Fluka formaldehyde solution 36% (formalin)
- Merck absolute ethanol solutions
    - 100%
    - 95%
    - 70%
- Merck xylene
- Whatman® Paper
- BD falcon® sterile tubes
- Bio plastic embedding devices
    - Bio cassettes
    - Disposable plastic embedding molds
- BHD Gurr® fibrowax (paraffin)

Methods:

Mice were sacrificed by CO2 inhalation using dry ice. A piece of the back skin of the mouse was cut and incubated overnight in a falcon containing formalin 36%. The

next day the skin was put in a bio cassette and left in water for 30 minutes. After the passage in water, passages in ethanol were done:

- 3 hours in 70% ethanol
- 1 hour in 95% ethanol
- overnight in 100% ethanol
- 4 hours in xilene
- 3 hours in paraffin at 60°C.

At this point the cassettes were opened, skin pieces were included in paraffin and after solidification, stored at -20°C or at room temperature.

Paraffin sections

Before the paraffin can be sectioned, its cooled at -20 to be firmer, and so easier to cut. Paraffin sections are cut to view the tissue. Different tissues or staining methods desire different thickness of the sections.

Materials:

- Microtome blades
- Superfrost ® plus slides

Methods:

Cold (-20°C) paraffin blocks were placed in the microtome, and they were cut at 10 - 7 micron. The sections were put floating in a water bath at 40°C and then transferred onto a superfrost slide, until each slide contained 3 paraffin sections. The slides were dried for at least 1 hour at 37°C, and were stored for immunohistochemistry.

Haematoxylin & Eosin staining

Haematoxylin and eosin stain is a popular staining method in histology. The staining method involves application of the basic dye haematoxylin, which colors basophilic structures with blue-purple hue, and alcohol-based acidic eosin Y, which colors eosinophilic structures bright pink. The basophilic structures are usually the ones containing nucleic acids, such as the ribosomes, the chromatin-rich cell nucleus, and the cytoplasmatic regions rich in RNA. The eosinophilic structures are generally composed of intracellular or extracellular protein. The Lewy bodies and Mallor bodies are examples of eosinophilic structures. Most of the cytoplasm is eosinophilic. Red blood cells are stained intensely red.

Materials:

- Merck xylene
- Merck absolute ethanol solutions
    - 100%
    - 70%
    - 50%
    - 25%
- Sigma® Haematoxylin
- Sigma® Eosin
- Eukitt® mounting medium

Methods:

The slides were deparaffinized in xylene for 3 changes of 5 minutes, following by 2 hydration passages in absolute ethanol for 10 minutes each. After these, there were passages of 2 minutes each in descendant ethanol succession from 70%-50%-25% to pure H_2O. Then the slides were stained in haematoxylin for 1 minute and 30 seconds. After the staining, the slides were rinsed in H_2O for 5 minutes. The next step was an eosin staining for 20 seconds, followed by another wash step in H_2O for 2 minutes. Next was an ascendant succession for 1 minute each in 25%-50%-70%and 100% ethanol. The final step before mounting was constituted by other 2 changes of xylene for 5 minutes each. To mount the coverslips onto the slides, the mounting medium Eukitt from Bio-Optica was used.

Proliferating Cell Nuclear Antigen staining

This staining method is generally used to stain the proliferating cell nucleus in paraffin sections. In my experiment, proliferating cells reside only in certain areas of the epidermis.

Materials:

- Merck xylene
- Merck absolute ethanol solutions
    - 100%
    - 95%
    - 80%

- Sigma hydrogen peroxide 30%
    - 3%
    - 0,3%
- PBS
- Citrate
    - Sigma sodium citrate 10mM
    - PH 6 with Merck citric acid 1M
- Goat serum
- Labvision ® Primary antibody PCNA Ab-1 mouse
- Vector ® Secondary antibody biotinylated anti mouse IgG
- Vector ® Vectastain ABC peroxidase standard kit
- AEC mix
    - Sodium acetate 3M PH 5,7
    - Sigma® hydrogen peroxide 30%
    - AEC chromogen
- Sigma haematoxylin
- Sigma ® Clarion mounting medium

Methods:

The slides were de-paraffinized by sequential immersion in 3 changes of xylene for 5 minutes each. The hydration steps were done in 3 descendant changes of ethanol. The incubation time in 100% ethanol was 6 minutes, the others 3 minutes each. The slides were then rinsed in H_2O for 5 minutes and subsequently immersed in 3% hydrogen peroxide for 10 minutes. Then the slides were washed for 6 minutes in PBS. During the antigen retrieval the slides were put in a Citrate buffer at 89°C for 10 minutes, and allowed to cool down to room temperature in 40 minutes. The Slides were washed again in PBS, this time for 3 minutes. The primary antibody was diluted 1:200 in 5.5% goat serum and applied to cover all the tissue on the slides. The incubation of the primary antibody was 30 minutes in a humidified chamber, and afterwards the slides were washed for 9 minutes in PBS. The secondary antibody and wash protocol was exactly the same as the primary antibody. Vectastain streptavidin peroxidase drops were added to cover the tissue sections and incubated for 20 minutes in a humidified chamber, followed by a wash step of 9 minutes in PBS. The AEC mix was made 20 minutes before application and stored at 4°C. The tissue sections on slides were covered by adding drops. The incubation was 15 minutes in a humidified chamber. Afterwards the slides were washed using PBS for 3 minutes.

During counterstaining the slides were immerged in haematoxylin for 2 seconds and washed in water several times. The slides were dried and mounted with coverslips using a clarion aqueous mounting medium.

BrdU staining

The purpose of BrdU staining using anti-BrdU antibody is to visualize the cells which incorporated BrdU.

Materials:

- DB Pharmingen™ BrdU In-Situ Detection Kit
    - Biotinylated Anti-BrdU Antibody
    - Diluent Buffer
    - BD™ Retrievagen A
        - Solution 1
        - Solution 2
    - DAB Chromogen
    - DAB buffer
    - Streptavidin-HRP
- Hydrogen peroxide 30%
    - 3% in PBS
- Merck xylene
- Merck absolute Ethanol solutions
    - 100%
    - 95%
- PBS
- Sigma haematoxylin
- Eukitt mounting medium

Methods:

The slides were deparaffinized by 2 changes of 5 minutes in xylene, and hydrated by 100% and 95% ethanol for 6 minutes each and then stained following BrdU kit instructions.

Protein extraction from formalin-fixed paraffin-embedded tissue.

To know what proteins are responsible for the differences between uPAR wt and uPAR ko mice we used a method to isolate the proteins from the FFPE material. These proteins were used for western blotting.

Materials:

- Samples in paraffin
- Microtome blades
- Eppendorf tubes
- Merck xylene
- Merck methanol
- Tris-HCL (PH 7-9) 2% SDS
- Heating block

Methods:

From each paraffin block 10 sections of 5 micron each were sliced. These sections were put into an Eppendorf tube and 1ml of xylene was added. The tubes were agitated using a vortex and incubated for 10 minutes at room temperature. Then 75µl of methanol was added, the tubes were shaken and then centrifuged for 10 minutes at 4000 RPM. Supernatants were removed and the samples were dried for 5 minutes under a hood. Subsequently 50 µl of Tris-Hcl (PH 7-9) 2% SDS was added and the samples were heated to 100°C for 20 minutes. As a final step the samples were incubated at 60°C for 2 hours, and stored at -20 [72].

Protein dosage measurement

To measure the amount of extracted proteins, I did the protein dosage according to Bio-Rad instructions.

Materials:

- Bio-rad DC protein assay kit containing
    - Reagent A
    - Reagent B
    - Reagent S
- 96 well plate
- protein samples
- Bovine Serum Albumin (BSA)
- Tris-HCL (PH 7-9) 2% SDS
- Microplate spectrophotometer

Methods:

The BSA was diluted in 6 different concentrations starting from 2, 1, 0.5, 0.25, 0.125 and 0.006 mg/ml. Protein samples were diluted 1:2 in Tris-HCL 2% SDS. The bio-rad A' solution was made by adding 20µl of reagent S to 1 ml of reagent A. In every well 25µl of A' was pipetted, together with 5 µl of the sample and 200µl of solution B. Each sample was done in triplicate and the blank contains only Tris-HCL 2% SDS solution.

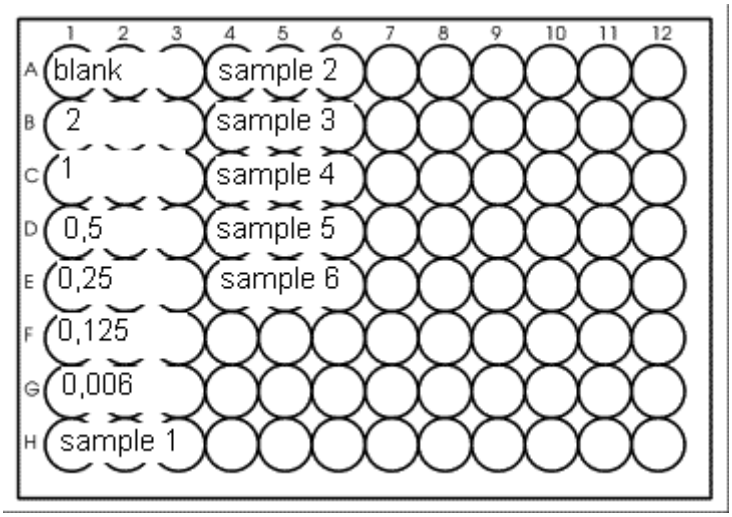


Fig 3 The samples and controls loaded in the 96 well plate for protein dosage.

The 96 well plate was shaked for 15 minutes on a rocking platform. Then the plate was measured at 570nm using a spectrophotometer and a computer equipped with the microplate manager software.

RAC activation assay

To identify the quantity of active RAC1 (RAC1-GTP) a glutathione (GST) pulldown method is used. The GST-agarose beads are bound to P21 activated kinase (PAK-1) containing amino acids 79-84 of the N-terminal regulatory region. This region is known as the P21 binding domain and interacts specifically with the activated RAC1. By this method activated RAC1 can be isolated from cellular lysates to be used in western blots.

Materials:

- cell lysates
- PAK1 bound with GST agarose beads.
- PBS
- Protease inhibitors
    - 1 tablet Roche complete EDTA free inhibitor cocktail.
    - Leupeptin
    - Aprotinin
    - EDTA
    - Sodium vanadate

Methods:

Proteins were extracted from paraffin-embedded sections of uPAR wt and ko skins both in anagen and telogen phase. The tablet of inhibitors was dissolved in 50 ml of PBS. This mix was added to the protein samples to a final volume of 500µl. 20µl of the PAK-GST agarose beads were added. The samples were centrifuged for 5 minutes at 2000 RPM and the supernatants were removed until about 30µl remained. The reducing sample buffer was added to the 30µl samples with RAC1-PAK-GST beads, these were boiled for 5 minutes at 95°C and were loaded in a 15% acrylamide gel to perform a Western blot.

Western blotting

A **western blot** (alternately, **immunoblot**) is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

Materials:

- transfer buffer 10X
    - 144gr glycine
    - 30gr Tris
    - 1 litre H_2O
- transfer buffer 1X
    - 100 ml transfer buffer 10X
    - 100 ml methanol
    - 800 ml H_2O

- running buffer
    - 100 ml 10XTGS
    - 900 ml H_2O
- TBS solution 10X
    - 500 ml Tris 1M
    - 300 ml NaCl 5M
    - 200 ml H_2O
- TBS1X-tween
    - 100 ml TBS 10X
    - 900 ml H_2O
    - 2 ml tween 20
- TBS1X
    - 100 ml TBS 10X
    - 900 ml H_2O
- Protein samples
- Sample buffer
- Blocking solution
    - 2.5gr milk powder
    - 50ml TBS-tween
- Millipore immobilon nitrocellulose membrane
- Methanol
- Acrylamide gels
    - 30% acrylamide
    - Tris-HCl PH 8.8 (for resolving gel)
    - Tris-HCl PH 6.8 (for stacking gel)
    - 10%SDS
    - 10% Ammonium persulfate
    - H_2O
    - TEMED
- Acrylamide gel electrophoresis running camber
- Filter paper
- Western blot transfer chamber
- Primary antibody
    - Upstate RAC1 mouse IgG antibody
    - Santa Cruz Actin (I-19) IgG antibody diluted 1:5000
    - Santa Cruz involucrin (M-15) igG antibody diluted 1.500
- Secondary HRP antibody
    - HRP anti-goat diluted 1:5000
    - HRP anti-mouse diluted :5000
- Pierce Grasp the proteome^{TM} ECL and ECL plus

- films

Methods:

The gel was made depending on the size of the protein of interest. First, resolving gel was made and after this was solidified, the stacking gel was made on top of it. The sample comb was inserted during the solidification of the stacking gel. Meanwhile 5µl of the reducing sample buffer was added to the samples and the samples were boiled to 95°C for 5 minutes. The gel was inserted in the running chamber and the chamber was filled with running buffer. The samples plus the protein marker were loaded into the wells of the gel. A continuous electric field was connected and the negative charged proteins migrated trough the gel to the positive pole.

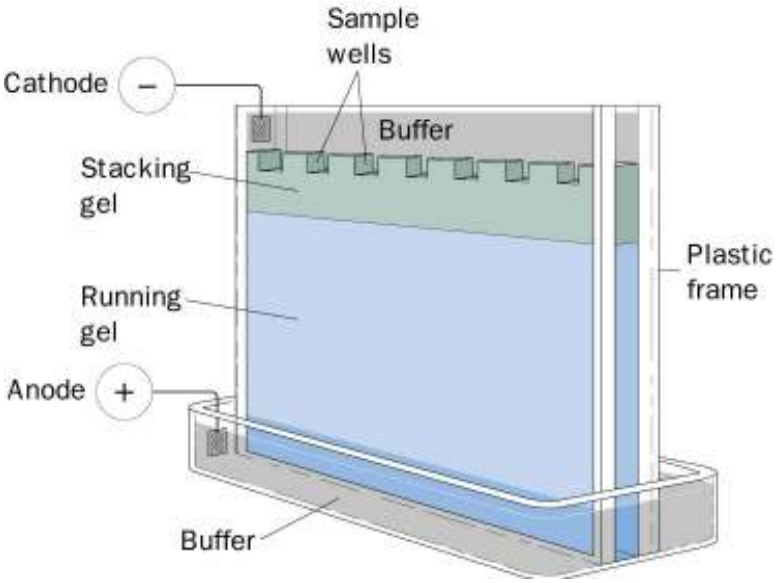


Fig 4 In the running gel the proteins migrated downwards by electrophoresis.

When the protein samples are migrated far enough to be distinguished, the running was stopped. The gel, the sponges, and the whatman papers were put in transfer buffer. The membrane was cut in the size of the gel and immersed in methanol for 10 seconds before it was put in the transfer buffer. The transfer “sandwich” was made by putting a sponge, a filter paper, the membrane, the gel, another filter paper, and another sponge into the plastic support. This was put into the transfer chamber with the gel at the negative, and the membrane at the positive pole. A continuous electric field was sent for at least 1 hour to do the protein transfer.

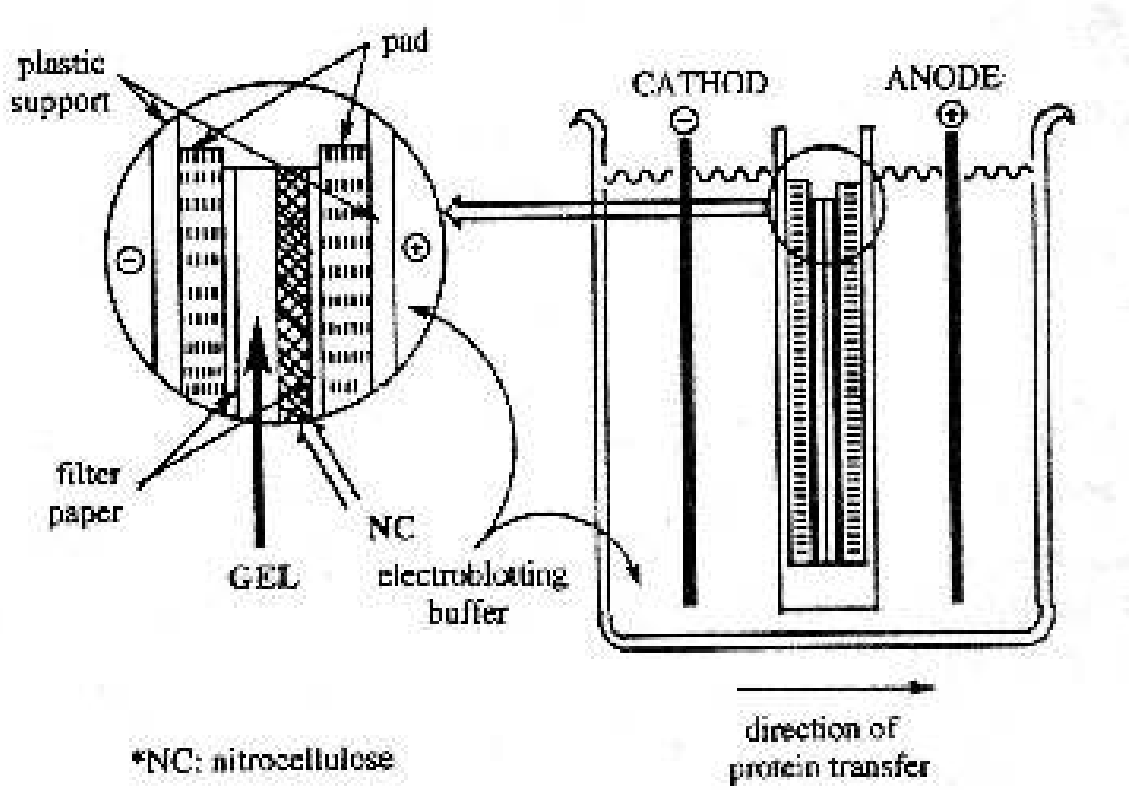


Fig 5 The transfer sandwich of the western blot, the proteins migrate through the gel onto the nitrocellulose membrane.

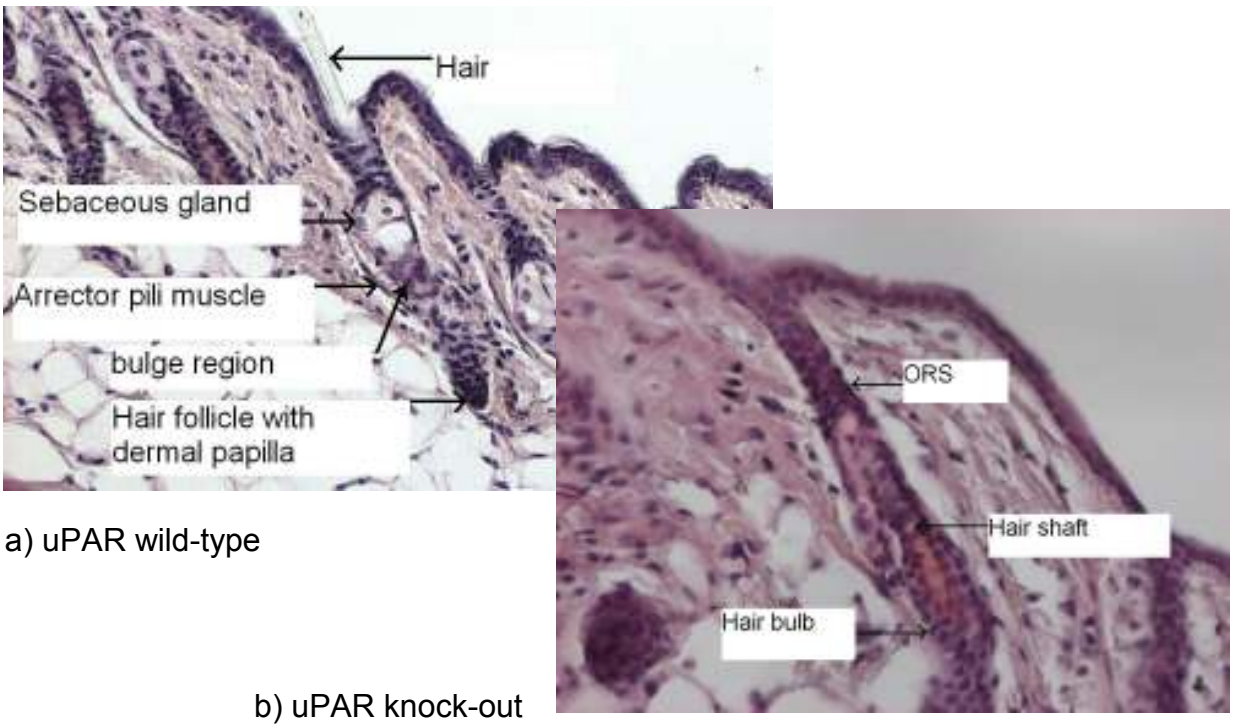
After the running of the transfer, the transfer sandwich was opened, and the membrane was blocked in blocking solution for 45 minutes on a rocking platform. After this incubation the primary antibody which was diluted in blocking solution was added, and put on a rocking platform for 1 hour and 30 minutes. The membrane was then washed for 15 minutes each in 2 changes of TBS 0,02% tween and 1 in TBS without tween. Then the secondary HRP-conjugated antibody was also diluted in blocking solution and the membrane incubated for 1 hour at room temperature. The washing step was repeated, and then the bands were detected by ECL developing reagents. Blots were kept in the ECL-developing solution for 1 min, exposed on film for different time points, and then developed.

Densitometric analysis of the bands were done with ImageQuant software and results were normalized with the housekeeping protein (actin)and reported as percentage of control (100%).

Results

Characterization of uPAR KO mouse skin

The skin of uPAR ko mice (n=4) (Fig.1b) was compared with that of wild-type mice (n=4) (Fig.1a) to determine if a skin phenotype was apparent in untreated quiescent uPAR ko epidermis. As shown in Fig.1, H&E-stained sections from 7-week-old uPAR ko mice (telogen phase) showed normal morphology, spacing and orientation of hair follicle and normal epidermal thickness. Skin morphology can be perceived from these images: the pilo-sebaceous unit contains a hair follicle, with its hair shaft, the dermal papilla and the outer root sheath (ORS), sebaceous gland and interfollicular epidermis. Skin stem cell compartment resides within the bulge region, next to the arrector pili muscle. Resident basal stem cells reside also in proximity of the basement membrane, but in a minor percentage.



a) uPAR wild-type

b) uPAR knock-out

Fig 1 Skin from 7-wk-old wt and ko mice was fixed in 10% formalin and stained with H&E.

When the follicle is induced to grow (anagen phase) and the epidermis is activated, even after an injury or a chemical treatment or simply by shaving the skin, the cells of the hair germ swell, undergo mitosis, grow downwards into the deep dermis, engulfing the follicular papilla, and ultimately differentiating into the complex, multilayered structure of the hair producing bulb [73]. In the mean time, keratinocytes hyperproliferate and generate a multilayered epidermis. In Fig.2b, H&E-stained sections from shaved 7-week-old uPAR ko mice (n=4) (anagen phase) showed a reduce hyperproliferation of the hair germ which is moving downwards into the dermis, compared to the wild-type mice (n=4) (Fig.2a). Epidermis thickness, which is more pronunciated compared to the telogen phase, looks similar in both genotypes. This is probably because epidermis has also is own source of stem cells, even if it is a small percentage. These results were observed in at least 6 mice per genotype and were reproducible. The images suggest that uPAR plays a role in the skin homeostasis in particular when the tissue is “activated”.

a) b)

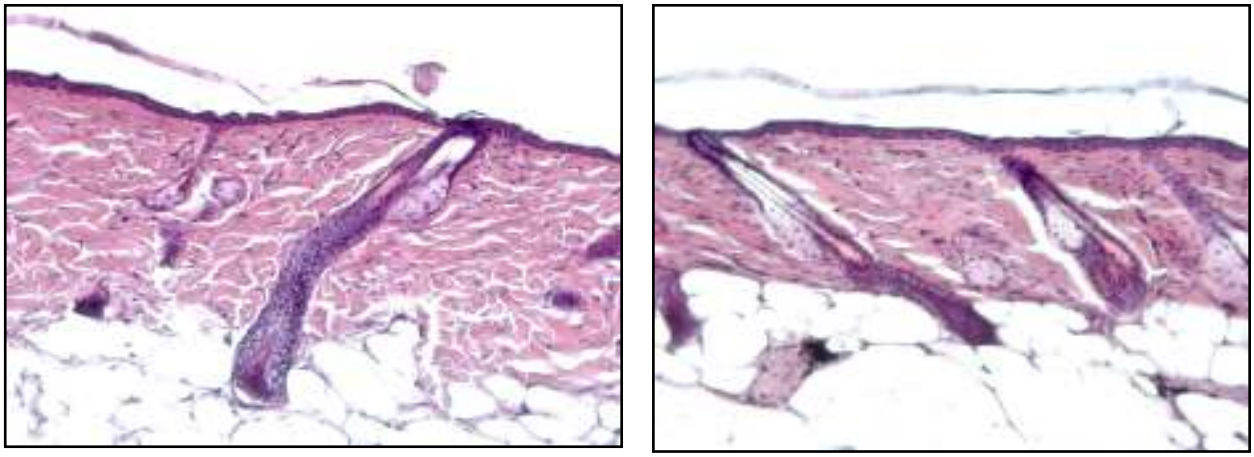


Fig.2 *Skin from 7-wk-old wt (a) and ko mice (b) were shaved to induce the anagen phase. After 5 days, when skins have reached the anagen IV phase, they were fixed in 10% formalin, included in paraffin and sections of 7-µm were stained with H&E (100x magnification).*

Skin wound healing is delayed in uPAR-knock out mice

To reinforce this hypothesis, uPAR-wt and ko skins were “activated” and induced to enter in the anagen phase by an injury. Cutaneous wound repair is in fact an

excellent model system to study proteinase function and regulation, because it comprises several processes requiring proteinase action: invasion of inflammatory cells as well as migration of fibroblasts and keratinocytes, angiogenesis, contraction and finally remodelling of the scar tissue [74-76].

Standardized 10 mm long, full thickness, incision wounds were generated in wild-type (n=10, for each time point) and uPAR-deficient mice (n=13, for each time point). The wounds were examined grossly by visual inspection, and the wound lengths measured three times a week. They are characterized by a period with a red wound field, which lacks epidermal covering and often granulation tissue protrudes between the two wound edges. Lesions were scored as fully healed when there was a complete closure of the incision interface with restoration of the epidermal covering. The mean time to complete healing for uPAR wild-type mice is 11.2 ± 1.4 days. In uPAR-deficient mice the value increased to 15.9 ± 2.1 days, a significant delay in skin wound healing compared to the wild-type mice (P= 0,00028) (Fig.3b).

Following wounding, the average wound lengths of uPAR-deficient mice were significantly increased compared to the wild-type (Fig.3a).

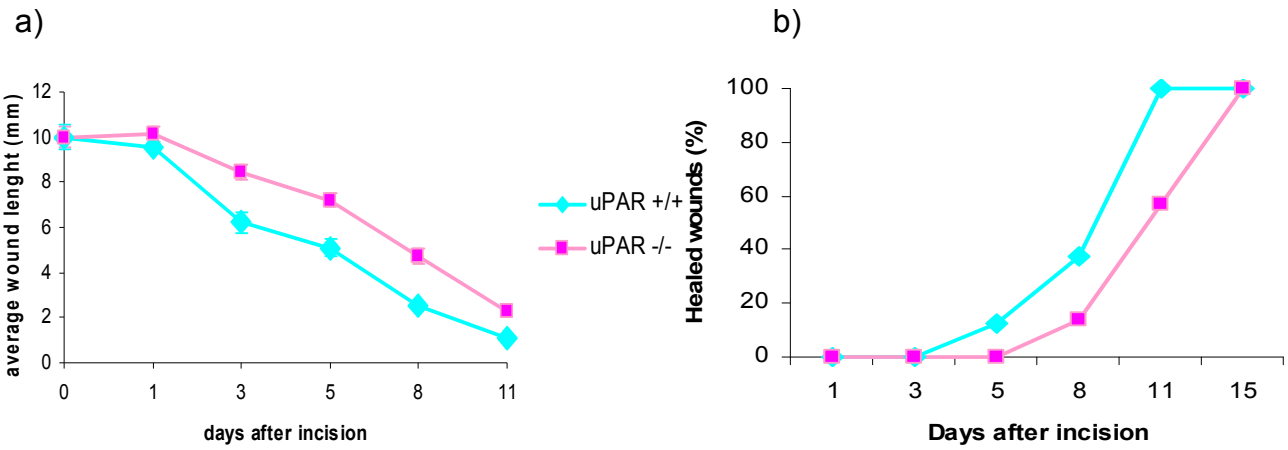


Fig.3 *Standardized 10 mm long, full thickness, incision wounds were generated in wild-type (n=10, for each time point) and uPAR-deficient mice (n=13, for each time point). The wounds were examined grossly by visual inspection at different time points(b), and the wound lengths measured three times a week(a).*

These results demonstrates that uPAR is necessary for efficient wound closure after incision injury, probably regulating keratinocytes biological functions and confirm our data in which uPAR plays a role in maintaining skin homeostasis.

UPAR deficiency up-regulates a differentiation marker in anagen phase

When epidermis is activated, and keratinocytes are induced to proliferate contributing to hair follicle growth and epidermis regeneration, a differentiation step is necessary at the end of this process. We tested whether uPAR could influence this step checking by Western Blotting the expression of a keratinocytes differentiation marker, involucrin, during anagen (wound healing or skin shaving) or telogen phase in uPAR wild-type(n=4) and ko mice (n=4).

As shown in Fig.4, uPAR ko mice express higher levels of involucrin, a marker of later differentiation both in telogen and in anagen phase compared to wild-type mice. This confirm previous results obtained by D' Alessio et al (unpublished data) where keratin-14, another differentiation marker, is overexpressed in uPAR ko mice after wounding (anagen phase), compared to wt.

Unfortunately, standard deviations and T-Test are not indicated, since the graph is the result of a single experiment.

These results, together with previous data, suggest that uPAR deficiency in an "activated" skin, not only reduces keratinocytes proliferation and delays epidermis and hair follicle regeneration, but accelerates the terminal differentiation, as well.

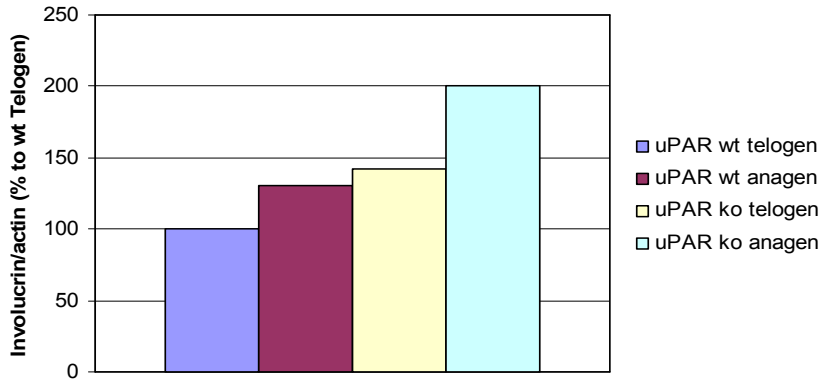


Fig. 4 *Cell extracts were obtained from paraffin-embedded tissues of uPAR wt (n=4) and ko mice (n=4) in anagen and telogen phase, as described in material and methods, and a Western Blotting for involucrin were performed. Bands were quantified by a densitometer and results are expressed in percentage respect to uPAR wt telogen.*

Emigration of upper follicular epithelial cells into uPAR wt and ko neonatal epidermis

The location of stem cells within the skin was identified by taking advantage of their relative quiescence. Despite their reduced mitotic index, epithelial stem cells can be labelled by continuous administration of nucleotide analogs (pulse) such as BrdU for a prolonged period. During the subsequent chase period, cells dilute their label through cell divisions. Those cells that divide less frequently during the chase period retain the label and hence have been referred to as label-retaining cells (LRCs) [77]. LRCs within the skin reside in the bulge area of the hair follicle [78].

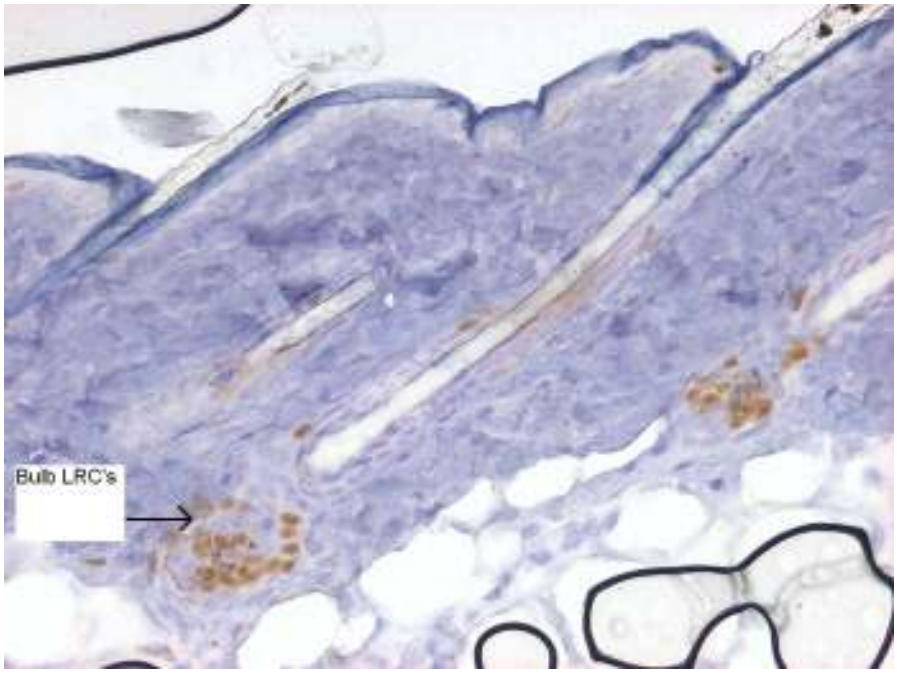
Cotsarelis and colleagues in 1990 speculated in their original “bulge activation hypothesis” that the progeny of bulge stem cells may migrate into the upper follicle and play a role in the long-term maintenance of the epidermis. Besides, in view of their intrinsically long-term persistence in the epidermis and hair follicles and high proliferative potential, they could be the target cells in skin carcinogenesis. Since uPAR ko mice showed reduced papilloma formation, after chemically-induced carcinogenesis, we tested whether uPAR plays a role in the mobilization and the behaviour of mouse skin stem cells. We will verify this hypothesis by two independent approaches.

At first we tried to detect and quantify the number of skin stem cells in the bulge region. For this purpose, we injected neonatal uPAR wt (n=4) and uPAR ko (n=4) C57BL/6 mice subcutaneously beginning at day 4 of life, when the epidermis was in the anagen phase, with 5-bromo-2-deoxyuridine (BrdU; 50 μg/g bodyweight) twice daily for 3 days. Cells retaining the label after 8-10 weeks were identified as LRCs. Unfortunately, after several attempts we failed to obtain any results, probably because subcutaneous injection didn’t work properly.

In another approach we wanted to selectively tag some of the cells in the upper follicle in order to follow their fate. To do this, we exploited the fact that keratinocytes have a heterogeneous cycle time [79, 80] and we used a double-labeling technique in which newborn mice (4-5 days postnatal) received a single subcutaneous injection of BrdU. After 18 h from injection, considering that during the anagen phase the cell cycle time is approximately between 10-30 h these mice were sacrificed at different time points: 0hr, 6hr, 18hr, 24hr and 30hr. Paraffin-skin sections were stained for BrdU, PCNA or both (Fig.2a and b) and the numbers of nuclei in upper follicular

epithelium and epidermis that were labelled by BrdU, PCNA or both, were counted (Fig. 3a and b) .

a)



b)

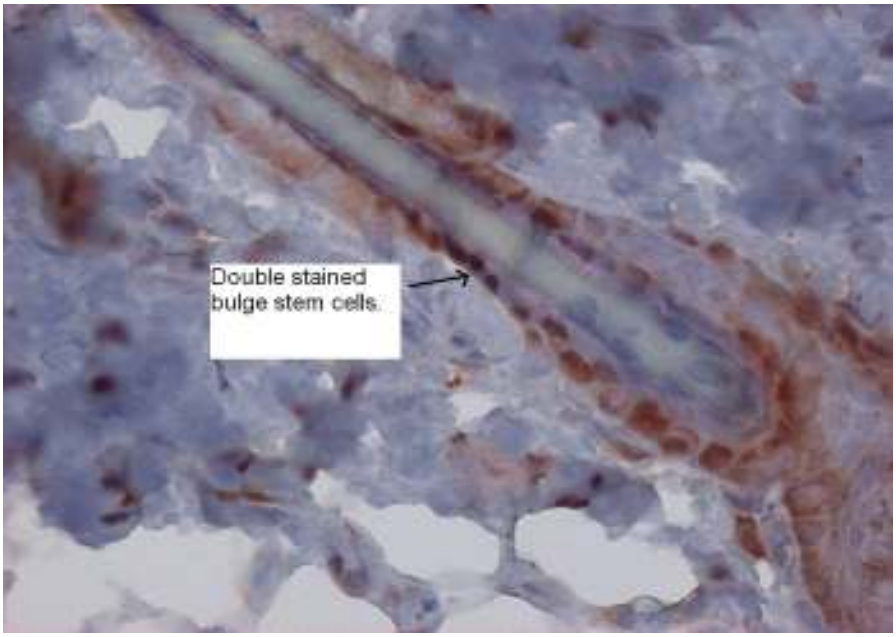
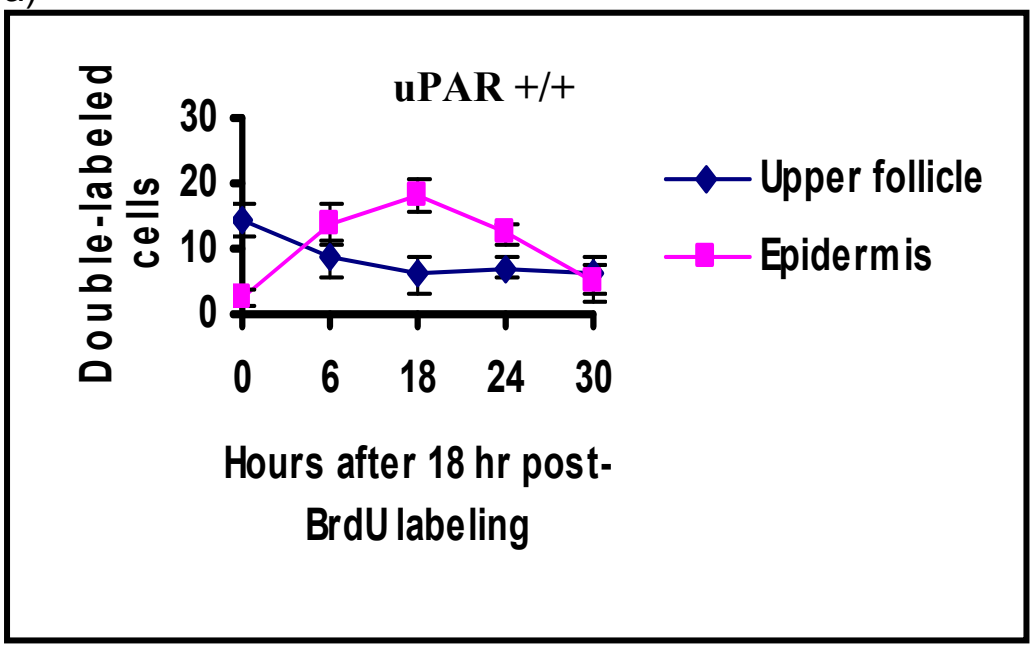


Fig 5 Skin paraffin sections (magnification 400x) at different time points after BrdU injection, were processed for BrdU (a), PCNA and BrdU/PCNA (b) immunohistochemistry as described in materal and methods.

After 18 hr post-BrdU labeling, many (15) of the uPAR-wt and uPAR ko BrdU-labeled infundibular cells became double-labeled with PCNA (Fig. 3a and b). The "background" of double-labeled cells in the epidermis was extremely low, i.e., less than 3 of the BrdU-labeled epidermal cells were double-labelled. As shown, there was a large number of double-labeled cells in the upper follicle, with scarcely any in the epidermis, in both genotypes. The fate of the double-labeled follicular cells was followed after chase periods of 0, 6, 18, 24 and 30 hr. A progressive decrease in the number of double-labeled cells in the upper follicle, accompanied by a dramatic increase of such cells in the epidermis (Fig. 3a), clearly demonstrated an emigration of the upper follicular cells into the epidermis. This migration is clearly decreased in uPAR ko skin. These results, unfortunately obtained from a single experiment, could demonstrate that uPAR contributes for populations of follicular keratinocytes to neonatal epidermis during a time of tissue expansion (anagen phase).

a)



b)

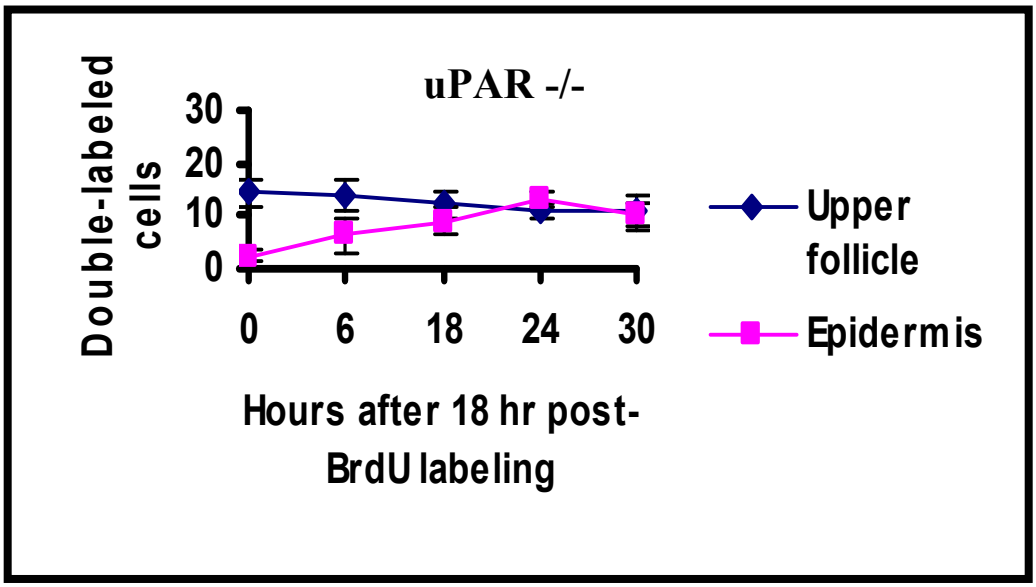


Fig 6 Quantification of double-labeled cells in uPAR wt (a) and ko (b) upper follicle versus epidermis. The results are expressed as number of double-labeled cells.

UPAR regulates Rac1 expression and activation

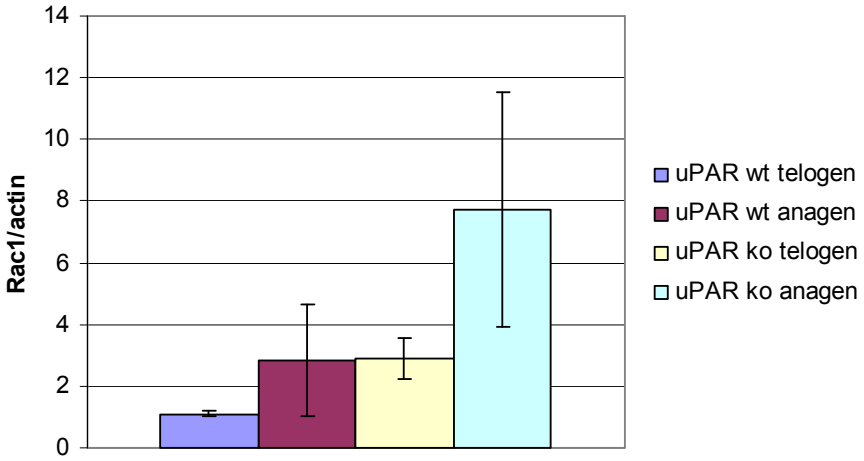
Proteins of the Rho family control signalling pathways that regulate the actin cytoskeleton and gene transcription [81, 82]Their expression was also found to be elevated in papillomas and squamous cell carcinomas, as for Rac1 [64].Since it is known that uPAR expression leads in several cell types to a dramatic reorganization of the actin cytoskeleton and that this receptor regulates cell motility at sites where Vitronectin and uPAR are co-expressed, such as malignant tumors, in a Rac1-dependent pathway [83], we analyzed Rac1 expression and activation in uPAR-wt (n=4) and ko (n=4) skin in anagen and telogen phase.

Results obtained from Western Blotting on protein extract isolated from paraffin-embedded skin sections, showed that Rac1 is overexpressed in uPAR ko skins both in telogen and in anagen phase (Fig.7a).

However, when we look at Rac1 activation the situation is completely inverted. In fact, Rac1 activation is strongly up-regulated in uPAR wt skin both in telogen and in anagen phase (Fig. 7b). Probably, uPAR ko cells overexpress Rac1 to compensate its poor activation.

These data suggest the possible mechanism by which uPAR regulates activated epidermis regeneration, which could be induced either after an injury or during tumor progression.

a)



b)

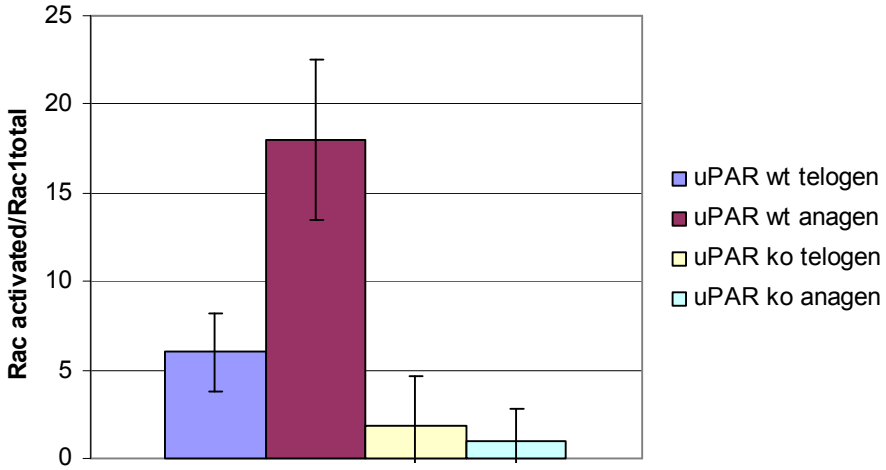


Fig. 7 Effect of uPAR on Rac1 expression (a) and activation (b) in cell extracts obtained from paraffin-embedded sections. The level of total cellular Rac1 and GST-PAK1 precipitated active Rac1 were determinate by SDS-PAGE on 15% gels followed by immunoblotting for Rac1 as described in material and methods. The ratio of Rac1 to actin and activated to total Rac1 was determined and results are expressed in arbitrary units. Results are mean ± SD from two independent experiments.

uPAR regulates Myc expression

It is known that one key mechanism by which Rac1 maintains epidermal stem cells is by negatively regulating Myc through PAK2 phosphorylation. Although Myc is a proto-oncogene, it promotes differentiation of epidermal lineages, disrupting adhesive interactions between stem cells and their niche. Since uPAR deficiency leads to accelerated terminal differentiation and since it down-regulates Rac1 activation in both anagen and telogen phase, compared to uPAR wt skins, we checked whether Myc was up-regulated in these same mice.

As shown in Fig.8, Myc is in fact overexpressed in uPAR ko epidermis both in anagen and in telogen phase, compared to the wt.

These data reinforce our demonstration that uPAR regulates activated epidermis regeneration by a Rac1-dependent mechanism.

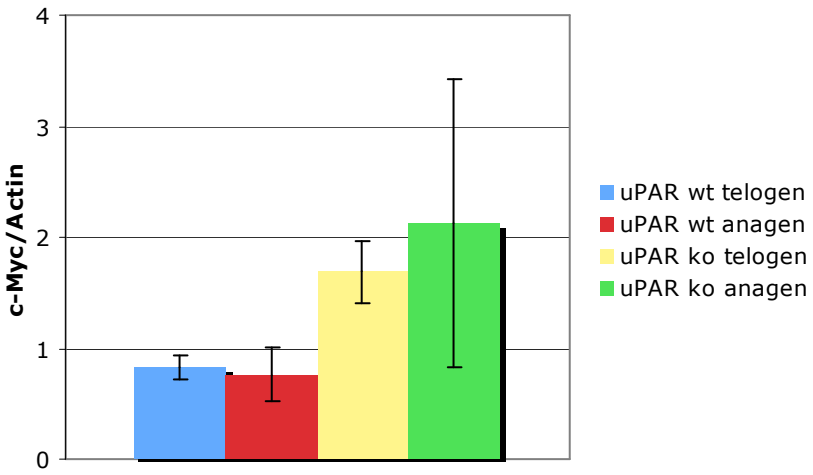


Fig. 8 *Effect of uPAR on Myc expression in cell extracts obtained from paraffin-embedded sections. The level of Myc was determinated by SDS-PAGE on 10% gels followed by immunoblotting for Myc. The ratio of Myc to actin was determined by a densitometer and results are expressed in arbitrary units. Results are mean ± SD from two independent experiment.*

Conclusions and discussion

We report here that the urokinase receptor (uPAR) is required for skin regeneration during an “activated” state (skin shaving, wound healing or skin tumor progression) and that influence this event by a Rac1-dependent pathway.

Our evidence suggests that the underlying mechanism governing the loss of tumor-forming capacity (D’alessio et al, unpublished data) and the delay in wound healing in uPAR knock-out mice involves disruption in the normal response of the hair follicle stem and progenitor cell population to proliferative signals elicited by anagen phase or injury. The first indication came up when we compared the skin of uPAR wild-type (wt) mice with that of uPAR knock-out (ko) mice in quiescent (telogen) and activated phase (anagen). As shown in Fig.1 of the results, H&E-stained sections from 7-week-old uPAR ko mice (telogen phase) showed normal morphology, spacing and orientation of hair follicle and normal epidermal thickness. But when the follicle was induced to grow (anagen phase) and the epidermis was activated simply by shaving the skin, uPAR ko mice presented a morphological reduced hyperproliferation of the hair germ which is moving downwards into the dermis, compared to the wild type mice (Fig.2a of the results). Epidermis thickness, which is more pronunciated compared to the telogen phase, looks similar in both genotypes. This is probably because epidermis has also is own source of stem cells, even if it is a small percentage. In most of the cases uPAR ko mice showed a thinner dermis and a thicker layer of subcutaneous fat tissue. This may be caused by impaired or delayed migration and proliferation of stromal cells in the dermis.

These data were confirmed by the wound healing experiment where a standardized 10 mm long, full thickness, incisional wound was generated in wild-type and uPAR-deficient mice. The mean time to complete healing for uPAR wild-type mice was 11.2 ± 1.4 days. In uPAR-deficient mice the value increased to 15.9 ± 2.1 days, showing a significant delay in skin wound healing compared to the wild-type (P= 0,00028) (Fig.3b). The average wound lengths of uPAR-deficient mice were also significantly increased compared to the wild-type (Fig.3a).

D’Alessio et al. (unpublished data) have already demonstrated that the absence of the urokinase plasminogen activator receptor delays keratinocytes migration,

proliferation and laminin-5 production *in vitro*, in particular under permissive conditions. Our results confirm this aspect *in vivo* since uPAR is necessary for efficient wound closure after incisional injury, probably regulating the biological functions of keratinocytes. Indeed, in other experiments still in progress, results show that keratinocytes hyperproliferation and migration during wound healing is clearly increased in uPAR wt mice compared to ko mice.

Successive experiments demonstrated that uPAR deficiency in an “activated” skin, not only reduces keratinocytes proliferation and delays epidermis and hair follicle regeneration, but accelerates also the terminal differentiation. In fact, when epidermis is activated, and keratinocytes are induced to proliferate contributing to hair follicle growth and epidermis regeneration, a differentiation step is necessary at the end of this process. As shown in Fig.4(results), uPAR ko mice express higher levels of involucrin, a marker of later differentiation both in telogen and in anagen phase compared to wild type mice. This confirms previous results obtained by D’Alessio et al (unpublished data) where keratin-14, another differentiation marker, is overexpressed in uPAR ko mice after wounding, compared to wt.

The role of uPAR in cancer and tumor progression is well known and has been studied for many years. In terms of skin tumors, D’Alessio et al. (unpublished data) have demonstrated a reduced papilloma formation, after chemically-induced carcinogenesis, in uPAR ko mice. Although all keratinocytes in the cutaneous epithelium can receive and sustain formation of carcinogen-DNA adducts and resulting mutations, the stem cells are the most likely targets due to their longstanding persistence in the tissue and their high proliferative potential. Several important studies have clearly shown that stem cell progeny migrate out of the bulge and form the lower hair follicle [52, 53, 84]. Bulge cell progeny have also been shown to populate the basal layer of the interfollicular epidermis on wounding [53, 57, 85, 86]. In addition, it has been shown that bulge cells transiently proliferate at anagen onset [87] and are stimulated to proliferate following chemical carcinogen exposure [87, 88]. On the lights of these evidences, we studied the role of uPAR in the mobilization from the upper follicle to the epidermis of mouse bulge stem cells, tagging some of the cells in the upper follicle by double-labeling technique in order to follow their fate.

After 18 hr post-BrdU labeling, many (15) of the uPAR-wt and uPAR ko BrdU-labeled infundibular cells became double-labeled with PCNA (Results fig. 6a and b). As

shown, there was a large number of double-labeled cells in the upper follicle, with scarcely any in the epidermis, in both genotype. A progressive decrease in the number of double-labeled cells in the upper follicle, accompanied by a dramatic increase of such cells in the epidermis (Results fig. 6a) demonstrated emigration of the upper follicular cells into the epidermis. This migration is clearly decreased in uPAR ko skin (Results fig. 6b). These results, unfortunately obtained from a single experiment, could demonstrate that uPAR contributes for populations of follicular keratinocytes to epidermis regeneration during a time of tissue expansion (anagen phase).

Proteins of the Rho family control signalling pathways that regulate the actin cytoskeleton and gene transcription [81, 82]. Evidence implicating aberrant Rho signalling in cancer has been obtained from *in vitro* studies that focused on specific aspects of tumor cell biology and more recently, *in vivo* studies using recombinant mice lacking or overexpressing Rho signalling proteins have provided direct evidence for the involvement of Rho proteins in cancer [89, 90]. In terms of skin tumors, their expression was found to be elevated in papillomas and squamous cell carcinomas, such as for Rac1 [64]. The same group showed that deletion of Rac1 in adult mouse epidermis stimulated stem cells to divide and undergo terminal differentiation, leading to failure to maintain the interfollicular epidermis, hair follicles and sebaceous glands. Since it is known that uPAR expression leads in several cell types to a dramatic reorganization of the actin cytoskeleton and that this receptor regulates cell motility at sites where Vitronectin and uPAR are co-expressed, such as malignant tumors, in a Rac1-dependent pathway [83], we analyzed Rac1 expression and activation in uPAR-wt and ko skin in anagen and telogen phase.

Results obtained from Western Blotting on protein extract isolated from paraffin-embedded skin sections, showed that Rac1 is overexpressed in uPAR ko skins both in telogen and in anagen phase (Results fig.7a). However, when we look at Rac1 activation the situation is completely inverted. In fact, Rac1 activation is strongly up-regulated in uPAR wt skin both in telogen and in anagen phase (Results fig. 7b). Probably, uPAR ko cells overexpress Rac1 to compensate its poor activation. These data suggest the possible mechanism by which uPAR regulates activated epidermis regeneration, which could be induced either after an injury or during tumor progression.

Rac1 exerts its effect in the epidermis by negatively regulating Myc through PAK2 phosphorylation. Although Myc is a proto-oncogene, it promotes differentiation of epidermal lineages, distrupting adhesive interactions between stem cells and their niche. Since uPAR deficiency leads to accelerated terminal differentiation and since it down-regulates Rac1 activation in both anagen and telogen phase, compared to uPAR wt skins, we checked whether Myc was up-regulated in these same mice and we found, in fact, that Myc is overexpressed in uPAR ko epidermis both in anagen and in telogen phase, compared to the wt (Results fig.8). These data reinforce our demonstration that uPAR regulates activated epidermis regeneration by a Rac1-dependent mechanism.

The consequence of uPAR deletion, particularly in the epidermis demonstrates that this receptor regulates not only tissue organization, probably by the specific Rac1-Myc-dependent signalling pathway, but also differentiation. Furthermore, they suggest that in addition to promoting invasion, increased expression of uPAR in epithelial tumors may stimulate expansion of the stem cell compartment, migration of these same skin stem cells within the tissue and inhibit differentiation. Since bulge cell progeny are required for skin tumor development in mice, these findings offer an opportunity for gaining insight into the earliest stages of neoplastic development.

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Since the beginning of my study at the Hogeschool Utrecht I have wished to do my final internship in Italy. After some first meetings with Mario van Berlo about possibilities abroad I went looking for a lab at the end of February 2007. Immediately I got a promising response from Prof. Blasi of San Raffaele in Milan. We arranged my stay in the lab over email and at 23 April I started my internship in his lab as a member of the subgroup of Silvia D'Alessio.

Thanks to some friends I already had in Milan I was able to find a nice place to live during my stay. The first weeks were quite difficult, I was adapting to the new environment and new situations. After these first couple of weeks I managed to get used to living here and speaking Italian. Additionally I had a major opportunity to learn a lot about working in a research lab.

In the end I am thankful to Prof. Blasi for the opportunity to do my internship abroad. Of course my special thanks go out to Silvia D'Alessio for her kind supervising and her help with my thesis. I'm very grateful for all the effort she did to help me during this internship. Besides that, I would also like to thank her and Laura Gerasi for the help and explanations to improve my skills in the lab.

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