Capturing epidermal stemness

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Abstract

Regenerative medicine aims to replace or regenerate tissues or organs to re-establish their normal function. In 1975, Rheinwald and Green developed a technique to isolate and amplify epidermal stem cells. Their discovery led to the development of cultured epidermal autografts (CEA), the first regenerative therapy using cultured cells.

Adult stem cells are the working force behind tissue homeostasis and repair. Through constant division and specialization, they produce enough daughter cells to maintain tissue architecture and function. This process is orchestrated by an elegant cross-talk between the stem cells and their microenvironment.

By using irradiated feeder cells (3T3-J2 cells), Rheinwald and Green were able to artificially instruct epidermal cells to grow *in vitro*. Later on, they discovered that these cultured cells could regenerate a functional epidermis when transplanted on patients. However, Barrandon and Green demonstrated that clonogenic keratinocytes lose progressively their growth potential *in vitro*. This process is called clonal conversion.

The 3T3-J2 cells are mouse embryonic fibroblasts. The molecules produced by these cells are necessary to promote self-renewal of keratinocyte stem cells *in vitro*. If the quality of the culture system is not monitored, clonal conversion can occur rapidly and the therapeutic potential is lost. Although the system is now used in the clinics for the treatment of large burns and cornea injuries, the regulatory affairs express genuine concerns towards the animal origin of the feeder cells. Ultimately, we would like to replace the current 3T3-J2 culture system by a fully defined alternative, devoid of animal products, for the production of CEA. In this thesis, we describe two strategies to address this challenge.

First, we developed a large scale RNAi strategy to investigate the cellular cross-talk between

feeder cells and human keratinocytes. We have identified several putative “feeder genes”. One of these genes is *FURIN*, a serine protease.

Second, we investigated the impact of ROCK inhibition on the procurement and culture of human keratinocytes. We found that it promoted the adhesion and proliferation of freshly isolated human keratinocytes. In opposition to what was described previously, we did not observe evidences of cellular immortalization or reprogramming when keratinocytes where treated with Y-27632 (ROCK inhibitor).

Together, the results of our two approaches provide new leads for the further development of a new culture system for human keratinocyte stem cells.

Keywords: 3T3, cell culture, epidermal stem cells, high-throughput screening, ROCK

Résumé

La médecine régénérative a pour but de remplacer ou régénérer les tissus et les organes afin de restaurer leur fonction. En 1975, Rheinwald et Green ont développé une technique pour isoler et amplifier les cellules souches épithéliales de l'épiderme. Cette découverte a abouti au développement des greffes cultivées autologues, la première thérapie de médecine régénérative utilisant des cellules amplifiées en laboratoire.

Les cellules souches adultes sont responsables du maintien et de la réparation de nos tissus. En se divisant et se spécialisant continuellement, elles produisent suffisamment de cellules filles pour maintenir l'architecture et la fonction de nos tissus. Ce processus est orchestré par un élégant dialogue entre les cellules souches et leur microenvironnement.

En utilisant des cellules nourricières irradiées (3T3-J2), Rheinwald et Green ont pu instruire artificiellement les cellules de l'épiderme à proliférer *in vitro*. Plus tard, ils ont découvert que ces cellules pouvaient régénérer un épiderme fonctionnel une fois transplantées sur des patients. De plus, Barrandon et Green ont démontré que les kératinocytes clonogéniques perdaient progressivement leur potentiel de croissance en culture. Ce processus s'appelle la conversion clonale.

Les cellules 3T3-J2 sont des fibroblastes de souris embryonnaires. Les molécules qu'ils produisent sont nécessaires pour promouvoir l'auto-renouvèlement des cellules souches de l'épiderme. Si la qualité du système de culture n'est pas suivie, la conversion clonale peut avoir lieu rapidement et le potentiel thérapeutique des cellules est perdu. Bien que le système des 3T3-J2 soit utilisé en clinique pour le traitement des grands brûlés et de certaines pathologies de la cornée, les affaires régulatrices expriment des préoccupations concernant l'origine animale des cellules nourricières. Idéalement, nous souhaiterions remplacer le système actuel

des 3T3-J2 par une alternative définie, sans aucune trace de composantes animales. Dans ce travail de thèse, nous avons développé deux stratégies pour aller dans cette direction.

Dans un premier temps, nous avons mis au point une approche à haut débit utilisant l' ARN interférence pour comprendre le dialogue cellulaire entre les 3T3-J2 et les cellules épithéliales. Nous avons ainsi identifié plusieurs gènes responsables de ce dialogue. L'un de ces gènes est la *Furin*, une protéase à sérine.

Dans un deuxième temps, nous avons étudié l'impact de l'inhibition de ROCK sur l'isolation et la culture des kératinocytes humains. Nous avons démontré que l'ajout de Y-27632, un inhibiteur de ROCK, au milieu de culture améliorait l'adhésion et la prolifération de cellules fraichement isolées. De plus, en opposition avec ce qui est décrit dans la littérature, nous n'avons pas trouvé de preuves témoignant de l'immortalisation ou de la reprogrammation de ces cellules par Y-27632.

Ensemble, les résultats de ces deux approches apportent de nouvelles pistes pour le développement d'un nouveau système de culture pour les cellules souches de l'épiderme humain.

Mots-clés : 3T3, culture cellulaire, cellules souches de l'épiderme, criblage à haut débit, ROCK

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1 Introduction

1.1 Stem cells

1.1.1 Definition

Stem cells are responsible for the development and renewal of our tissues and organs. They are defined by two properties, they can continuously self-renew and they have the ability to produce specialized cells. Self-renewal implies that when a stem cell divides, it always produces at least one daughter cell equivalent to the mother cell. When a stem cell enters commitment, it stops to self-renew and it starts to differentiate.

The potency of a stem cell defines the lineage choices available for commitment. There are five levels of potency. Stem cells can be pluripotent and generate all tissue lineages (with some extraembryonic lineages). Multipotent stem cells can form all the lineages of an entire tissue. Oligopotent stem cells can only form some, but not all lineages within a tissue. Unipotent stem cells can commit to only one lineage. Finally, only the zygote and the morula are totipotent and sufficient to form an entire organism (Smith, 2006).

1.1.2 Adult stem cells

Adult stem cells are responsible for tissue renewal, growth and repair. Although many hypothesized their existence, they were first identified in the hematopoietic system (Becker et al., 1963; Wu et al., 1968; Till and McCulloch, 1961). Now, we know that many other tissues (such as the

skin, the cornea or the intestine etc.) have their own reservoir of stem cells. Stem cells from different tissues do not have the same potency. For example, the hematopoietic stem cell is a multipotent stem cell capable of regenerating all myeloid and lymphoid lineages, whereas the interfollicular keratinocyte stem cell will only differentiate in corneocytes during homeostasis.

Not all tissues have the same turnover or the same prevalence to injury. Stem cells from different tissues display different behaviors. Some divide frequently to produce enough daughter cells for the tissue to function, while others only display increased mitotic activity during repair or regeneration. However, all adult stem cells can make the same choices. Stem cells can divide or remain quiescent. When they divide, they can give rise to two identical daughter cells (symmetric division) or to two different daughter cells (asymmetric division). They can also differentiate directly or die (Fig. 1.1). All stem cells within our body continuously choose between one of these outcomes with different frequencies.

The fate of the stem cell, the "output", is decided by the integration of all intrinsic and extrinsic inputs. The intrinsic components include several factors, such as the mRNA levels or protein levels of various genes, the activity of different stress responses (DNA damage, infection, metabolic, oxydative), the concentration of metabolites and nutrients within the cell. In the other hand, the extrinsic signals influencing stem cell fate are integrated in the stem cell microenvironment.

1.1.3 The microenvironment

The microenvironment, or the stem cell niche, encompasses all the extrinsic signals influencing stem cell fate. This concept was introduced in a seminal paper by Schofield in the late 1970s (Schofield, 1978). The microenvironment is dynamic and composed of different types of signals. Their sources are the interactions between the stem cells and the extracellular matrix, growth factors and "niche" cells. The presence or absence of humoral factors, chemokines, metabolites, in adition with physical and chemical constraints also influence stem cell behavior. Nerve endings can also have a function in the microenvironment.

Not only do stem cells respond to their microenvironment, they also remodel it. As the organism develops, stem cells specialize and differentiate. During this process and throughout

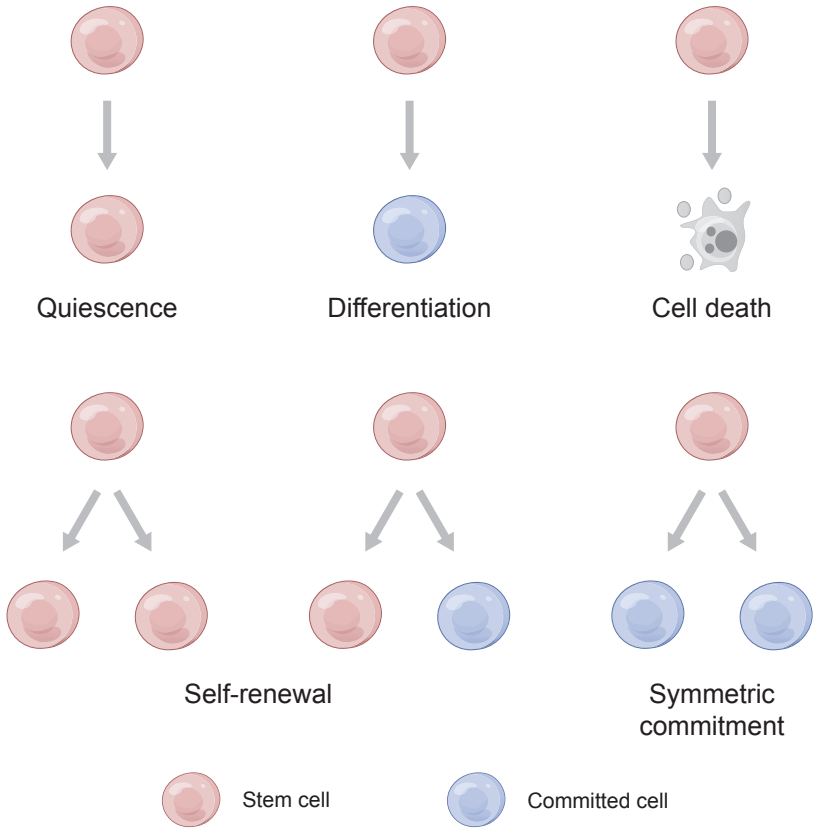

Figure 1.1 – Stem cell fates. Stem cells can remain quiescent, differentiate or die. They can also self-renew or produce committed daughter cells through symmetric or assymetric cell division.

homeostasis, cells reshape the surrounding extracellular matrix to provide corresponding signals required for proper development. When they differentiate, stem cells give rise to specialized cells which feedback directly with the stem cell through cell-cell interactions and other signaling molecules (Scadden, 2014).

1.1.4 Cellular plasticity

Cellular plasticity is of particular importance for stem cell biology and regenerative medicine. This concept describes the fact that some cells can display an increase in potency in peculiar conditions (Smith, 2006). The fundamental discovery of nuclear reprogramming demonstrated that the differentiated state of a cell is not fixed and no genomic information is lost during development, with the exception of VDJ recombination events (Gurdon et al., 1958; Takahashi

and Yamanaka, 2006). Somatic nuclear transfer, forced expression of transcription factors and cell fusion experiments are all elegant examples of nuclear reprogramming which lead to an increase in potency of the targeted cell (Blau et al., 1985). However, cellular plasticity is not limited to the artificial environment of the laboratory. It is also displayed by several plant and animal cells *in vivo* (Sánchez Alvarado and Yamanaka, 2014).

In mammals, cellular plasticity can refer to three different phenomena. First, some adult stem cells can transdifferentiate in stem cells of recipient tissues when transplanted in a different microenvironment. In rats, thymic epithelial cells have the capacity to behave as bonafide multipotent epidermal stem cells when challenged by a skin reconstitution assay (Bonfanti et al., 2010). In humans, oral mucosa epithelial cells can be expanded in vitro and transplanted on the cornea stroma to regenerate an epithelium. In both cases, transplanted cells and their progeny adopt similar morphologies and gene expression patterns of the tissue resident stem cells (Nishida et al., 2004).

The second case of plasticity has been revealed through lineage tracing experiments in mice. During repair, some tissues have different strategies to restore integrity. In epithelia of the lung, the stomach and the intestine, specialized cells can dedifferentiate and replace the stem cells lost after injuries or targeted depletion (Stange et al., 2013; Tata et al., 2013; van Es et al., 2012). In the liver, hepatocytes can transdifferentiate in biliary epithelial cells after injury (Michalopoulos et al., 2005). In mice hair follicles, epithelial cells adjacent to the bulge migrate and adopt the fate of the stem cells if those are ablated (Rompolas et al., 2013).

The third occurrence of cellular plasticity comes from clinical observations. Epithelial metaplasia is a pathology characterized by a switch of cellular phenotype. For example, Barett's metaplasia is characterized by a switch of eosphagus epithelium into an intestinal epithelium (Bonfanti et al., 2012). A dramatic change in the local microenvironment is often the underlying cause. The complete molecular mechanisms behind this phenomenon remains to be identified (Slack, 2007).

Altogether, these evidences demonstrate that cells can adopt a different fate in response to a change in their microenvironment. Whether they are transplanted in a different organ or facing local perturbation, cells can hijack lineage restriction and restore local tissue function.

Interestingly, R. Schofield already suggested in his 1978's paper that specialized cells could become "fixed stem cell" if they had found a niche that would provide them with the appropriate signals. It will be of interest to know if these properties extend to other tissues and cells of the human body.

1.1.5 Capturing stemness

Stem cell therapy aims to harness the potential of stem cells to rebuild tissues and organs to restore their normal functions. Stem cells (somatic or pluripotent) are often rare and prone to differentiate in response to stress. To circumvent these limitations, scientists have developed culture systems to both promote stem cell self-renewal and prevent differentiation *in vitro*. These artificial microenvironments provide the required signals to capture stem cells and their potential, allowing the study of developmental and regenerative processes and the development of new regenerative therapies.

One of the most popular model system for developmental biology are the embryonic stem (ES) cells or pluripotent stem cells (PSC). These cells were first derived from the inner cell mass (ICM) of a mouse embryo and cultured in presence of a layer of irradiated feeder cells (mouse embryonic fibroblasts, MEF) in medium containing fetal calf serum (Martin, 1981; Evans and Kaufman, 1981). These cells can grow indefinitely *in vitro* and form teratocarcinomas when grafted to adult mice. More importantly, they can contribute to all tissues of a chimeric mouse when transplanted in a recipient developing blastocyst (Bradley et al., 1984). This was the first demonstration of a culture system that could capture pluripotency of normal cells *in vitro*. Few years later, the same approach was used to derive human embryonic stem cells isolated from the ICM of a developing blastocyst produced by *in vitro* fertilization (Thomson, 1998).

Feeder cells (mitotically inactivated cells) were previously used to support the growth of Hela cells and normal epithelial cells (Puck et al., 1956; Cieciura et al., 1956). Later on, the same approach used to support the growth of teratocarcinoma cell lines that paved the way to embryonic stem cell culture (Martin and Evans, 1975; Rheinwald and Green, 1975a). A similar approach was used to develop a culture system that supports hematopoiesis with the OP9 stromal cell line (Kodama et al., 1994). Although feeder cells enable the cultivation of cells of

various nature, they also have drawbacks. First, their animal origin often raises concerns for further clinical applications. They also add an additional level of complexity to the molecular mechanisms underlying growth, self-renewal and differentiation of stem cells *in vitro*. The same is true for the serum. Feeder cells also require to be cultivated in parallel with their own medium.

There is an ongoing effort to develop feeder-free and serum-free culture systems for both mouse and human pluripotent stem cells. By screening conditioned media, they identified LIF (leukemia inhibitory factor) as a key molecule to inhibit mouse ES cells differentiation (Smith et al., 1988). In addition, LIF can replace the use of feeder cells for some, but not all mouse ES cell lines. The addition of BMP4 (bone morphogenetic protein 4) further alleviates the use of the serum by blocking neural differentiation of mouse ES cells (Ying et al., 2003). The system was further improved by the development of 2i (2 inhibitors) to target MEK (mitogen-activated protein kinase/ERK kinase) and GSK3 (glycogen synthase kinase 3). Inhibition of these two proteins, in combination with LIF, enables the derivation of mouse ES cells from previously non permissive mice strains in feeder-free and serum-free conditions (Wray et al., 2010).

Unlike mouse ES cells, human pluripotent stem cells require different culture conditions. Human ES cells do not depend on LIF to promote self-renewal *in vitro*. Instead, they require the addition of FGF2 (fibroblast growth factor 2 or bFGF) to the culture medium in addition to 20% fetal calf serum or 20% Knockout Serum Replacer (Amit et al., 2000). By studying the components of the serum, S1P (sphingosine-1-phosphate) and PDGF (platelet-derived growth factor) were identified as being sufficient to promote self-renewal of hES cells in serum-free condtions (Pébay and Pera, 2009). However, the culture system for human pluripotent stem cells was still dependent on MEF. The first report of feeder-free culture of hES cells used a combination of Matrigel® and feeder conditioned medium (Xu et al., 2001). While they can replace the feeder layer, these two components are not xeno-free. Matrigel® is a mixture of extracellular matrix proteins and growth factors directly extracted from mouse sarcoma tumors (Kibbey, 1994). Matrigel® is also susceptible to batch variation due to its nature. Laminins are one of the main constituent of Matrigel®, they are also the first extracellular matrix proteins expressed during development. Culture dishes coated with Laminins can limit the use of Matrigel®. Further trials identified recombinant human LN-511 (Laminin 511) and

its E8 fragment as the best alternative to Matrigel® and feeder cells for both human ES and IPS cells culture (Domogatskaya et al., 2008; Rodin et al., 2010; Miyazaki et al., 2012).

The development of mouse and human ES cells culture systems are great examples of the empirical approaches used to build systems that efficiently capture stemness *in vitro*. While the use of feeders initially permitted ES cells culture, it had flaws. But, it allowed researchers to study self-renewal and the signaling pathways underlying stem cell fates choices. By progressively understanding the role of some key proteins and pathways, they were able to build xeno-free and defined culture systems.

1.2 The skin

1.2.1 Structure of the skin

The skin is the largest organ of the body. It acts as a barrier to protect the organism from the environment and dehydration. The skin is also important for thermoregulation, nutrients retention and vitamins synthesis. This organ is subdivided in three different layers: the epidermis, the dermis and the hypodermis. The epidermis, the outermost layer of the skin, is a stratified squamous epithelium formed by keratinocytes. The dermis is the underlying connective tissue that provides elasticity to the skin. The innermost layer, the hypodermis, acts as an energy reservoir and thermal barrier by storing fat. Hair follicles, sebaceous glands and sweat glands are epidermal appendages which extend into the dermis (Fig. 1.2). These structures arise during development and have distinct functions. Their formation results from the interaction between epidermal cells and the underlying mesenchyme.

1.2.2 The epidermis

The epidermis is very thin in comparison to the dermis and hypodermis.It renews constantly with the division of basal epidermal cells and their progressive outwards migration and differentiation (Fig. 1.3). These basal cells rest on a basement membrane produced by both keratinocytes and dermal fibroblasts. Basal keratinocytes can be identified by the expression of KERATIN 5 and 14 (KRT5 and KRT14). When they leave the basal layer, they enlarge and

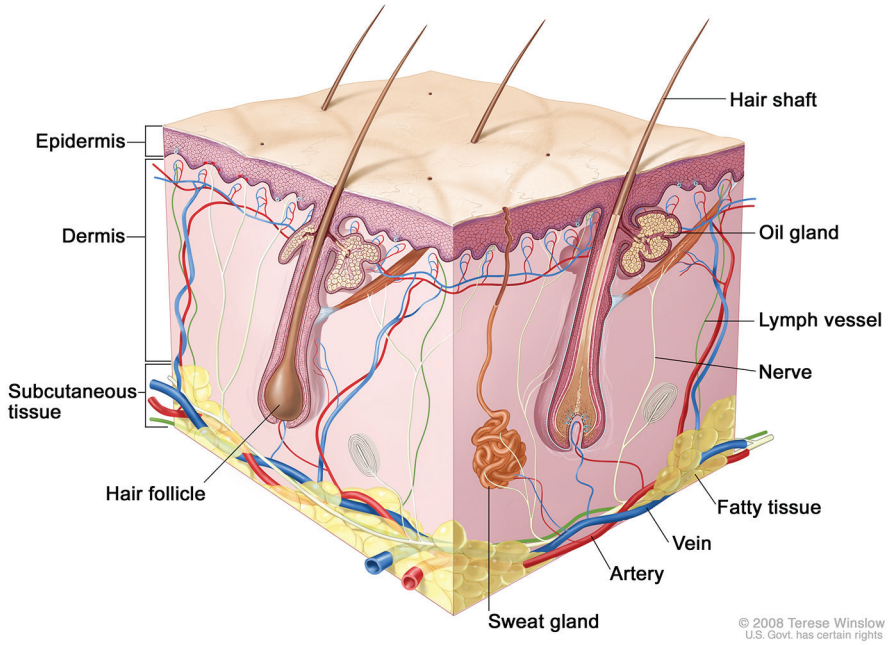

Figure 1.2 – Schematic representation of human skin. (For the National Cancer Institute © 2008 Terese Winslow, U.S. Govt. has certain rights.)

connect with adjacent cells through desmosomes. It results in the formation of the spinous layer. At the same time, these cells start to differentiate and switch the expression of KRT5 and KRT14 to KRT1 and KRT10. The granular layer rests above the spinous layer and precedes the stratum corneum. The latter is formed by terminally differentiated cells that have lost their nuclei. These cells possess a cornified envelope produced by the crosslinking of precursor proteins, such as IVL (involucrin) and LOR (loricrin), with several lipids (fatty acids, sterols and ceramids) by transglutaminases.

The epidermis also hosts other cells that contribute to skin function. Melanocyte stem cells and differentiated melanocytes are localized within hair follicles and throughout the basal layer of the epidermis. They provide photo-protection by producing melanosomes which are transferred to keratinocytes (Nishimura et al., 2002). Merkel cells are also located in the basal layer of the epidermis and hair follicles. They are of epithelial origin and participate in mechanotransduction (Van Keymeulen et al., 2009). Langerhans cells and resident T lymphocytes are also present within the epidermis and provide additional defense mechanisms to the skin immune system (Pasparakis et al., 2014). These cells produce multiple cytokines that can

impact keratinocyte stem cells (Pasparakis et al., 2014).

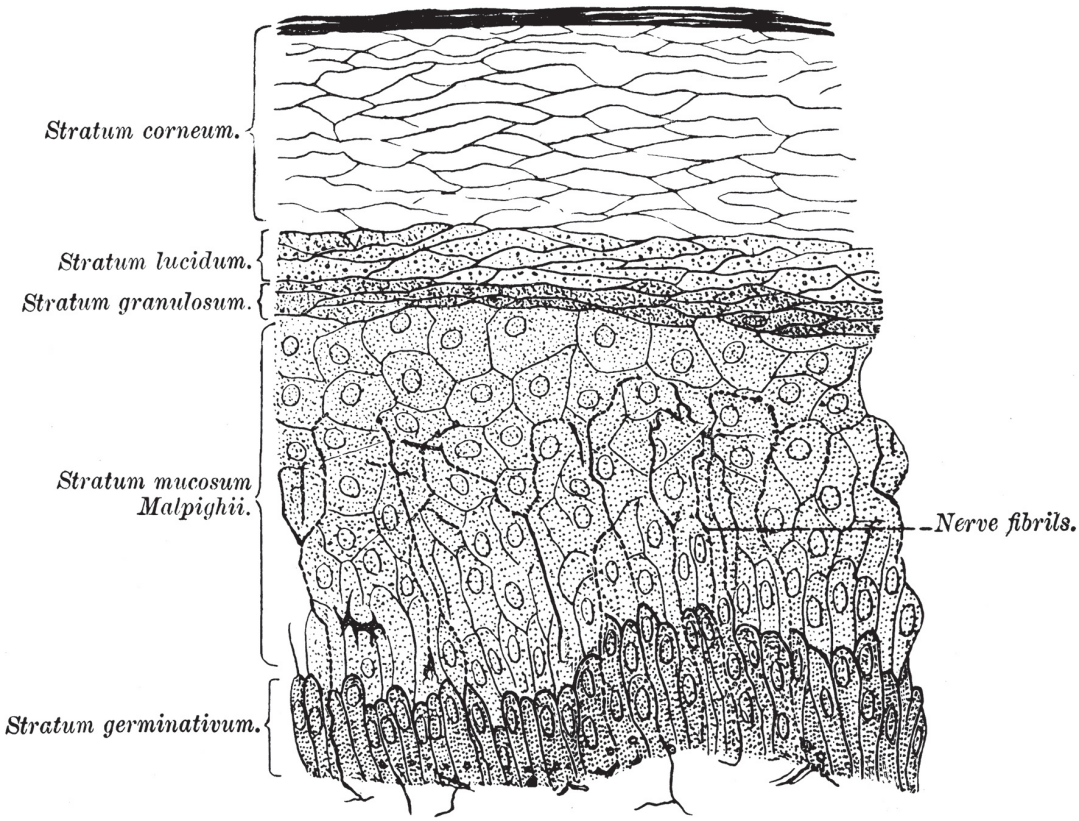

Figure 1.3 – Illustration of the epidermis. Gray, Henry Gray's Anatomy: *Descriptive and Applied* (Philadelphia: Lea & Febiger, 1913) 1154. Copyright © 2004–2016 Florida Center for Instructional Technology.

1.2.3 Culture of human epidermal stem cells

In 1975, Rheinwald and Green made the seminal discovery that keratinocytes can be serially cultivated *in vitro* on a feeder layer of lethally irradiated 3T3-J2 cells (Fig. 1.4) (Rheinwald and Green, 1975b). These cells were derived from mouse embryonic fibroblasts by the same laboratory (Todaro and Green, 1963). Using this culture system, keratinocytes can grow and form an epithelium that can regenerate a fully functional epidermis when transplanted on the back of a recipient athymic mouse (Banks-Schlegel and Green, 1980). This discovery was quickly translated in a clinical application to produce cultured epithelium autografts (CEA) for burn patients (Fig. 1.5). In 1984, two young large burn patients had their lives saved by CEA derived from tiny skin biopsies (O'Connor et al., 1981; Gallico et al., 1984). CEA are now

successfully used in competent clinics for the treatment of 3rd degree burned patients and for the treatment of specific cornea injuries.

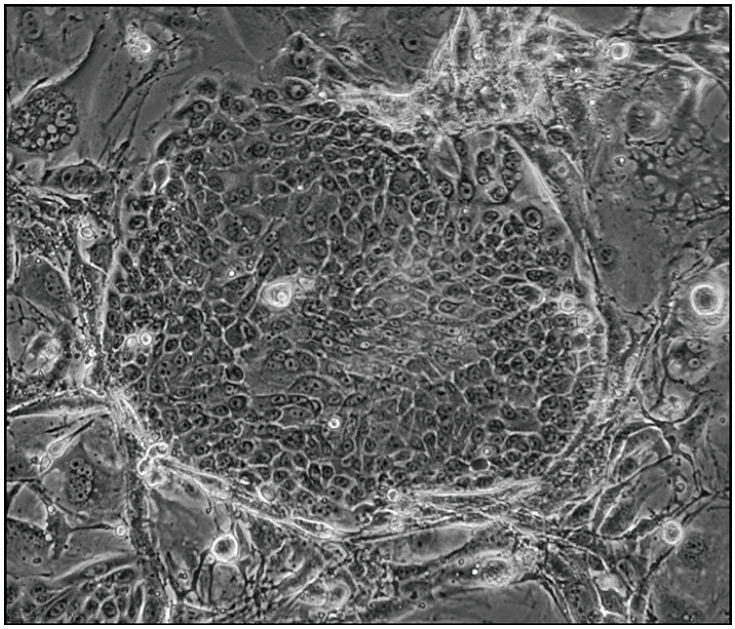

Figure 1.4 – A colony of human keratinocytes (center) surrounded by irradiated 3T3-j2 cells.

The regenerated epidermis can self-renew for several years (>20 years). This observation confirms that CEA include epidermal stem cells, as no remaining epithelial cells are present on the wound beds (burns are excised to the muscle fascia). It also demonstrates that stem cells were able to self-renew and maintain their potency during *in-vitro* expansion (Green, 2008; Claudinot et al., 2005). However, CEA are unable to recapitulate the developmental processes required for epidermal appendages formation. No hair follicles or gland are present in the regenerated epidermis.

The phenotype of cultured keratinocytes is different from their *in vivo* counterpart. Cultured epithelial cells form stratifying colonies of 2 to 3 layers (Rheinwald and Green, 1975b). The basal-like cells express KRT5, KRT14 and KRT17. The latter is normally expressed during wound repair (Lindberg and Rheinwald, 1990; Pastar et al., 2014). The suprabasal cells express markers of terminal differentiation, such as IVL (involucrin) and LEKTI (Simon and Green, 1985; Barrandon and Green, 1987b). The serial cultivation of normal keratinocytes is dependant on the density of feeder cells (Rheinwald, 1980). Moreover, addition of EGF or

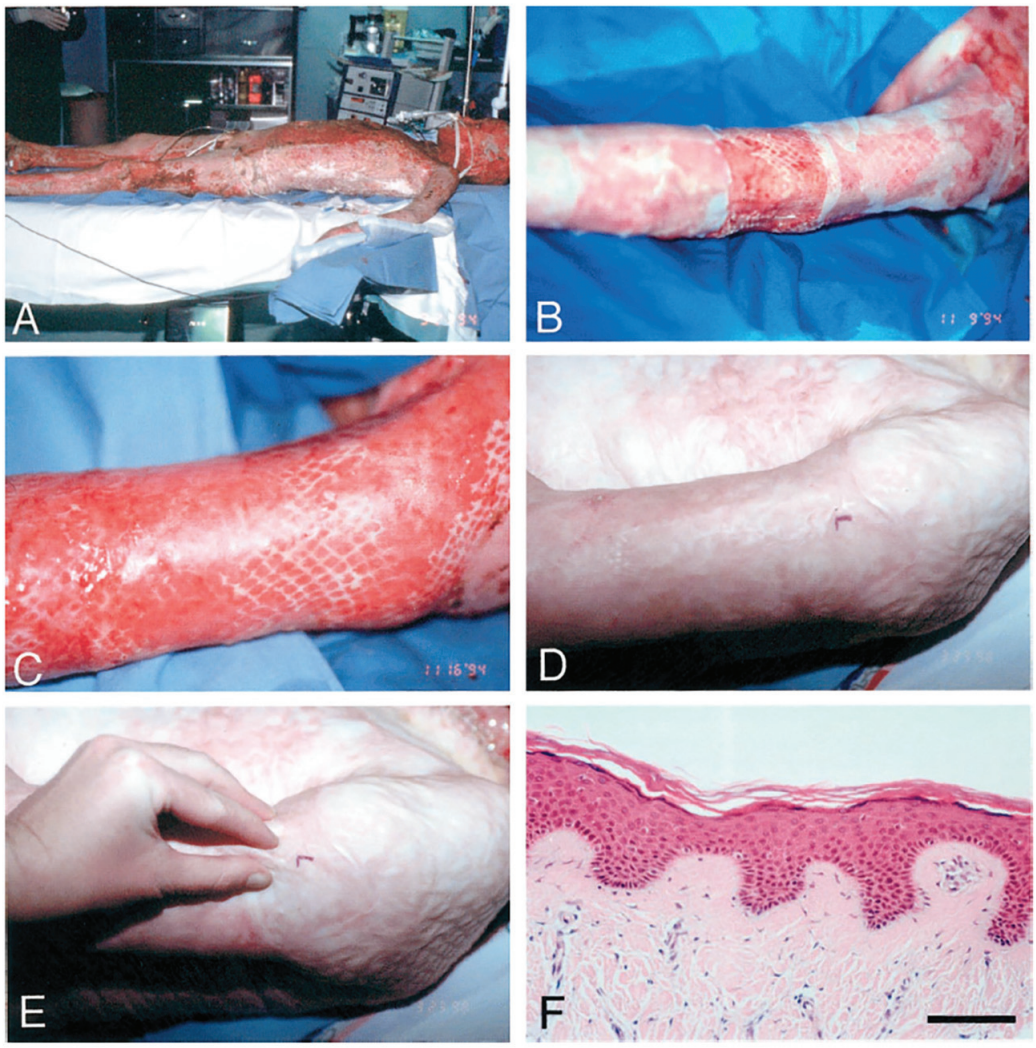

Figure 1.5 – "Long-term follow-up of cultured epithelia transplanted on a fibrin matrix. 9-year-old boy was burned by flames over 95% of his body. A, Admission at Percy Burn Centre a month after injury. B, Transplantation of cultured epithelia grown on a fibrin matrix on the left arm. C, Appearance of the transplanted area at take-down. D and E, Clinical appearance of the skin 3.5 years after the transplantation. The skin is elastic when pinched and has a smoother appearance than the neighboring split-thickness skin autografts. F, Histological appearance of the skin 3.5 years after transplantation. The epidermis is histologically normal. Note the presence of rete ridges and that of a superficial neodermis with vascular arcades. Similar results were obtained with cultured epithelia grown in absence of fibrin. Bar: 100 μm". Figure and legend from Ronfard et al. (2000)

TGFα to the culture medium greatly enhances keratinocytes migration and proliferation. It also improves keratinocytes colony forming efficiency in subcultures and enhances culture lifetime overall (Barrandon and Green, 1987a; Rheinwald and Green, 1977).

Cultured keratinocytes are not identical. Indeed, Barrandon and Green demonstrated that clonogenic keratinocytes can be classified in three different categories based on the growth potential of their progeny (see Fig. 1.6). Holoclones have the highest growth potential. They are the *in vitro* phenotype of epidermal stem cells (Rochat et al., 1994). Paraclones are cells with the lowest growth potential. Their progeny mostly form aborted colonies that express marker of differentiation such as IVL. Meroclones are composed of cells with intermediate growth capacity (Barrandon and Green, 1987b). Keratinocytes progressively lose their growth potential *in vitro*. At each passages, the number of holoclones diminishes while the number of meroclones and paraclones increases. This process is defined as clonal conversion (Barrandon and Green, 1987b). It highlights the fact that the 3T3-J2 culture system, in its current iteration, cannot capture epidermal stemness indefinitely. This process is accelerated if the culture conditions are not appropriate or if the cells were isolated from an old patient (Barrandon et al., 2012). Clonal conversion is also independent from replicative senescence as it can occur in few cell divisions (Barrandon et al., 2012). Rapamycin, a small molecule inhibitor of mTOR, can slow down clonal conversion. However, it also slows down keratinocyte proliferation (Mosig, 2013).

Although the culture system developed by Rheinwald and Green allows for long term keratinocytes cultivation and CEA production, it raises important safety concerns due to the animal origins of the 3T3-J2 cells and the serum. Several groups and companies have developed alternative culture systems for human keratinocytes. However, those systems either rely on the use of irradiated human fibroblasts or they require high cell seeding densities (Higham et al., 2003). Unfortunately, neither the feeder-free, serum-free and other commercially available media can recapitulate the performance of the 3T3-J2 culture system and its ability to produce CEA (Lamb and Ambler, 2013).

We know from the work of Rheinwald and Green that 3T3-J2 cells secrete growth factors and other soluble molecules that promote the survival and proliferation of human keratinocytes. To some extent, a conditioned medium can promote the growth of human keratinocytes. However, the system only reaches its optimal performance when keratinocytes are co-cultured directly with the lethally irradiated feeders (Rheinwald, 1980). This suggests that the physical contacts with the feeders play a positive role in the system. Moreover, it could also mean that

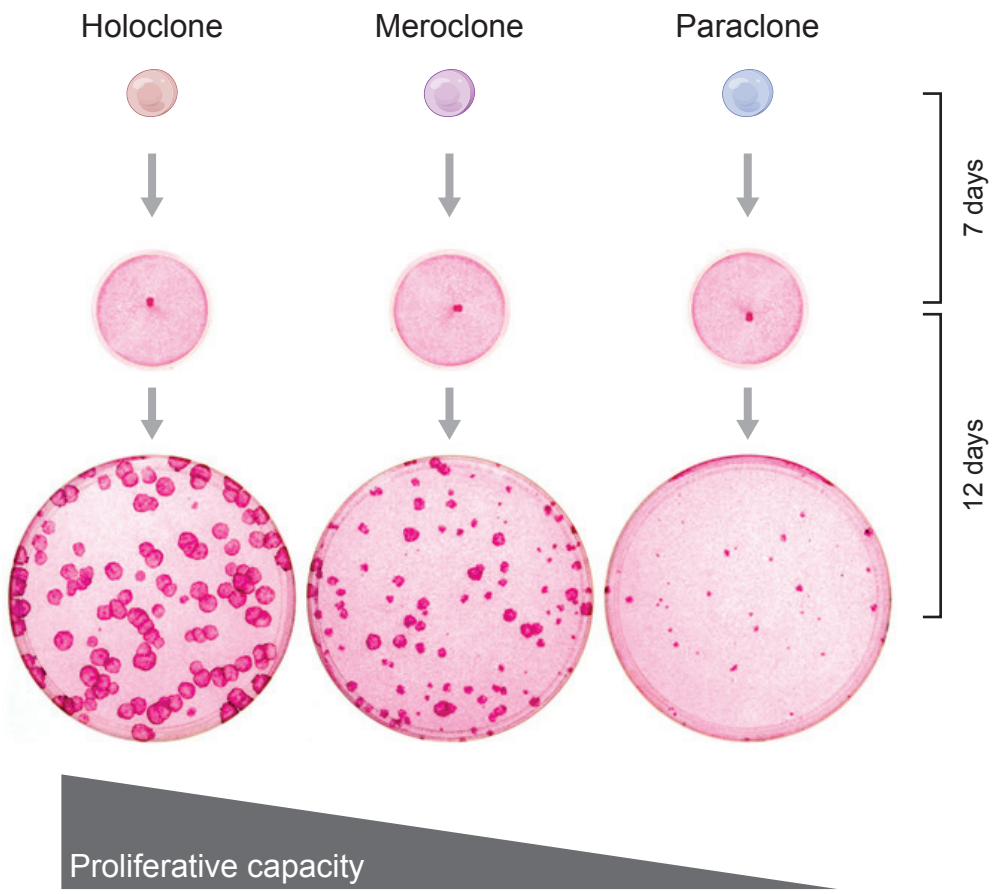

Figure 1.6 – Clonal analysis of human clonogenic keratinocytes. Holoclones generate daughter cells with high proliferative capacity. Paraclones mostly give rise to cells that form aborted colonies. Meroclones produce cells with both high and low proliferative capacity. Modified from Barrandon et al. (2012).

some soluble factors have limited diffusion properties and that the feeders might act as a buffer for inhibitory signals (Rheinwald, 1980).

To understand the function of the 3T3-J2 cells, several groups have investigated the proteins they produce and their molecular mechanisms. 3T3-J2 cells produce several proteins of the extracellular matrix in culture, such as COL4 (collagen type IV), laminins and FN1 (fibronectin) (Alitalo et al., 1982). These proteins are normally expressed in the basement membrane of the epidermis and act as scaffolds and ligands for cell adhesion and cell signaling. Multiple laminins (such as Laminin-332 or Laminin-511) bind to integrins ($\alpha 6\beta 4$, $\alpha 3\beta 1$) expressed by basal keratinocytes to promote cell survival and cell proliferation (Sugawara et al., 2008).

Blocking the internalization process of ITGB1 (integrin β1) with a targeting monoclonal antibody inhibits terminal differentiation of human keratinocytes *in vitro*. Fibronectin binds to ITGB1 (integrin β1) and can also inhibit terminal differentiation in methylcellulose suspension. The effect is even more potent when laminins and COL4 are mixed with Fibronectin (Adams and Watt, 1989, 1990; Watt et al., 1993). These results strongly suggest that the extracellular matrix produced by the 3T3-J2 cells play a key role in their function. However, neither collagen-coated or laminin-coated culture dishes can replace the feeder cells.

Other signaling molecules have been identified as effector of the 3T3-J2 cells' function. Barreca and colleagues demonstrated that 3T3-J2 cells secrete IGF1 (insulin like growth factor I) which can positively influence keratinocytes proliferation (Barreca et al., 1992). The paracrine function of the 3T3 cells is not unidirectional. Together, human keratinocytes and feeder cells communicate through a double paracrine mechanism. Keratinocytes produce IL1A and IL1B (interleukin 1 α and β) which bind the IL1R (interleukin 1 receptor) on the cell surface of 3T3 cells to induce the production of KGF (keratinocyte growth factor). Blocking the function of either IL1A, IL1B, IL1R or KGF with neutralizing antibodies greatly impair keratinocytes proliferation (Maas-Szabowski et al., 1999). In addition, HGF (or scatter factor) is produced by 3T3-J2 cells and can promote growth and cellular migration of epithelial cells *in vitro* (Stoker et al., 1987; Panos et al., 1993). Recently, DACT1 (Dishevelled-binding antagonist of Beta-Catenin 1) was identified as a mediator of the 3T3-J2 function. DACT1 promotes keratinocyte proliferation through attenuation of WNT-induced production of TGF-β2 by the 3T3-J2 cells (Suzuki and Senoo, 2015).

While some pieces of the puzzle have been identified, other molecular mechanisms remain to be elucidated in order to replace the 3T3-J2 cells. Recently, a study highlighted the potential of SMAD inhibition to enable the long term cultivation of basal epithelial cells (P63 positive cells). They found that dual inhibition of TGFβ and BMP signaling could replace the feeder cells in human and mouse keratinocytes culture (Mou et al., 2016). It will be of interest to see if this allows clonal analysis and CEA production in feeder-free condition.

1.2.4 Epidermal homeostasis

The ability of the epidermis and its appendages to self-renew is ensured by the presence of multiple adult keratinocyte stem cells with different potency. Unipotent keratinocyte stem cells are located within the basal layer of the epidermis (VANSCOTT and EKEL, 1963; Penneys et al., 1970; Barrandon and Green, 1987b). In hair follicles, multipotent keratinocyte stem cells contribute to both hair follicle, sebaceous gland and merkel cell lineages. They can also contribute to the interfollicular lineage during wound repair. In human hairs, multipotent stem cells are located below the bulge of the follicle (Rochat et al., 1994). In rodents, the bulge region of the follicle is the main location of multipotent stem cells. However, stem cells can also be isolated from the lower part of the follicle during the growing phase of the hair cycle (Oshima et al., 2001; Blanpain et al., 2004; Claudinot et al., 2005).

Although the presence of epidermal stem cells has been confirmed (Blanpain and Fuchs, 2006; Green, 2008), several laboratories continue to disagree on a unified model for epidermal homeostasis. Initially, Potten argued for a hierarchical model, the epidermal proliferative unit (EPU). This model describes the epidermis as an assembly of small columnar units, each with one slowly dividing stem cell that gives rise to committed amplifying progenitors (Potten, 1974). However, lineage tracing experiments in mice did not confirm the EPU model (Clayton et al., 2007). When genetically labelled, single basal cells give rise to clones of various size and shape. The clone-size distributions could be predicted by a model involving only a population of equipotent progenitors (Clayton et al., 2007; Jones et al., 2007; Doupé et al., 2010). These cells can divide both symmetrically and asymmetrically with different frequencies to maintain the epidermis. Interestingly, the authors believe that those cells are distinct from stem cells (Clayton et al., 2007). This model was recently challenged by a study from Mascre and colleagues. Using a similar approach, they found a second population of cells within the basal layer, with reduced chances of long term survival (Mascré et al., 2012). Their analysis suggests that stem cells give rise to committed progenitors during homoeostasis. However, their data indicate that the fraction of committed progenitors within the basal layer is small. Additional work would be required to confirm a hierarchical relationship between the two basal cell populations. Although these studies disagree, they suggest that the basal layer of mouse epidermis has more stem/progenitor cells than expected. It would be of interest to

know if it is the same in human epidermis.

In human, keratinocyte stem cells are thought to express high amount of integrin α6 (ITGA6 or CD49f) and β1 (ITGB1). Cells with high amount of CD49f and low amount of transferin receptor (TFRC or CD71) have high colony forming efficiencies *in vitro* (Jones and Watt, 1993; Kaur and Li, 2000; Li et al., 1998). LRIG1, a transmembrane protein, has also been described as a putative marker for epidermal stem cells (Jensen and Watt, 2006). LRIG1 expression influences the mitotic activity of epidermal cells by modulating EGF signaling. When skin sections are labelled for one of these markers, the majority of basal cells are positive. It is currently not known if all positive cells are stem cells.

1.2.5 TP63

In 1998, Yang and colleagues identified TP63 as a new member of the P53 family of transcription factors (Yang et al., 1998). *TP63* encodes 6 different isoforms which can be distinguished by their different combination of N-terminal (TA and ΔN) and C-terminal (α, β, γ) ends. P63 isoforms share identical DNA-binding and oligomerization domains. The trans-activating domain is restricted to the TA isoforms. Similarly, the sterile alpha motif region is unique to the α isoforms (Yang et al., 1998). It has been shown that the main isoform expressed in mammalian epidermis and cultured human keratinocytes is ΔNP63α (referred as P63 for rest of this document) (Fig. 1.7). Genetic ablation of *P63* in mice results in a lethal phenotype. Newborn mice display a strong failure to develop and maintain different ectoderm and mesenchymal structures which require proper ectodermal-mesenchymal interactions, such as the skin and its appendages. The thymus and the limbs also fail to develop properly (Yang et al., 1999; Mills et al., 1999; Crum and McKeon, 2010; Senoo et al., 2007).

Initially, there was a controversy surrounding the proposed function for P63 in mammalian epidermis and other epithelia. Some believed that P63 was responsible for stratification and differentiation of keratinocytes while others were claiming that it was required for stem cell maintenance (Yang et al., 1999; Mills et al., 1999; Koster et al., 2007). However, recent studies indicate that P63 might be fulfilling both functions (Truong et al., 2006; Sen et al., 2012). High P63 expression is prominent in the basal layer of mammalian epidermis and corneal epithelia.

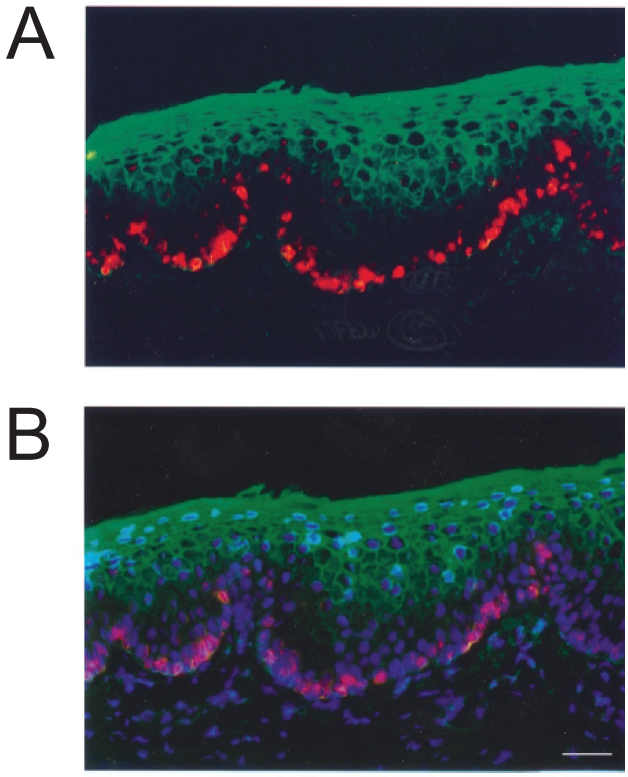

Figure 1.7 – Distribution of P63 and INVOLUCRIN in normal human epidermis. Frozen sections of human foreskin were fixed and stained for P63 (red) and INVOLUCRIN (green) (A), and for DNA (blue)(B). INVOLUCRIN appears in the spinous layer beginning several layers above the first layer lacking P63 and is typically located in the peripheral cytoplasm, close to the cell membrane. P63 is located mainly in the nuclei of the basal layer and immediately suprabasal layers of the epidermis. As in the case of the mRNA, cells with the most abundant P63 protein appear to be clustered in patches. The green color staining in the dermis is non-specific. Scale bar: 50 μ. Figure and legend from Parsa et al. (1999).

It also correlates positively with proliferative colonies in vitro (Parsa et al., 1999; Pellegrini et al., 2001; Senoo et al., 2007). Knockdown experiments have shown that P63 controls cellular adhesion and proliferation of epithelial cells, confirming its importance for maintaining stem cell properties (Carroll et al., 2006; Senoo et al., 2007). It is still unclear how P63 can achieve to regulate different processes important for both stem/progenitor and differentiating cells.

1.2.6 ROCK inhibition & clonal fate

In 2003, McMullan and colleagues described that human keratinocyte differentiation is regulated by RHO/ROCK (rho associated kinases) signaling (McMullan et al., 2003). ROCK-I and

ROCK-II belong to the family of serine-threonine kinases. They act downstream of RHO GTPases, which are regulators of various processes, such as cell adhesion, cytoskeleton dynamics and cell cycle (Jaffe and Hall, 2005). Using a ROCK inhibitor (Y-27632), they could inhibit keratinocyte differentiation and increase cell proliferation *in vitro*. They also observed an increase in keratinocyte colony forming efficiency in presence of Y-27632.

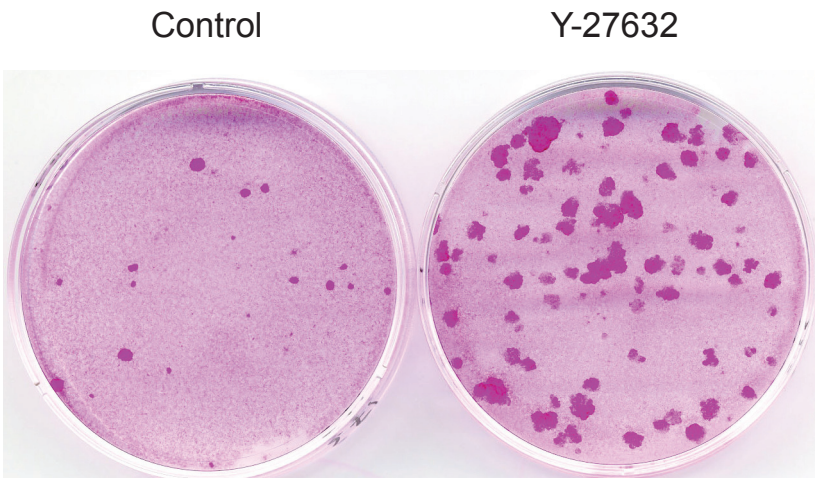

Figure 1.8 – The effect of Y-27632 on the procurement of human epidermal cells. Y-27632 improves the number of colony forming cells from freshly dissociated biopsies.

Few years later, Sasai and colleagues published a seminal paper on the impact of Y-27632 treatment for the culture of human embryonic stem cells (Watanabe et al., 2007). This study brought back the ROCK inhibitor under the spotlight and in 2010, Terunuma and colleagues reported that a RHO-associated protein kinases (ROCK) inhibitor (Y-27632) could greatly improve the culture of primary human keratinocytes (Terunuma et al., 2010). Colony forming efficiency of freshly isolated keratinocytes was efficiently increased (50 fold) when cells were cultured in presence of Y-27632 (Fig. 1.8). With these information in mind, Terunuma and colleagues hypothesized that the ROCK inhibitor could enhance the survival of keratinocyte stem cells and/or promote progenitor cells to exhibit a stem cell behavior.

Other studies confirmed the effect of Y-27632 on epithelial cells' survival and proliferation *in vitro* (Chapman et al., 2010; Suprynowicz et al., 2012). Initially, they interpreted their results as an observation of cellular immortalization. Later, they refined their conclusion and postulated that Y-27632 could induce a conditional reprogramming of cultured epithelial cells which can

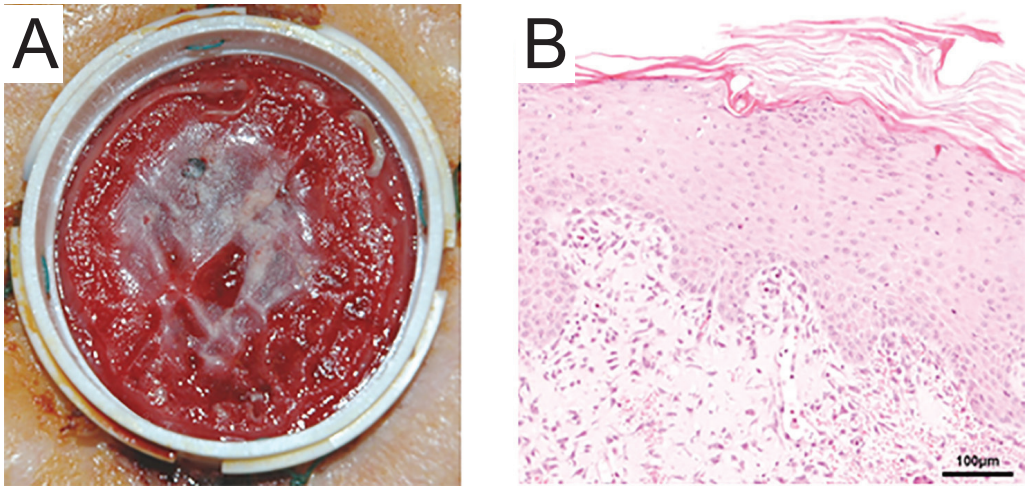

Figure 1.9 – Pig keratinocytes were cultured in presence of Y-27632 and transplanted on the muscle fascia of a pig wound model (A). After 14 days, a biopsy confirmed the ability of the transplanted cells to regenerate all the layers of the epidermis (N. Grasset & F. Gorostidi, unpublished data).

be reverted by removal of the ROCK inhibitor from the medium (Liu et al., 2012).

The effect of Y-27632 on epidermal cell culture is very promising for both basic research and regenerative medicine (Fig. 1.9). Our group has studied its effects on long term culture and single cell behavior (A. Rochat & F. Gorostidi, unpublished data). They were able to confirm that Y-27632 promotes a holoclone-like behavior in single cells that have successfully adhered to the culture dish (Gorostidi, 2012). Moreover, Nanba and colleagues demonstrated that ROCK inhibition was insufficient to restore the growth potential of a paraclone (Nanba et al., 2013). This result was also confirmed by our laboratory (unpublished data, Y. Barrandon & A. Rochat).

The effect of ROCK inhibition is highly reproducible, but the molecular mechanism underlying this phenomenal phenotype is still poorly understood. We know that ROCK regulate various cellular processes. Actin filaments stabilization and actin network assembly are regulated by the activity of ROCK through myosin light chain (MLC) and LIMK respectively. Moreover, ROCK mediate the signal transduction from focal adhesion to actin filaments rearrangements. In consequence, ROCK regulate cell migration and establishment of cell polarity

(Riento and Ridley, 2003). Recently, it has been demonstrated that ROCK integrate multiple physical cues, such as local stiffness, cellular crowding and integrin signaling to control cellular proliferation *in vivo* and *in vitro*. These outcomes are often mediated trough changes in the activity of the Hippo signaling pathway (Halder et al., 2012). We currently do not know if any of these pathways are involved in the phenotype described earlier. A better understanding of the molecular pathway involved in ROCK inhibition would allow us to better understand and target clonal conversion.

2 Aims of the thesis

It has been more than 40 years since Rheinwald and Green discovered the 3T3-J2 culture system. Their discovery paved the way for the birth of therapy with cultured cells. Recently, new culture systems have been developed for the *in vitro* expansion of other epithelial stem cells (Sato et al., 2009; Wang et al., 2015). Although these systems have different compositions, they all rely on the ability of epithelial cells to tolerate artificial microenvironments. However, there are two obstacles that prevent the general use of these culture systems in both basic research and regenerative medicine. First, the underlying components of these systems are both undefined and from animal origin (feeder cells, animal serum, Matrigel®). Second, the quality of these systems can affect their ability to capture epithelial stemness and promote self-renewal *in vitro*. These two issues raise strong concerns towards the regulatory affairs, the scientific community and the clinicians.

The aim of this thesis is to provide new leads for the development of a better, animal free, culture system for human epithelial stem cells. With the development of high throughput technologies (Moffat and Sabatini, 2006), we now have the tools to investigate the molecular crosstalk between feeder cells and human epithelial cells. Moreover, the recent discovery of the effect of ROCK inhibition on the procurement of human keratinocytes suggests that a new implementation of the Rheinwald and Green culture system could be possible.

First, we developed a high-throughput approach using RNAi to investigate the cellular crosstalk between 3T3-J2 cells and human keratinocytes. We designed an assay to measure the

impact of a single feeder gene knockdown on keratinocytes. We then screened the 3T3-J2 cells with a siRNA (small interfering RNA) library for the druggable fraction of the mouse genome. We identified a small number of candidate genes and confirmed the biological impact on human keratinocytes for one of them. Although the strategy that we used was unable to identify genes with redundant functions or genes that were not covered by the siRNA library, we validated our experimental approach.

Second, to assess the compatibility of ROCK inhibition with the Rheinwald and Green culture system, we studied its impact on human keratinocytes. We confirmed that ROCK inhibition did not trigger cellular immortalization or reprogramming to a stem-like state. We also confirmed that Y-27632 treatment could inhibit keratinocyte differentiation and enhance the number of freshly isolated colony forming cells, as previously described in the literature. However, we could not identify the underlying molecular mechanisms behind this phenotype. Nevertheless, our results suggested that Y-27632 could improve the current culture system for human keratinocytes.

3 Materials & Methods

3.1 Cell culture

3T3-J2

3T3-J2 cells were propagated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 8% Bovine Serum (BS, Thermo Scientific) and incubated in 10% CO_2 atmosphere at 37°C). Cells were inoculated every 7 days at low density (1 to 5 x 10^5 cells per 162 cm^2 flasks). The culture medium was replaced every 3 to 4 days. For keratinocytes propagation, 3T3-J2 cells were irradiated with a dose of 60 Gy and then plated at a density of 2.5 x 10^4 cells/cm^2.

Human keratinocytes

Human keratinocytes were propagated on top of the irradiated 3T3-J2 cells with cFAD culture medium, a 3:1 ratio of DMEM and Ham's F12 culture medium (Amimed), supplemented with insulin (5 μg/mL, Sigma), Triiodothyronine (2 x 10^{-9} M, Sigma), hydrocortisone (0.4 μg/mL, Calbiochem) and cholera toxin (1 x 10^{-10} M, ICN). Cells were incubated in 10% CO_2 humid atmosphere at 37°C. For serial amplification, pre-confluent keratinocytes were trypsinized (0.05% trypsin and 0.1% EDTA) and seeded at appropriate density once a week. The culture medium was changed every 2 to 3 days for mass culture or every 4 days for colony forming efficiency. EGF was added at each feeding (10 ng/mL, Upstate Biotechnology Inc.). For

ROCK inhibition experiments, the small molecule Y-27632 (ROCK inhibitor) was added to the culture medium (10 μM, Tocris) from cell seeding and at each feedings. For FURIN inhibition experiments, the Proprotein Convertase Inhibitor (50 μM, Calbiochem) was added daily in cFAD without EGF to mimic the screening conditions. Similarly, recombinant human IGFI (100 ng/uL, Peprotech) was added daily in cFAD minus EGF for some experiments.

3.2 Human skin dissociation & cell sorting

Human skin samples were obtained from adult women (age 30-50). Skin biopsies were incubated overnight at 4°C in DMEM supplemented with 8% Bovine Serum (BS) prior treatment. The majority of the hypodermis and the dermis were removed with surgical scissors. Then, the tissue was cut in small pieces (0.5-1 cm^2) and incubated overnight at 4°C in DMEM/BS supplemented with 1mg/mL Collagenase/Dispase or 1 mg/mL Dispase (Roche). The next day, the tissue was incubated up to 2 hours at 37°C with agitation in the same medium. Fresh Collagenase/Dispase was added to the medium if the epidermis did not start to peel off after 1 hour of incubation. After incubation, the epidermis was removed from the remaining dermis and incubated in trypsin/EDTA for 5 minutes at 37°C. The epidermis and cells in suspension were centrifugated and resuspended in HBSS supplemented with 2% BS and 20 mM HEPES (FACS buffer). Cells were filtered using a 100 μm cell strainer (Millipore).

For cell sorting, cells were incubated with a primary antibody against ITGA6 (CD49f) conjugated with Alexa Fluor® 647 (1:100, clone GoH3, Biolegend) for 30 minutes at RT in FACS buffer. Cells were then washed and re-suspend in FACS buffer. Dead cells were stained with DAPI (1:1000). The sort was performed by the Flow cytometry core facility (FCCF) using a MoFlo Astrios (Beckman Coulter). Debris and doublets were excluded based on the physical parameters (forward and side light scatter). DAPI positive cells were excluded. Live cells were then separated in $CD49f^{high}$ and $CD49f^{low}$ fractions. The purity of the samples was checked by second flow cytometry analysis. Sorted cells were then used to perform colony forming efficiency analysis in presence or absence of Y-27632 (10 μM, Tocris).

3.3 Flow cytometry

Cells were trypsinized and re-suspended in cold Hank's balanced salt solution (HBSS) supplemented with 2% BS (Flow buffer). Then, the cells were incubated with conjugated antibodies on ice for 30min. After incubation, cells were washed once and re-suspended in 200 μL of HBSS/2%BS. The samples were filtered with a 70 μM cell strainer (Millipore) and analyzed with a BD™ LSR II cytometer (BD Biosciences). Cell viability was assessed using either DAPI or PI (1:1000).

3.4 cDNA synthesis

RNA extraction was performed using the RNeasy® Mini Kit (Qiagen). The RNA was either directly used for cDNA synthesis or stored at -80°C. Total RNA concentration was measured using a nanodrop spectrophotometer (Thermo Scientific). First strand cDNA synthesis was performed with the SuperScript® III reverse-transcriptase (Invitrogen) according to the manufacturer's protocol. 1 μg of total RNA and random primers were used for cDNA synthesis. The cDNA was then diluted ten times in nuclease free water (QIAGEN) prior downstream application.

3.5 Real-time quantitative PCR

Real-time quantitative PCR was performed according to the Taqman® or the Power SYBR® manufacturer's protocols (Life Technologies). The cDNA was amplified on the 7900HT QPCR system (Life Technologies). Reactions were run in triplicates. The delta CT method was used to measure relative gene expression. The reference CT was calculated as the average CT from the endogenous controls. We used commercially available Taqman® assays from Life Technologies or Integrated DNA Technologies to assess gene expression. Comparisons were performed using the paired t test (two-tailed).

3.6 Protein extraction

Total protein extraction

Pre-confluent 60 mm dishes were washed twice with cold PBS and lysed on ice with 1% Triton X-100, 50 mM Tris pH 8, 150 mM NaCl and protease inhibitors (Roche). Cells were then scrapped and harvested in eppendorf tubes and left for 45-60 min on ice for complete cell lysis. Cell debris were centrifugated (13'000 rpm, 10 min, 4°C) and the supernatant containing the proteins was then stored at -80°C.

Subcellular protein fractionation

Pre-confluent culture dishes were placed on ice and lysed according to the ProteoExtract® subcellular protein extraction kit's protocol (Merck Millipore). Samples were then stored at -80°C.

Protein quantification

Protein concentration was determined with the Pierce® BCA protein assay kit (Thermo Scientific). Protein samples were diluted 3 or 5 times before protein quantification to get accurate measurements.

3.7 Western blot

Protein samples in SDS-PAGE sample buffer (50 mM Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.02% bromophenol blue) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then blocked with 5% skimmed milk in TBS or TBST for 30-60 min and incubated with diluted primary antibodies (O/N at 4°C). After overnight incubation, membranes were washed and incubated with corresponding secondary antibodies (HRP-linked) for 1 h at room temperature (RT). Detailed informations of the primary antibodies are listed on Table 3.1.

Target	Clone	Manufacturer	Cat. No.	Dilution
P63	4A4	Sigma Aldrich	P3737	1:5000
YAP	-	Cell Signaling	4912	1:1000
p-YAP	-	Cell Signaling	4911	1:1000
TAZ	-	Abcam	ab84927	1:1000
FRIZZLED6	D16E5	Cell Signaling	5168	1:2500
FURIN	-	Abcam	ab3467	1:1000
H3	D1H2	Cell Signaling	4499	1:50000
CALNEXIN	-	BD transduction	610523	1:5000
pan-CADHERIN	-	Cell Signaling	4068	1:5000
GAPDH	6C5	Abcam	ab8245	1:50000
pRB	G3-245	BD pharmingen	554136	1:1000

Table 3.1 – Primary antibodies used for Western blot.

3.8 Immunocytochemistry

For immunocytochemistry, cells were grown on coverslip (12 well plate) for 7 to 10 days and fixed with 4% paraformaldehyde (PFA) for 15 min. The coverslips were then washed three times with PBS and permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 10 min. After another three consecutives washes with PBS, the cells were blocked with 2% bovine serum albumin (BSA) in PBS for 45 min. Cells were incubated overnight at 4°C with primary antibody diluted in blocking solution. After three washes with PBS, the cells were incubated with corresponding conjugated secondary antibodies diluted in PBS for 45 min at RT. DNA was counterstained with DAPI for 10 min. Coverslips were washed twice with PBS and mounted on microscope slides.

3.9 High-throughput screening of 3T3-J2

The mouse druggable genome siRNA library was first pooled (4 siRNAs per gene) by the Biomolecular Screening Facility (BSF) and then spotted on 96 well plates (BD Falcon) in duplicates. Briefly, 10 μL of pooled siRNAs (QIAGEN) were spotted (40 nM final concentration) with 0.4 μL of RiboCellIn transfection reagent (BioCellChallenge) and 10 μL of OptiMem (Life Technologies) with the Caliper Sciclone platform (PerkinElmer). The first and last columns were spotted with scramble siRNA (negative control) and mouse specific cell death cocktail

siRNAs (positive control) respectively. After 15 min incubation at RT, 10'000 irradiated 3T3-J2 cells (in 80 μL of DMEM/BS) were seeded in each well using a Multiflow liquid dispenser (Biotek). Assay plates were then incubated overnight in 10% CO_2 atmosphere at 37°C. The next day, the medium was removed using an ELx405 (Biotek) and 600 normal human epidermal cells (strain YF29) in 100 μL of cFAD were dispensed in each well with the Multiflow. The plates were then incubated for one week in 10% CO_2 atmosphere at 37°C with one medium change (cFAD without EGF) on the third day after seeding using the ELx405 and the Multiflow (Biotek). After 7 days of culture, the plates were fixed for 15 min with 3.4% formaldehyde (Sigma-Aldrich) and stained for 10 min with 3% Rhodamine B (Sigma). Next, the plates were washed 9 times with PBS using the ELx406 (Biotek) and the remaining Rhodamine B was then re-suspended in 100 μL of PBS by 20 min incubation at RT on an orbital shaker at 90 rpm. Finally, the total fluorescence of the Rhodamine B was measured using an Infinite F500 plate reader (Tecan). The acquisition parameters are listed on Table 3.2.

Parameter	Value
Excitation wavelength	485 nm
Emission wavelength	590 nm
Excitation bandwidth	20 nm
Emission bandwidth	10 nm
Gain	26
Number of flashes	5
Integration time	40 μs

Table 3.2 – Acquisition parameters for rhodamine B fluorescence measurements.

The mean (μ) and the standard deviation (σ) of the fluorescence signal for the positive and negative controls were used to calculate the Z' (equation 3.1) to assess the assay's quality both during the assay development and the screening campaign (Zhang et al., 1999).

$$Z' = 1 - \frac{3 * (\sigma_{\text{pos}} + \sigma_{\text{neg}})}{|\mu_{\text{pos}} - \mu_{\text{neg}}|} \tag{3.1}$$

$$Z' = \begin{cases} 1 & \text{SD = 0 - an ideal assay} \\ 1 > Z' \geq 0.5 & \text{separation band is large - an excellent assay} \\ 0.5 > Z' > 0 & \text{separation band is small - a double assay} \\ 0 & \text{no separation band - a poor assay} \\ < 0 & \text{both signals overlap - screening impossible} \end{cases} \tag{3.2}$$

If the Z' was below 0.5 (equation 3.2), the experiment (96 well plate) was repeated or excluded from the analysis. All the dispensing and read-out data were integrated in the managing software of the BSF. During the screen, the results were selected as putative hits when both duplicates produced a signal below the threshold given by this formula:

$$\text{Signal} < \mu_{\text{neg}} - 3 * \sigma_{\text{neg}} \tag{3.3}$$

3.10 CRISPR-Cas9 mediatd 3T3-J2 knockouts

To generate *Furin* knockout feeder cells, we transfected 3T3-J2 cells with a plasmid containing a guide RNA to target *Furin*, the hSPCas9 enzyme and EGFP (gift from the Constam lab). The plasmid was transfected using Lipofectamine® 3000 Reagent (Thermo Scientific). After 3 days, GFP^{pos} cells were sorted by the FCCF. Single cells were sorted in a 96 well plates prepared with irradiated untransfected 3T3-J2 feeder cells at low density (0.5 x 10^4 cells/cm^2). After 14 days, confluent cells were dissociated and cultured for two passages without irradiated feeder cells. Later, the cells were dissociated and used for protein extraction. Candidate clones were identified by Western blot analysis for FURIN expression.

3.11 Telomere length analysis

Genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. The DNA was digested with HinfI and RsaI overnight at 37°C. The digested DNA was separated by pulse field agarose electrophoresis (0.6% agarose

gel, pulse speed of 1 to 6 s at 200 V overnight at 14°C). The gel was dried and denaturated. It was then used as a membrane and hybridized to radioactive labelled telomeric oligonucleotide probes. The signal was captured with a FLA-3000 (Fujifilm).

3.12 GAPDH activity assay

Cell lysates were used to measure GAPDH activity with the KDalert™ GAPDH Assay Kit (Thermofisher) according to the manufacturer's protocol. The protocol was performed on 96 well plates.

4 Results

4.1 Deconvolution of the 3T3-J2 culture system

4.1.1 Assay development

siRNA reverse transfection in 3T3-J2 cells

To establish our screening assay, we first had to set-up the knockdown protocol in the feeder cells. To optimize the siRNA reverse transfection in irradiated 3T3-J2 cells, we tried multiple siRNA transfection reagents and found that RiboCellIn reagent (BioCellChallenge) was the most effective. It was also the less toxic reagent for 3T3-J2 cells (data not shown). To measure the efficiency of siRNA mediated gene knockdown, we reverse transfected irradiated 3T3-J2 cells with either scramble or *Gapdh* (a well known housekeeping gene) targeting siRNAs. Then, we measured the activity of GAPDH in cell lysates 72 hours post transfection. The measurments showed that GAPDH could be selectively and efficiently knocked down by siRNA reverse transfection (Fig. 4.1).

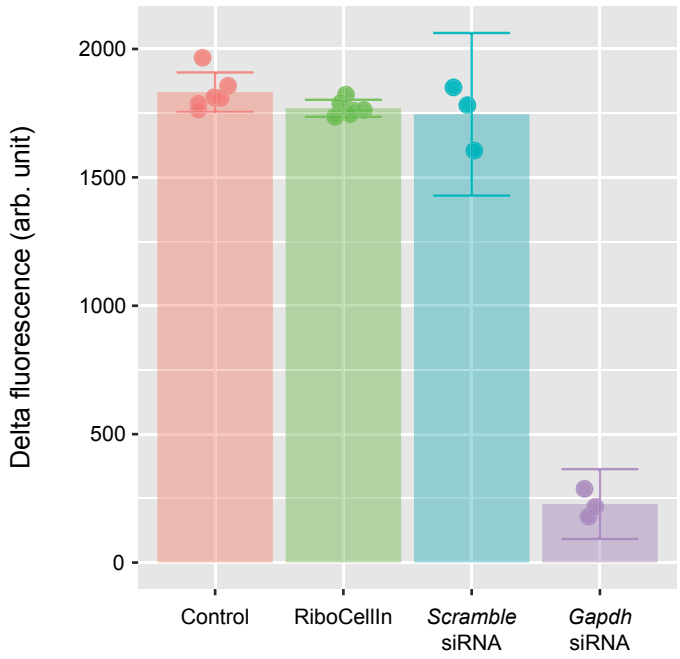

Figure 4.1 – Efficient knockdown of 3T3-J2 by reverse transfection of siRNAs. Irradiated 3T3-J2 cells were reverse transfected with scramble or *Gapdh* targetting siRNAs. After 72h, cells lysates were used to measure GAPDH activity. The delta fluorescence signal is proportional to the activity of GADPH. Error bars: 95% Student t-test confidence interval.

Screening assay

Next, we developed an assay to measure the impact of one feeder gene knockdown in the 3T2-J2 co-culture system. The assay had to be compatible for high-throughput screening (HTS). We decided to go for a single fluorescence measurement, instead of a high content assay (multiple measurments by imaging), to reduce the time of data acquisition and analysis. After few trials, we decided to adapt the reliable colony forming efficiency (CFE) assay for HTS (Fig. 4.2). We made the assumption that variation of the signal would reflect changes in human keratinocytes colony forming efficiency and proliferation. The Rhodamine B staining is also dependant on the keratinization of epithelial cells *in vitro* (Rheinwald and Green, 1975a). Stratifying colonies stain well with Rhodamine B. Therefore, the assay would allow us to detect genes that impact the system at different levels.

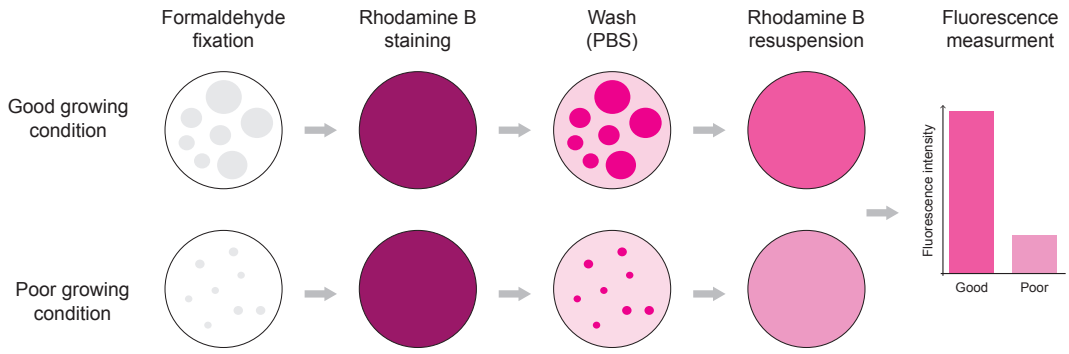

Figure 4.2 – Schematic representation of the rhodamine assay used to measure perturbation in the 3T3-J2 culture system.

Initially, 96 well plates were prepared with various densities of feeder cells on day 0. The next day, normal human keratinocytes (strain YF29) were seeded at different densities on top of feeder cells. Keratinocytes were then allowed to grow for 7 days with one medium change at day 4 (without EGF). Subsequently, the cells were fixed with 3.4% formaldehyde and stained with rhodamine B. After several washes, the remaining rhodamine B that attached to the cells was resuspended in PBS with strong agitation for total fluorescence measurement (Fig. 4.3). As expected, we measured a positive relationship between YF29's confluency after a week of culture and the amount of feeder cells initially seeded (Rheinwald, 1980). The signal intensity was also dependent on the number of keratinocytes that were seeded initially (Fig. 4.4). Moreover, fluorescence imaging confirmed the positive correlation between the fluorescence signal and the confluency of the wells (Fig. 4.5). Together, these results confirmed that this variation of the CFE assay could be adapted for HTS.

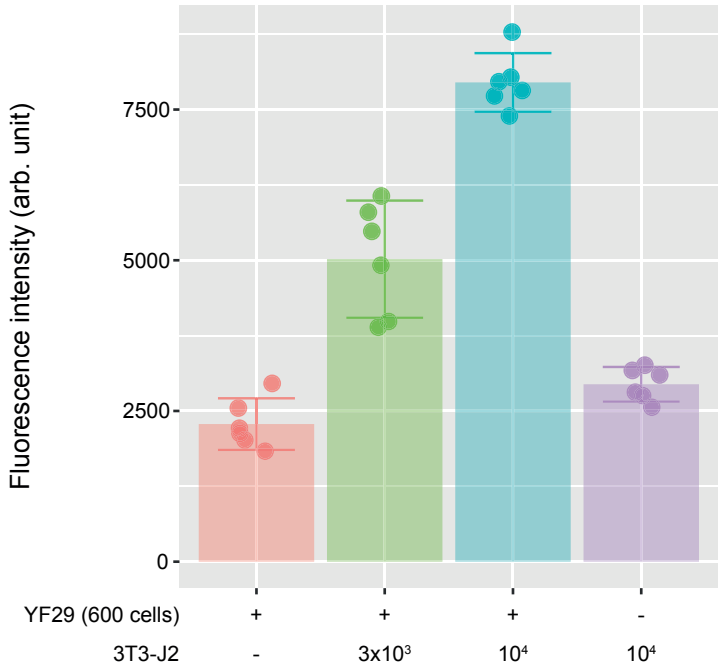

Figure 4.3 – The rhodamine assay detects variation in confluency after 7 days of culture with different densities of feeder cells. The assay measured the impact of the initial number of feeder cells (3T3-J2) on the proliferation of human keratinocytes (YF29). After a week of culture in a 96 well plate, the cells were fixed and then stained with rhodamine B. After several washes with PBS, the remaining rhodamine B was resuspended in PBS and total fluorescence measurements were performed with a plate reader. Error bars: 95% Student t-test confidence interval.

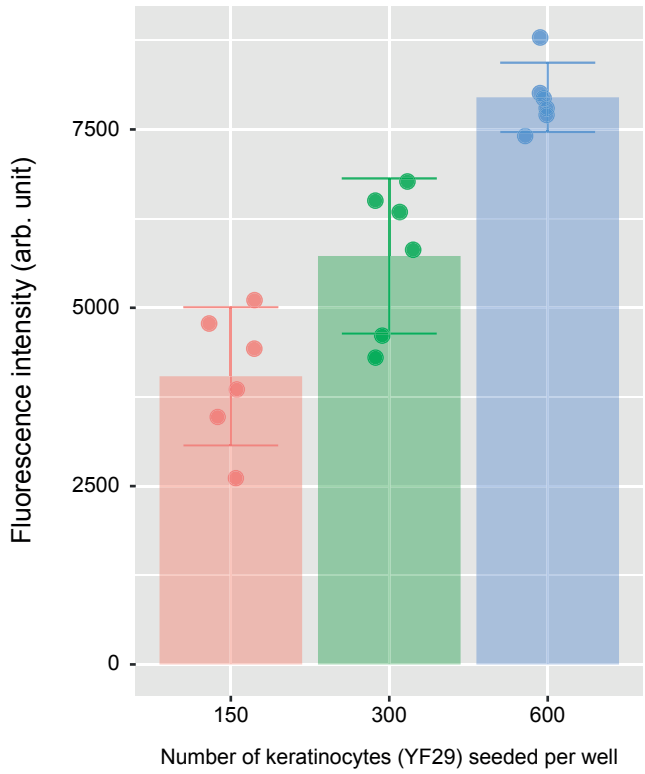

Figure 4.4 – The rhodamine assay detected variation of confluency after 7 days of culture with different initial densities of human keratinocytes (YF29). After a week of culture in a 96 well plate, the cells were fixed and then stained with rhodamine B. After several washes with PBS, the remaining rhodamine B was resuspended in PBS and total fluorescence measurements were performed with a plate reader. The assay could measure the positive relationship between the number of keratinocytes seeded per well (600, 300 or 150 cells/well) and the signal intensity after 7 days of culture. 10^4 feeder cells per well. Error bars: 95% Student t-test confidence interval.

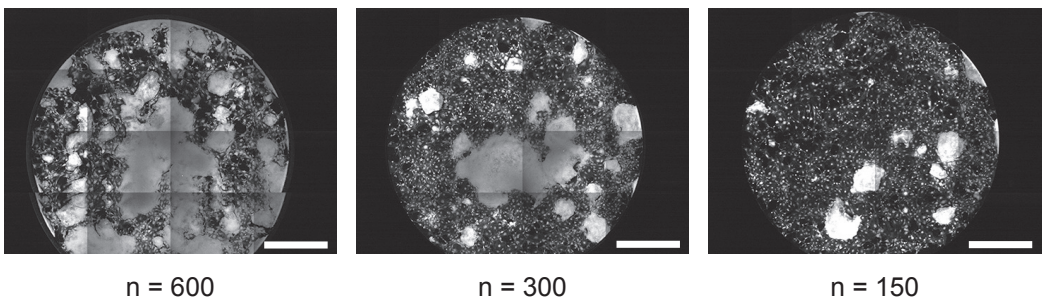

Figure 4.5 – Fluorescence imaging of Rhodamine B staining. Keratinocytes were seeded at different densities (600, 300 or 150 cells/well) in 96 well plates. After a week of culture, cells were fixed and then stained with rhodamine B. Large white/grey surfaces are human keratinocytes colonies. The dot-like shapes are remaining feeder cells. Scale bars: 1.58 mm.

Definition of the control conditions for HTS

After establishing the reverse transfection and read-out protocols, we researched a candidate positive control for the screen. To identify a putative positive control, we performed a small primary siRNA screen on a small list of genes (Table 4.1). We hypothesized that genes influencing keratinocyte proliferation or genes encoding for known components of the basement membrane would be ideal candidates as they contribute to the microenvironment of epidermal stem cells *in vivo*. We also made the assumption that our assay would allow us to identify non-redundant factors of the system. We included several growth factors, such as *Egf* and *Tgfα* (Barrandon and Green, 1987a). We also included several collagen and laminin genes, which are known components of the epidermal basement membrane. In addition, we also included a cell death inducing target (*Plk1*) and few randomly selected genes to the list. For each genes, we reverse transfected 3T3-J2 cells with a pool of siRNAs (4 siRNAs per target gene). A scramble siRNA was used as a negative control and a low serum (2% FBS) cFAD medium was used as a surrogate positive control.

Table 4.1 – List of genes for the primary screen.

Entrez gene id	Ncbi gene symbol	Entrez gene id	Ncbi gene symbol
11548	*Adra1b*	16177	*Il1r1*
12156	*Bmp2*	16178	*Il1r2*
12159	*Bmp4*	107527	*Il1rl2*
12770	*Ccr1l1*	16330	*Inpp5b*
12842	*Col1a1*	16179	*Irak1*
12843	*Col1a2*	65099	*Irak1bp1*
12828	*Col4a3*	16772	*Lama1*
12832	*Col5a2*	16773	*Lama2*
12836	*Col7a1*	16775	*Lama4*
329941	*Col8a2*	16776	*Lama5*
12765	*Cxcr2*	16779	*Lamb2*
13645	*Egf*	16780	*Lamb3*
14165	*Fgf10*	16905	*Lmna*
14179	*Fgf8*	17199	*Mc1r*
14182	*Fgfr1*	15235	*Mst1*
75296	*Fgfr1op*	18121	*Nog*
14290	*Fpr-rs3*	18817	*Plk1*
14309	*Fshr*	19035	*Ppib*
15234	*Hgf*	20423	*Shh*
54426	*Hgfac*	21802	*Tgfa*
15978	*Ifng*	117149	*Tirap*
15979	*Ifngr1*	21926	*Tnf*
16159	*Il12a*	22408	*Wnt1*
16161	*Il12rb1*	22415	*Wnt3*
16162	*Il12rb2*	22416	*Wnt3a*

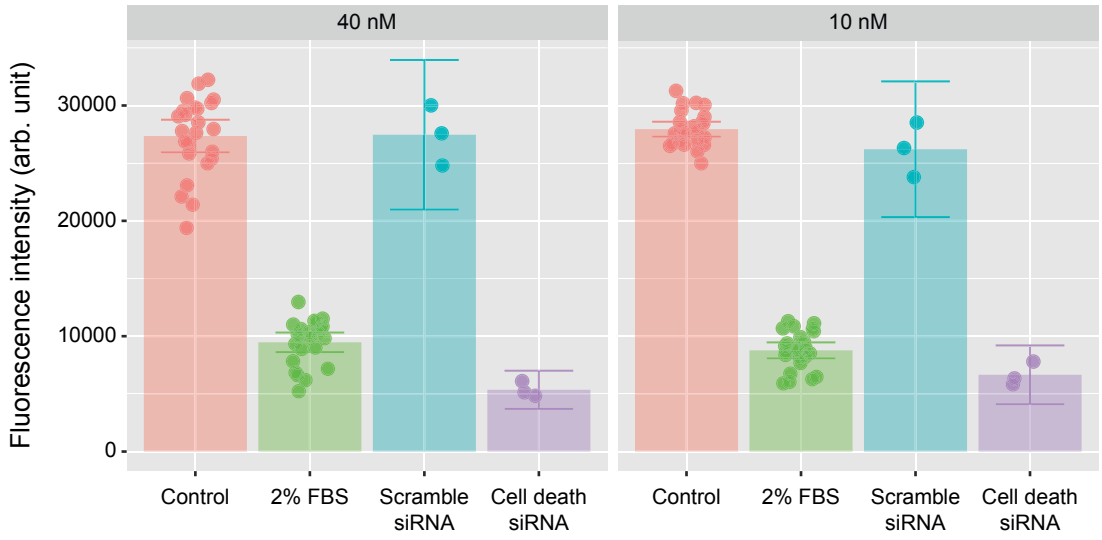

Figure 4.6 – Compatibility of the reverse transfection protocol and the rhodamine assay. Keratinocytes (YF29) were expanded on irradiated 3T3-J2 cells in normal cFAD or low serum medium. They were also expanded on irradiated 3T3-J2 cells that were previously reverse transfected with scramble or cell death cocktails siRNAs. After one week of culture, the rhodamine assay was performed. Both 40 nM and 10 nM siRNA final concentrations were reproducing the normal and low serum condition. Error bars: 95% Student t-test confidence interval.

None of the selected genes showed a significant reduction in the fluorescence signal when downregulated (data not shown). Therefore, we decided to use a cell death inducing cocktail of siRNAs (AllStars Mm/Rn Cell Death Control siRNA, Qiagen) as a candidate control. We repeated the experimental protocol with the new control siRNA. Both 40 nM or 10 nM siRNA final concentrations could mimic the normal and low serum conditions (Fig. 4.6). These results confirmed that the cell death cocktail could be used as a positive control for the screen. The fact that it also produced an effect at 10 nM suggested that, during the screen, one indivudual siRNA from a pool of 4 siRNAs (each at 10 nM) could still produce an effect. The knockdown efficiency is dependent on the targeted gene and the siRNA sequence. Usually, for a single gene, not all siRNAs have the same efficiency. Therefore, it is important to set-up the final siRNA concentration to a level that does not produce off-target effects, but that still allows at least one siRNA to be effective. Finally, we decided to use a final concentration of 40 nM for the screen.

Edge effects

Edge effects are a source of variation in cell based assay in HTS. Indeed, the increased evaporation at the edges of the assay plates can result in decreased cell proliferation during long period of incubation. In our assay, the fluorescence signal intensity was greatly reduced at the edges of the plate. This could impact the assay quality. To solve this issue, we manually filled the interstices between the wells at the periphery of the plates with 80 μL of PBS. Moreover, we had a dedicated incubator for the screen to prevent constant perturbation of the atmosphere due to frequent opening and closing of the incubator's door. This allowed us to use all the wells of the culture plates and it simplified the screening procedure.

The assay is compatible with HTS

To further assess the compatibility of the protocol and control conditions for HTS, the experiment was repeated using both the spotting robot of the Biomolecular Screening Facility (BSF) and the plate washer and dispenser for cell seeding and feeding. The fixation and staining protocols were also performed with a plate washer/dispenser. A Z' was computed to assess the quality of the screening procedure. The calculated Z'(0.568) confirmed the compatibility of the co-culture assay for HTS (Fig. 4.7).

Although our controls did produce a strong effect, it would have been preferable to have a single target control that would produce a signal closer to what one would expect from a hit. We also did not know if the duration of the knockdown in feeder cells would be sufficient to produce an effect on keratinocyte. Nevertheless, we decided to move on with the screen to identify putative "feeder genes".

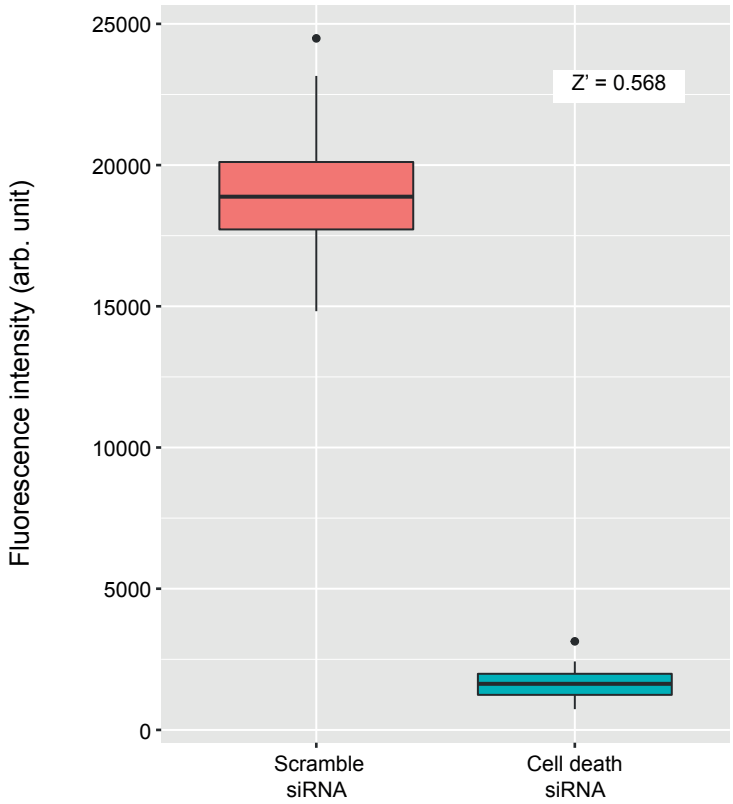

Figure 4.7 – The assay is compatible with HTS. The reverse transfection of the 3T3-J2 with a scramble siRNA or a cell death cocktail of siRNAs were performed in automation. Human keratinocytes (YF29) were seeded with a liquid handler. After 7 days, we performed the rhodamine assay. A Z' was computed from the results and it confirmed the quality and compatibility of the screening protocol for HTS.

4.1.2 Screening of the mouse druggable genome siRNA library

We decided to screen the 3T3-J2 cells with the Mouse druggable genome siRNA library from QIAGEN (Fig. 4.8). The library was already available at the BSF and covered about half of the mouse genome. This library covered 8'320 different genes, each with 4 different siRNAs. The four individual siRNAs were pooled together by the BSF and the screen was performed in duplicate. Every week, 24 x 96 well plates were spotted by the BSF with the siRNAs and transfection mix. The plates were then processed for cell culture and read-out acquisition in our laboratory. The screen lasted 15 weeks. We had to repeat 20 plates as one amplification of YF29 cells was not optimal.

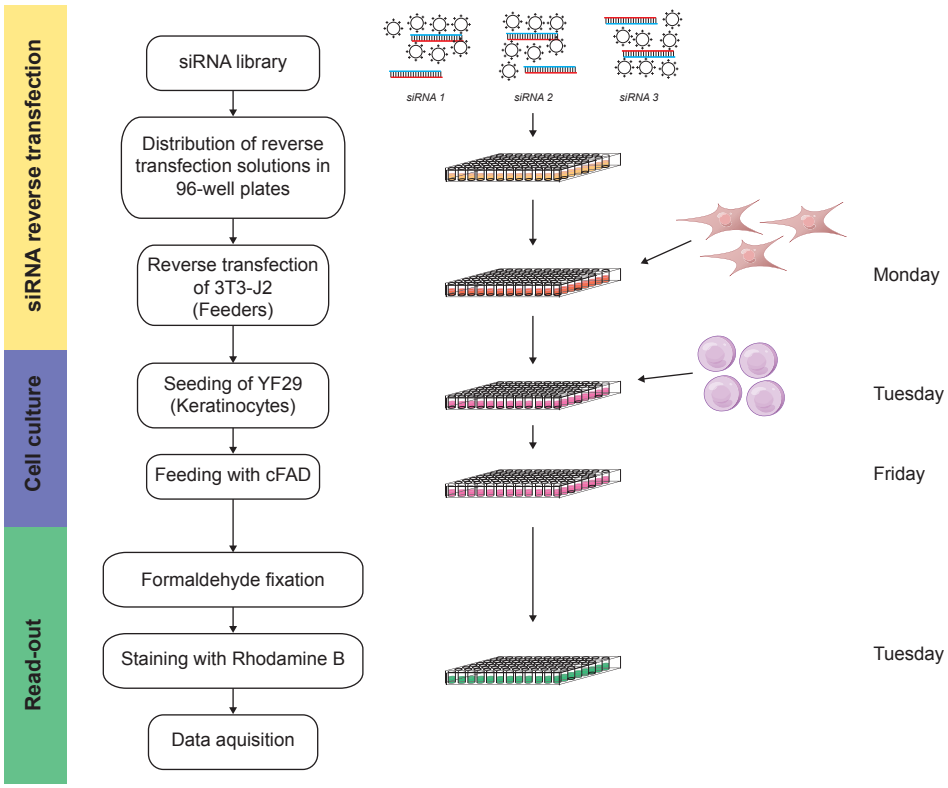

Figure 4.8 – Schematic representation of the screening procedure.

Out of the 8'320 pools of siRNAs, only 126 pools of siRNA did produce an effect in duplicates. The hits were located throughout the whole library and did not result from "plate effects". To further validate these hits, we decided to repeat the screening procedure with a deconvoluted library (single siRNA).

4.1.3 Secondary screening

To validate the initial hits, we repeated the screening procedure for the 126 genes using single siRNAs. We ordered the same 4 siRNAs for each genes separately. We also included 6 additional genes in the list. One of these genes, *Smc3*, was not detected as a hit in the screen because one duplicate was under the detection threshold. However, this gene encodes for a protein that is either targeted to the nucleus or secreted after the addition of several chondroitin sulfate chains. The secreted glycoprotein form is BAMACAN, one of the principal component of the epidermal basement membrane (Wu and Couchman, 1997). Proteoglycans play a a major

role in signaling as they can tether and modulate growth factors signaling activity (Yurchenco, 2011). *Fgf7* (*Kgf*, keratinocyte growth factor) was also added as it was previously described as one of the growth factors that 3T3 cells produce when co-cultured with keratinocytes. *F10* (coagulation factor X) was added as it was detected as a hit during the screening campaign, even though it was absent from the final report. Its absence from the list was likely due to a bug of the inventory software used to track the assay plates.

Since we also wanted to investigate genes that are known to activate the RHO/ROCK pathway, we included *Porcn*, *Wnt3* and *Tgfb1* (Tgfβ, transforming growth factor beta). The Wnt/Planar cell polarity pathway and Tgfβ pathway can signal through ROCK (Kobune et al., 2007; Vasilaki et al., 2010). We wanted to know if either one of these genes could impact keratinocyte proliferation (positively or negatively) in the context of the second project described in this thesis.

All the 4 siRNAs for the 131 genes were tested independently. In addition, the single siRNAs were used to generate new pools of siRNAs for the validation. Each siRNA or pools were tested in duplicates. The procedure for the validation was identical to the first screen.

Table 4.2 – Results of the secondary screen. The score represent the percentage of inhibition compared to the negative control (scramble siRNA).

Name	Score	ScoreSD	GeneID	GeneName
Mm_Psmb4_4	0.871	0.104	19172	Psmb4
Mm_Gpx7_3	0.849	0.030	67305	Gpx7
Mm_Psmc5_6	0.795	0.169	19184	Psmc5
Mm_Itgav_3	0.793	0.002	16410	Itgav
Mm_Pgam2_2	0.739	0.000	56012	Pgam2
Mm_Sars1_3	0.728	0.024	20226	Sars
Mm_Fkbp8_4	0.716	0.029	14232	Fkbp8
Mm_Psmc5_1	0.700	0.005	19184	Psmc5
Pooled_Smc3	0.689	0.047	13006	Smc3
Mm_Csnk1a1_3	0.687	0.040	93687	Csnk1a1
Mm_Ddx39_3	0.650	0.029	68278	Ddx39
Mm_Ddx39_1	0.649	0.059	68278	Ddx39
Mm_Gpd2_5	0.645	0.017	14571	Gpd2
Mm_Rrm1_7	0.642	0.019	20133	Rrm1
Pooled_Psmc5	0.626	0.020	19184	Psmc5
Mm_Gne_1	0.618	0.076	50798	Gne
Mm_Lifr_1	0.616	0.331	16880	Lifr
Mm_Rpl38_3	0.612	0.026	67671	Rpl38
Pooled_Xpo1	0.603	0.016	103573	Xpo1
Mm_V1ra2_2	0.600	0.001	22297	Vmn1r45
Mm_Rrm2_3	0.589	0.046	20135	Rrm2
Mm_Eif3s8_1	0.588	0.149	56347	Eif3c
Mm_LOC545756_4	0.559	0.049	545756	Gm5867
Mm_Psmc1_2	0.549	0.283	19179	Psmc1
Pooled_Psmb4	0.528	0.050	19172	Psmb4
Mm_Gsn_5	0.514	0.063	227753	Gsn
Mm_Psmb4_2	0.484	0.013	19172	Psmb4
Mm_Pdlim7_3	0.473	0.161	67399	Pdlim7
Mm_Rpl4_6	0.472	0.101	67891	Rpl4
Mm_Papln_1	0.465	0.009	170721	Papln
Pooled_Ddx54	0.463	0.065	71990	Ddx54
Mm_Mmp1b_4	0.462	0.029	83996	Mmp1b
Mm_Ddx58_4	0.459	0.300	230073	Ddx58
Mm_Fkbp8_5	0.451	0.088	14232	Fkbp8
Mm_Il20_3	0.450	0.051	58181	Il20
Mm_Hs3st1_7	0.450	0.137	15476	Hs3st1
Mm_Gnb5_4	0.447	0.032	14697	Gnb5
Mm_BC048082_4	0.445	0.019	332110	Mapk15
Pooled_Itgav	0.439	0.044	16410	Itgav
Mm_Dmbt1_2	0.436	0.225	12945	Dmbt1
Mm_Efcbp2_4	0.432	0.194	117148	Necab2
Pooled_Ddx39	0.430	0.041	68278	Ddx39

Table 4.2 – Results of the secondary screen. The score represent the percentage of inhibition compared to the negative control (scramble siRNA).

Name	Score	ScoreSD	GeneID	GeneName
Mm_Xab2_6	0.428	0.107	67439	Xab2
Mm_A530016O06Rik_4	0.427	0.050	319660	Agmo
Mm_D10Ertd610e_4	0.420	0.059	52666	Arhgef25
Mm_Dll3_3	0.414	0.104	13389	Dll3
Mm_Lmo2_3	0.397	0.004	16909	Lmo2
Mm_Mllt6_4	0.391	0.100	246198	Mllt6
Mm_Pole_3	0.384	0.019	18973	Pole
Mm_Fbxw11_3	0.369	0.105	103583	Fbxw11
Pooled_Dll4	0.364	0.084	54485	Dll4
Mm_Emd_1	0.364	0.042	13726	Emd
Mm_Olfr731_3	0.360	0.149	258360	Olfr731
Mm_Prkwnk4_3	0.359	0.135	69847	Wnk4
Pooled_Efna1	0.354	0.116	13636	Efna1
Mm_Nelf_2	0.351	0.124	56876	Nsmf
Mm_Htr4_7	0.341	0.063	15562	Htr4
Mm_Eif3s8_5	0.340	0.040	56347	Eif3c
Mm_Tssk2_4	0.339	0.098	22115	Tssk2
Mm_Dctn1_5	0.338	0.077	13191	Dctn1
Mm_Ddx54_3	0.338	0.097	71990	Ddx54
Pooled_Fbxo34	0.338	0.040	78938	Fbxo34
Mm_Ctdsp2_6	0.334	0.074	52468	Ctdsp2
Mm_Ptk2_6	0.329	0.105	14083	Ptk2
Mm_Gpnmb_6	0.323	0.027	93695	Gpnmb
Mm_Pkia_1	0.319	0.030	18767	Pkia
Mm_Mllt6_3	0.318	0.129	246198	Mllt6
Mm_Eya3_4	0.312	0.036	14050	Eya3
Mm_Fbxo31_1	0.302	0.029	76454	Fbxo31
Mm_Pkn3_1	0.292	0.012	263803	Pkn3
Mm_Ewsr1_1	0.290	0.016	14030	Ewsr1
Pooled_Bzrap1	0.287	0.079	207777	Bzrap1
Mm_Xpo1_6	0.286	0.010	103573	Xpo1
Mm_1700011C14Rik_1	0.282	0.008	76571	Styxl1
Mm_Xpo1_5	0.274	0.104	103573	Xpo1
Mm_Rrm1_5	0.267	0.022	20133	Rrm1
Mm_Snrpd1_4	0.263	0.026	20641	Snrpd1
Mm_Dok1_1	0.259	0.019	13448	Dok1
Mm_Lmo2_4	0.244	0.025	16909	Lmo2
Mm_Ercc2_1	0.238	0.033	13871	Ercc2
Mm_Nefh_6	0.235	0.028	380684	Nefh
Mm_Egln1_3	0.231	0.015	112405	Egln1
Mm_Furin_1	0.225	0.064	18550	Furin
Mm_Psmd2_5	0.213	0.011	21762	Psmd2

Table 4.2 – Results of the secondary screen. The score represent the percentage of inhibition compared to the negative control (scramble siRNA).

Name	Score	ScoreSD	GeneID	GeneName
Mm_Cacna1e_8	0.209	0.039	12290	Cacna1e
Mm_Csnk1a1_2	0.209	0.028	93687	Csnk1a1
Mm_Dll4_3	0.204	0.006	54485	Dll4
Mm_Psmc5_5	0.202	0.019	19184	Psmc5

From the validation, only 70 out of the 131 genes were classified as hits (Table 4.2). The majority of the hits were reproduced with a single siRNA. Interestingly, only 11 pools of siRNAs reproduced their effects. We performed a functional annotation analysis to identify enriched KEGG pathways in the hit list using the DAVID bioinformatics ressource (Huang et al., 2009). Only 3 pathways were enriched in the list (Table 4.3). However, these were unlikely to represent the feeder function of the 3T3-J2 cells. In cancer and transformed cells, proteasome inhibition act as a radiosensitizer to promote cell death after irradiation (McBride et al., 2003). This could explain why genes encoding for proteasome subunits are enriched in our hit list.

Pathway (KEGG)	# genes	p-value
Proteasome	4	3.60E-03
Pyrimidine metabolism	4	2.50E-02
Glutathione metabolism	3	4.60E-02

Table 4.3 – Results of the KEGG pathways enrichment analysis.

In addition to the functional annotation analysis, we also used the GeneCards® database to retrieve the subcellular localization annotations for each gene. Out of the 70 genes, the majority encoded for intracellular proteins. However, 11 genes encoded for known secreted proteins, transmembrane ligands and receptor proteins (Table 4.4).

Interestingly, none of these genes were previously described in the 3T3-J2 culture system. However, nearly all of them were linked with epidermal homeostasis & wound repair. For example, IL20 is a cytokine that is upregulated in psoriasis (a disease characterized by a hyper proliferative epidermis) (Ouyang et al., 2011). MMP1, a collagenase, is also upregulated in wounded skin and facilitates the migration of keratinocytes (Rohani et al., 2014). FURIN is also known to play a key role during wound repair (Gurtner et al., 2008). However, we could

Entrez gene id	NCBI gene symbol	RT-PCR
13389	*Dll3*	-
54485	*Dll4*	+
12945	*Dmbt1*	-
13636	*Efna1*	?
18550	*Furin*	+
93695	*Gpnmb*	?
58181	*Il20*	+*
16880	*Lifr*	?
332110	*Mapk15*	?
83996	*Mmp1b*	?
170721	*Papln*	?
13006	*Smc3*	+

Table 4.4 – List of putative "feeder genes" identified in the secondary screen. The mRNA expression was confirmed by RT-qPCR for some of them (+ : detected, - : not detected, ? : not tested, * : *Il20* was only detected in 3T3-J2 after irradiation).

not identify growth factors such as EGF, KGF or TGFα. But, we did validate SMC3 (BAMACAN), one of the major proteoglycans of the basement membrane.

4.1.4 Investigating the role of FURIN in the culture system

To further confirm the results of the screen, we decided to study the role of one of the putative feeder genes in the culture system. Although all of the hits are interesting candidates on their own, we chose to focus on one gene due to time constraints. We decided to concentrate our efforts on FURIN (or PACE), a proprotein convertase (PC) responsible for the maturation of multiple targets. FURIN is a serine protease. It recognizes the consensus sequence (Arg-X-X-Arg) and cleaves after the last Arginine residue. While FURIN is mostly enriched in the *trans*-Golgi network, an active secreted form of FURIN has also been reported (Plaimauer et al., 2001; Mesnard et al., 2011). Also, FURIN has been reported to process several substrates at the cell surface and in early endosomes (Thomas, 2002).

In the epidermis, FURIN is expressed alongside three other PCs (PACE4, PC5/6 and PC7/8). FURIN is known to processes multiple protein precursors that influence epidermal homeostasis. TGFβ, a well-known FURIN substrate, can affect the proliferation and differentiation of epidermal keratinocytes. If TGFβ1 is not cleaved and activated by FURIN, it cannot bind

to its receptor. When activated, TGFβ1 inhibits keratinocytes proliferation *in vitro* (Pietenpol et al., 1990; Shipley et al., 1986). In addition, FURIN is also required for the processing and activation of the NOTCH1 receptor (Logeat et al., 1998). NOTCH1 is expressed in the suprabasal cells of the epidermis. Conditional deletion of NOTCH1 in the epidermis results in disturbed differentiation and expansion of the basal layer leading to hyperplasia (Rangarajan et al., 2001; Nicolas et al., 2003). Moreover, IGF1, a known growth factor produced by 3T3 cells, is processed by FURIN (Duguay et al., 1997). In addition, the receptor for IGF1, IGF-1R, is also processed by FURIN (Fu et al., 2012). These examples only represent a small fraction of all protein precursors processed by FURIN. There are other FURIN substrates, such as EDA (ectodysplasin A), BMP4, various MMPs (matrix metallopeptidase) and COL17A1 (collagen XVII), which can influence epidermal development and homeostasis (Tian et al., 2011). In the 3T3-J2 culture system, both 3T3 feeder cells and human keratinocytes express FURIN. Therefore, understanding the role and function of FURIN in the culture system for human keratinocytes is a very challenging question.

To further confirm the results produced by RNA interference, we first decided to generate a *Furin* knockout (KO) 3T3 cell line using the CRISPR/Cas9 technology. The plasmid containing the guide RNA and the Cas9 was kindly shared by Prof. D. Constam and Dr. S. Bessonnard. The guide RNA (target sequence: GGTTGCTATGGGTGGTCGCA) was designed by Dr. S. Bessonnard to target the coding sequence early after the start codon. The plasmid also included the elements to express both hSPCas9 and EGFP. Pre-confluent 3T3-J2 cells were transfected with the plasmid and allowed to grow for 3 days. Then, single GFP positive cells (1.5 %) were sorted by FACS in 96 well plates that were previously prepared with irradiated untransfected 3T3-J2 cells. After 14 days, the clones were serially amplified for two passages without irradiated cells. The clones were then screened by Western blot analysis for FURIN expression. Out of 40 different clones, we could identify 3 clones that did not express FURIN (expected band 95-100 kDa). By themselves, the knockout clones did not grow very well. To have enough cells for co-culture experiments with human keratincoytes, we also had to use irradiated feeder cells (10^4 cells/mL) to support the growth of the *Furin* KO cell lines. Therefore, we cannot exclude the presence of a very small number of normal irradiated feeder cells in our experiments with *Furin* KO 3T3 cells.

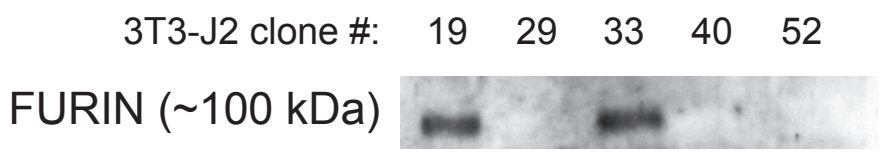

Figure 4.9 – Analysis of FURIN expression in 3T3-J2 clones. Western blot detection of FURIN in total cell lysates.

We first decided to repeat the rhodamine assay with the *Furin* KO 3T3 clone. We co-cultured human keratinocytes (YF29) with either wild-type (F4), *Furin* KO (c52) or control (c19) feeder cells in 96 well plates. After a week of culture, the plates were fixed and processed for rhodamine B staining and fluorescence measurements. The intensity of the rhodamine B fluorescence was reduced when human keratinocytes we co-cultured with irradiated clone 52 feeder cells (Fig. 4.10) and there were less keratinocytes compared to wild-type and control 3T3 cell lines (Fig. 4.11). The percentage inhibition of the signal (16% versus control untreated F4 3T3 cells, paired t test two-tailed p-value = $9.46E^{-5}$) was similar to the results of RNAi (score of 22% +/- 6%). It was previously shown that 3T3-J2 cells produce IGF1. Therefore, we hypothesized that the phenotype could be rescued by the addition of recombinant human IGF1. YF29 were co-cultured with either wild-type or *Furin* KO 3T3 cells lines with or without the addition of recombinant IGF1 (100 ng/mL, every day). Unfortunately, the addition of IGF1 did not result in an increase of the rhodamine signal (Fig. 4.12). This suggested that addition of recombinant IGF1 alone could not compensate for the loss of FURIN in the feeder cells, or that it was already compensated by the cFAD medium (containing recombinant human insuline).

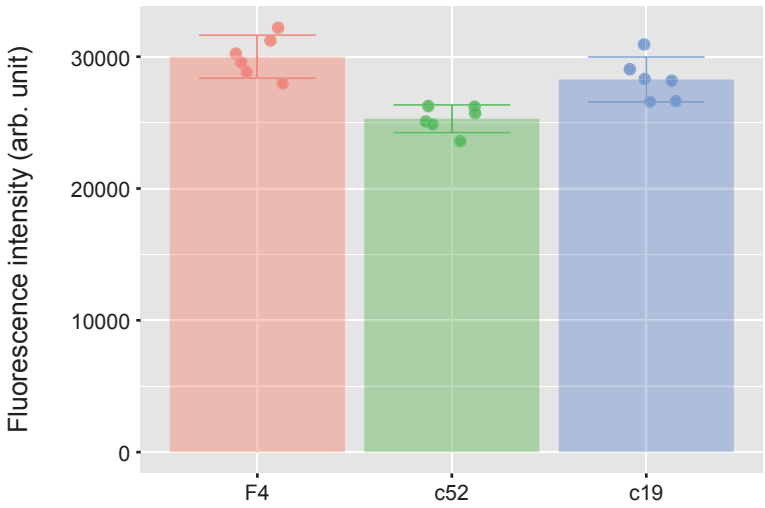

Figure 4.10 – Rhodamine assay with different 3T3 cell lines. YF29 cells were co-cultured with either wild-type (F4), *Furin* KO (c52) or control (c19) 3T3 cells lines for a week and analysed with the rhodamine assay. When co-cultured with 3T3 clone 52 cells, we could observe a decrease in total rhodamine B fluorescence intensity.

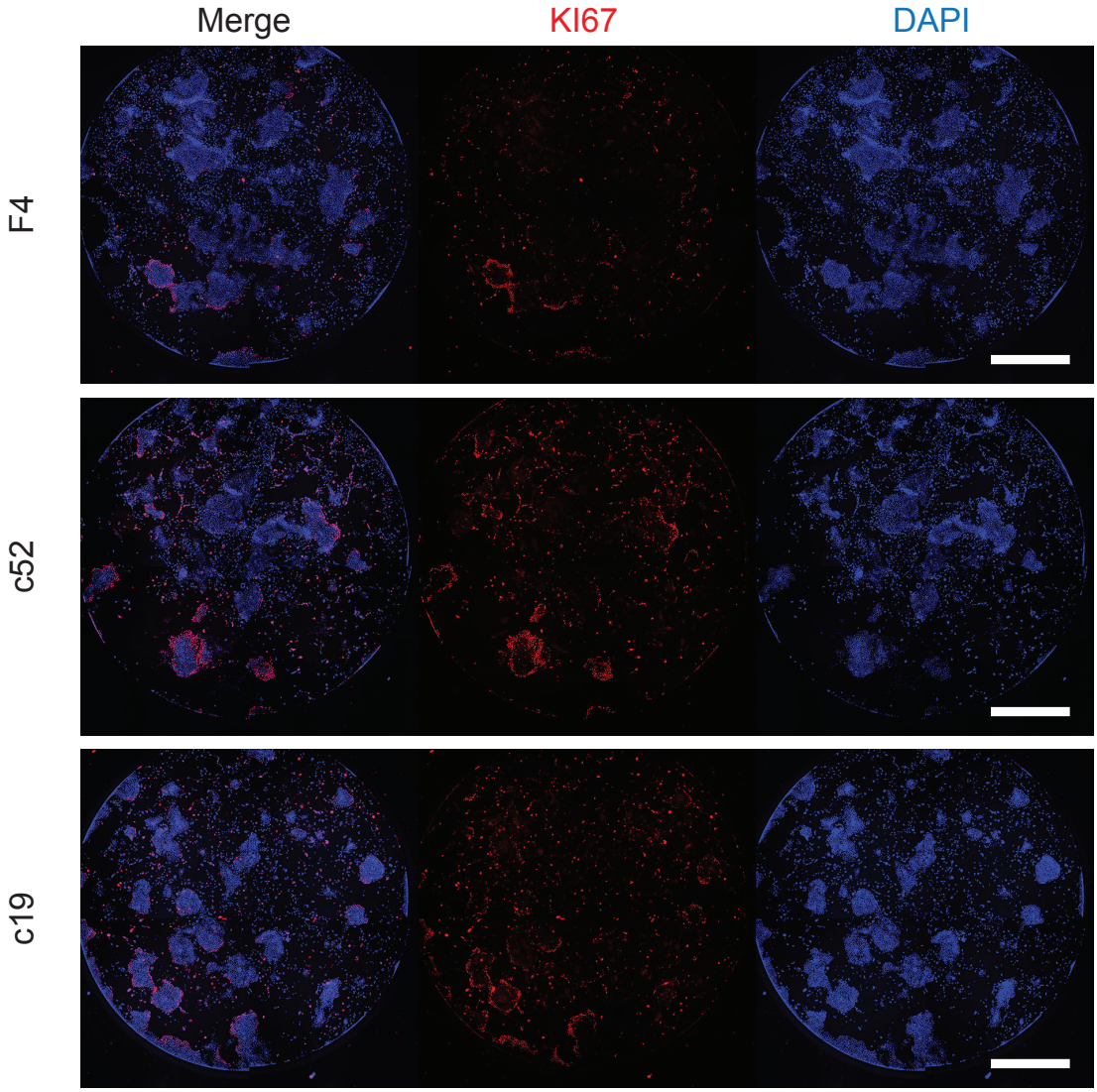

Figure 4.11 – Immunofluorescence analysis of KI67 expression. Human keratinocytes were co-cultured with irradiated wilde-type (F4), *Furin* KO (c52) and control (c19) 3T3-J2 cells for 7 days. The confluency was slightly lower when human keratinocytes were co-cultured with irradiated c52 cells. Scale bars: 1.58 mm.

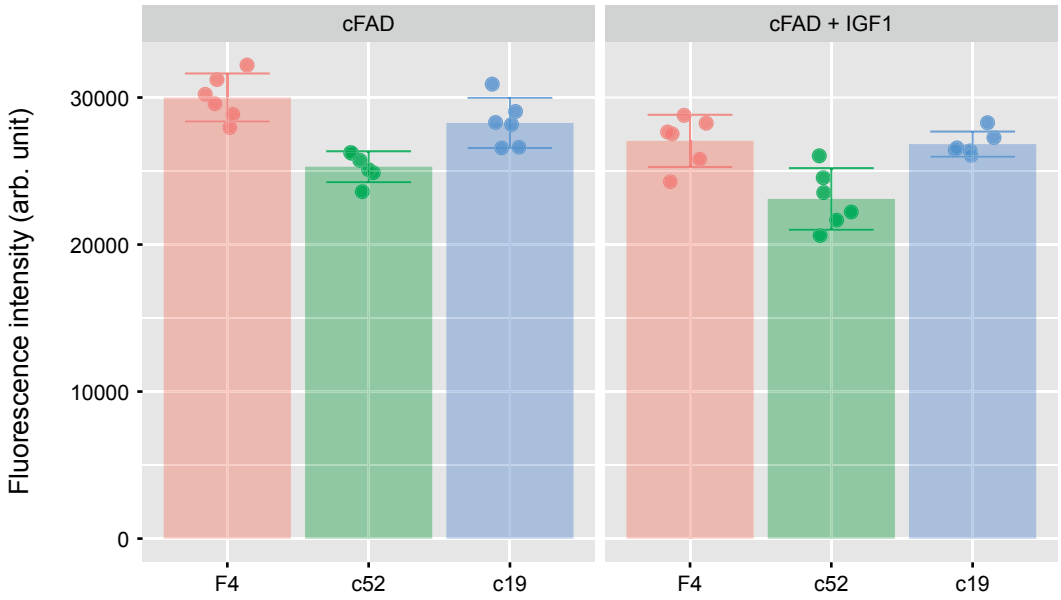

Figure 4.12 – IGF1 does not increase the rhodamine signal. Human keratinocytes were co-cultured with different 3T3 cell lines (F4, clone 52 and clone 19) in presence or absence of recombinant human IGF1 (100 ng/mL). After 7 days, the plates were fixed and analyzed with the rhodamine assay. Addition of IGF1 did not increase the signal of the assay.

In parallel to the CRISPR/Cas9 experiments, we also investigated the impact of FURIN inhibition with a soluble, cell permeable inhibitor (FURIN inhibitor or I24) (Becker et al., 2012). These experiments differed from the CRISPR/Cas9 approach as the inhibitor targeted both human keratinocytes and feeder cells.

First, we repeated the rhodamine assay with or without treatment with the FURIN inhibitor. While treatment with DMSO (carrier) did produce an increase in signal intensity, the signal was drastically lower when the FURIN inhibitor was added to the culture medium (Fig. 4.13). The effect was also stronger than what we observed with the *Furin* KO 3T3 clones. To investigate if it did impact keratinocyte proliferation, we performed an immunofluorescence analysis of KI67 expression (Fig. 4.14). To our surprise, we detected more keratinocyte colonies in presence of DMSO (carrier) or FURIN inhibitor compared to untreated cells. Moreover, there was a significant increase in the number of KI67 positive cells in presence of the FURIN inhibitor compared to DMSO (Fig. 4.15).

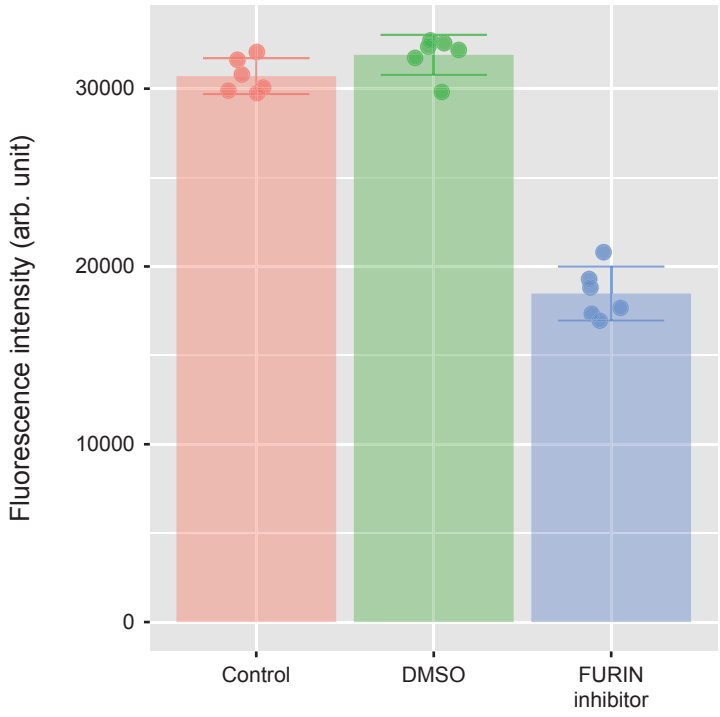

Figure 4.13 – FURIN inhibition results in a reduced signal of the rhodamine assay. Human keratinocytes were co-cultured with irradiated 3T3-J2 cells in presence or absence of a FURIN inhibitor (I24) for 7 days. FURIN inhibition drastically reduced the signal intensity of the rhodamine assay.

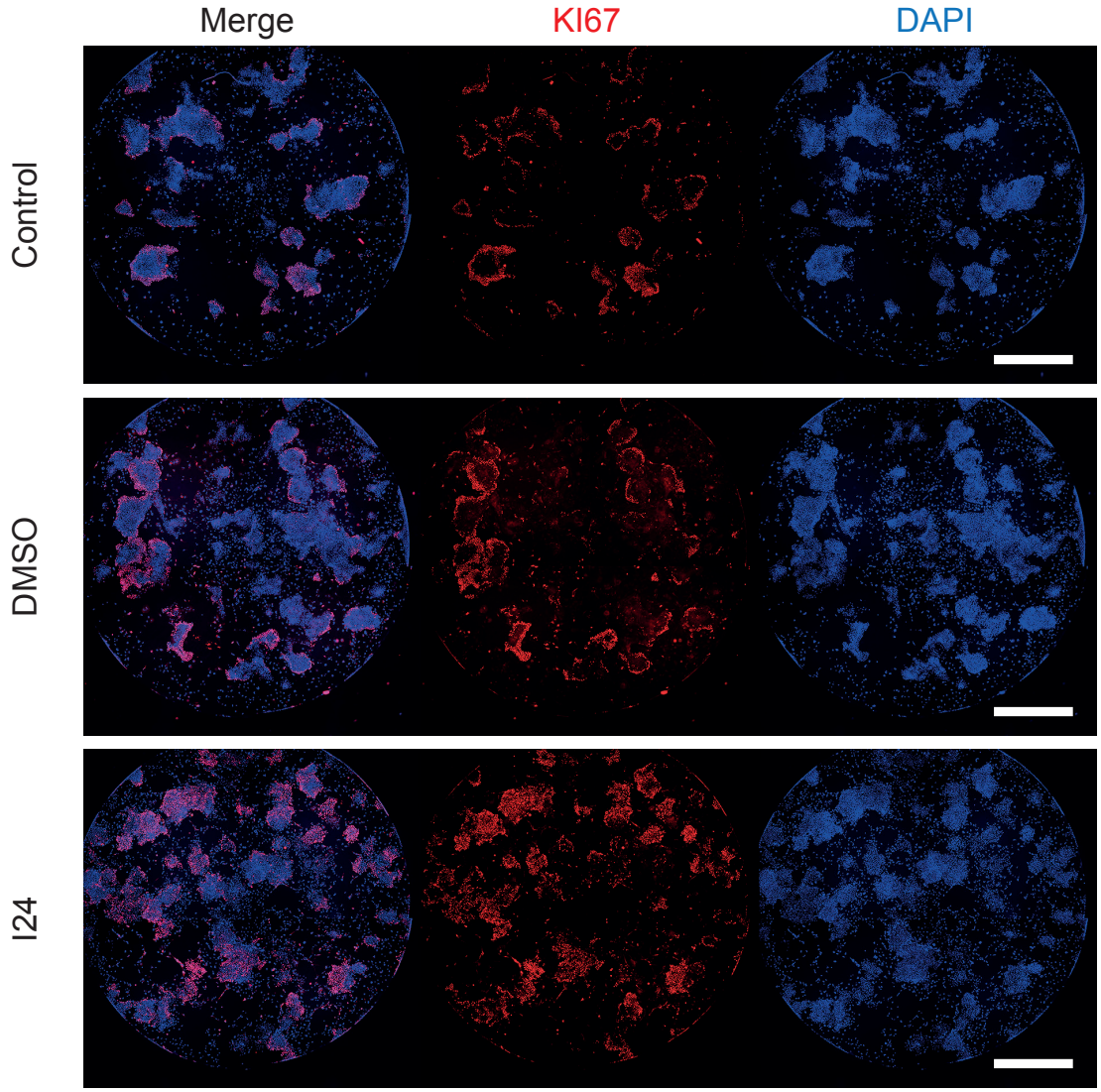

Figure 4.14 – Immunofluorescence analysis of KI67 expression. Human keratinocytes were co-cultured with irradiated 3T3-J2 cells in presence or absence of a FURIN inhibitor (I24) for 7 days. Scale bars: 1.58 mm.

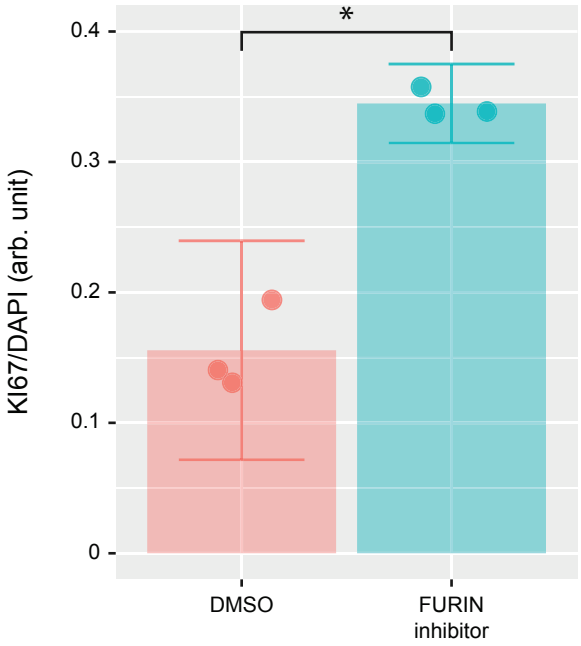

Figure 4.15 – Quantification of KI67 positive cells. Human keratinocytes were co-cultured with irradiated 3T3-J2 cells in presence or absence of a FURIN inhibitor (I24) for 7 days. The number of KI67 positive cells was increased in presence of the FURIN inhibitor. Immunofluorescence images (n=3) were anazyled with the Fiji software. Error bars: 95% Student t-test confidence interval. *: paired t test two-tailed p-value = $4.35E^{-3}$.

Keratinization (or stratification) increases the intensity of the rhodamine B staining (Rheinwald and Green, 1975a). Indeed, stratified colonies of epithelial cells stain well with rhodamine B while unstratified (or refringent) colonies do not (Pluchinotta, 2016). Human keratinocytes form stratified colonies *in vitro*. Thus, we investigated the effect of FURIN inhibition on the expression of genes known to be differentially expressed during keratinocyte differentiation and stratification by RT-qPCR. We observed no difference in the expression of *DNP63* (Delta N isoform of TP63), *HOPX* & *LEKTI*. However, we did observe an increase in *KRT1* expression and a decrease in *IVL* expression (Fig. 4.16). IVL is a marker of keratinocyte terminal differentiation. It is also the main component of the cornified envelope. In human keratinocytes, KRT1 is also a marker of differentiation. KRT1 expression also precedes IVL expression during keratinocyte differentiation *in vivo* (Hsu et al., 2014). Together, the qPCR and rhodamine assay results suggest that FURIN inhibition did disturb the late stages of keratinocyte differentiation and the formation of the cornified envelope.

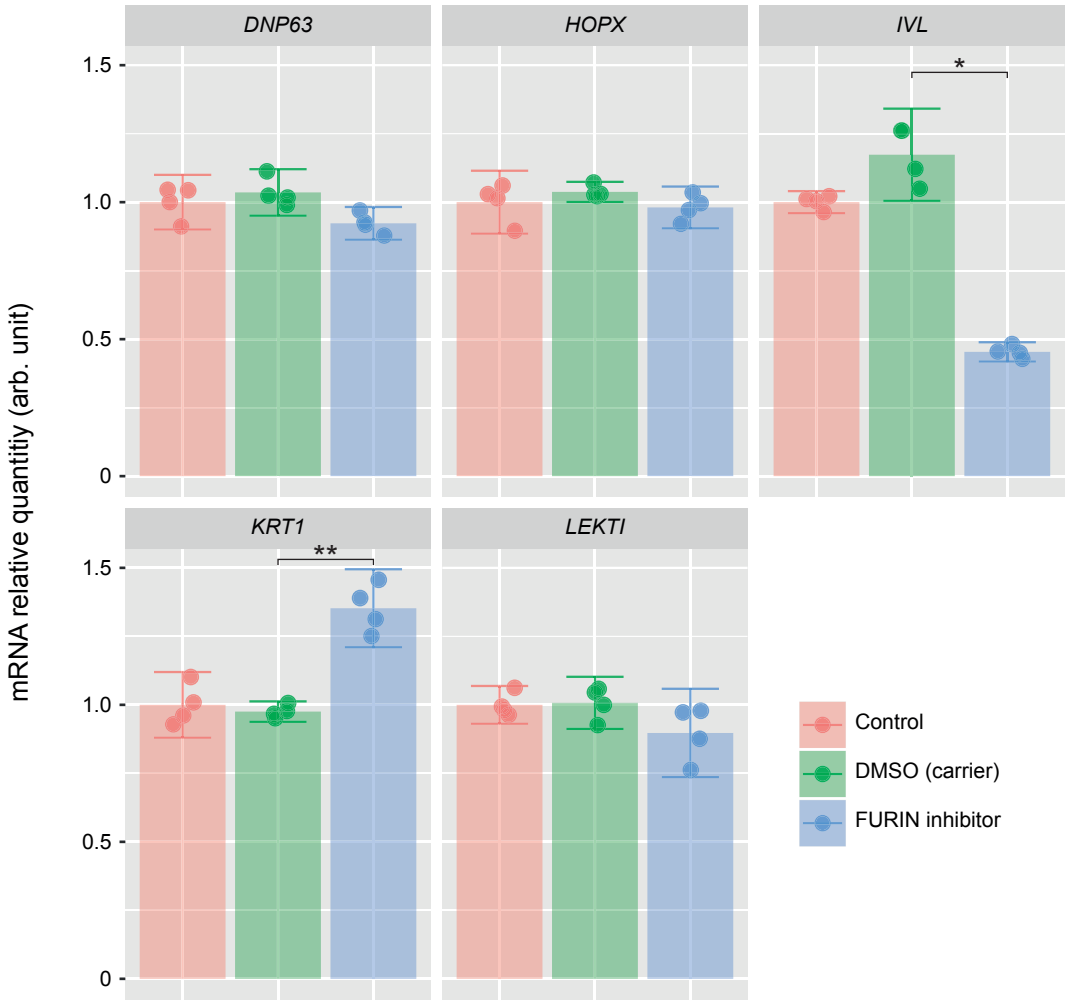

Figure 4.16 – FURIN inhibition impacts the transcription of several genes associated with keratinocytes differentiation. Human keratinocytes were co-cultured with 3T3-J2 cells in presence or absence of a FURIN inhibitor. Total RNA was extracted after 7 days and used for RT-qPCR. We analyzed the expression of *DNP63*, *KRT1*, *HOPX*, *IVL* and *LEKTI* (n=4). Endogenous controls: *EEF1A1*, *SDHA* and *TBP*. Error bars: 95% Student t-test confidence interval. *: paired t test two-tailed p-value = $8.01E^{-4}$. **: paired t test two-tailed p-value = $2.58E^{-3}$

4.2 The impact of ROCK inhibition on cultured keratinocytes

4.2.1 ROCK inhibition increases TP63 expression

Since Terunuma and colleagues (2010) published their paper on the impact of ROCK inhibition on primary keratinocytes, our laboratory repeated and confirmed their results on freshly isolated cells from multiple patients (unpublished data, A. Rochat). Keratinocytes treated with Y-27632 can still regenerate an epidermis when transplanted on an animal model (unpublished data, A. Rochat & F. Gorostidi). In addition, our laboratory also observed that freshly isolated cells form mostly holoclone- and meroclone-like colonies (Barrandon et al., 2012). Therefore, we thought to investigate if ROCK inhibition was improving stem cell survival during isolation and *in vitro* expansion or if it was promoting non stem cells to have a holoclone-like phenotype.

First, we hypothesized that the cellular phenotype produced by ROCK inhibition would also correlate with changes in gene expression in keratinocytes. The holoclone phenotype is positively correlated with the expression of P63 (Pellegrini et al., 2001). Human keratinocytes (strain YF29) were cultured in presence or absence of Y-27632 for both mRNA and protein extraction. In our culture conditions, P63 expression was increased after Y-27632 treatment. We also observed a decrease in *IVL* (involucrin) mRNA level and an increase in *TERT* mRNA level (Fig. 4.17). Although the qPCR data were not statistically significant, the western blot analysis did confirm the increased P63 expression (Fig. 4.18). Keratinocytes treated with Y-27632 did not stratify *in vitro* (unpublished data, A. Rochat). These results confirmed the effect of Y-27632 on P63 expression (Zhou et al., 2012; Suprynowicz et al., 2012; Palechor-Ceron et al., 2013a) and also suggested that Y-27632 prevented keratinocyte differentiation.

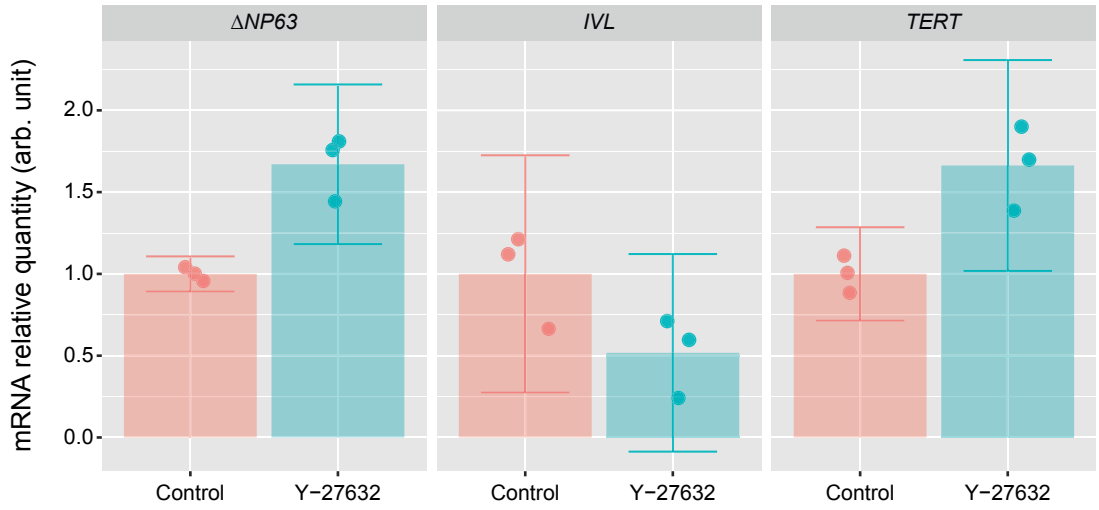

Figure 4.17 – RT-qPCR results for the expression of human *DNP63*, *IVL* and *TERT* (n=3). Keratinocytes were expanded on normal feeders (3T3-J2) in presence or absence or Y-27632. Endogenous controls: *EEF1A1*, *SDHA* and *TBP*. Error bars: 95% Student t-test confidence interval.

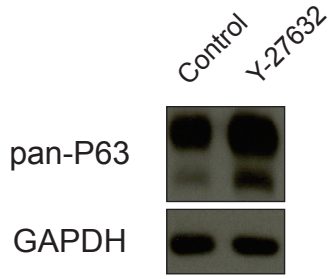

Figure 4.18 – Western blot analysis of TP63 expression. Keratinocytes were expanded in presence or absence of Y-27632. ROCK inhibition resulted in an increase of TP63 expression.

4.2.2 ROCK inhibition does not induce cellular immortalization

To further assess the cellular impact of Y-27632 treatment, we investigated two markers of cellular immortalization. First, YF29 cells were serially cultivated in presence or absence of Y-27632. The cells were cultivated over several weeks and genomic DNA was extracted at each passages. The telomere length was measured by Southern blot analysis of terminal restriction fragment lengths (Fig. 4.20). We compared three different passages (VII, X & XII) which covered around 35 cell doublings. Surprisingly, the average telomere length did diminish over time,

but very slowly. Y-27632 did not prevent telomere shortening in normal keratinocytes.

In addition, we investigated the phosphorylation state of the retinoblastoma protein (RB, RB1). The RB protein is often constantly hyper-phosphorylated in immortalized cell lines. The treatment with Y-27632 did not result in an increase of the hyper-phosphorylated form of RB (ppRB). Both ppRB and pRB showed similar levels by western blot analysis in both culture conditions (Fig. 4.19).

Together, these results argued against an induced cellular immortalization by ROCK inhibition. Moreover, when Y-27632 was withdrawn from the culture medium, a strong decrease in colony forming efficiency could be observed (Gorostidi (2012), unpublished data from A. Rochat).

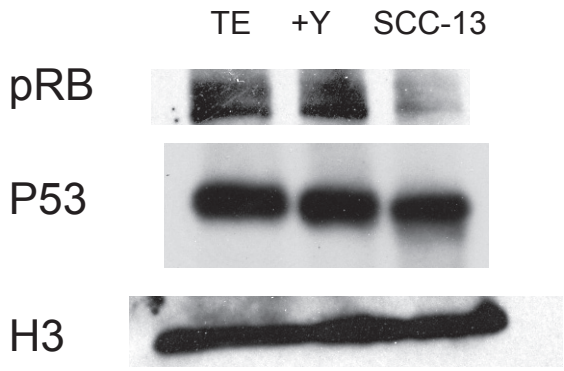

Figure 4.19 – Western blot analysis of pRB, P53 and H3 expression in normal human keratinocytes (YF29) or derived from a squamous cell carcinoma (SCC-13). Nuclear proteins were extracted from human keratinocytes cultured with (+Y) or without (TE) Y-27632 and from keratinocytes of the SCC-13 cell line. ROCK inhibition did not impact pRB phosphorylation in normal human keratinocytes.

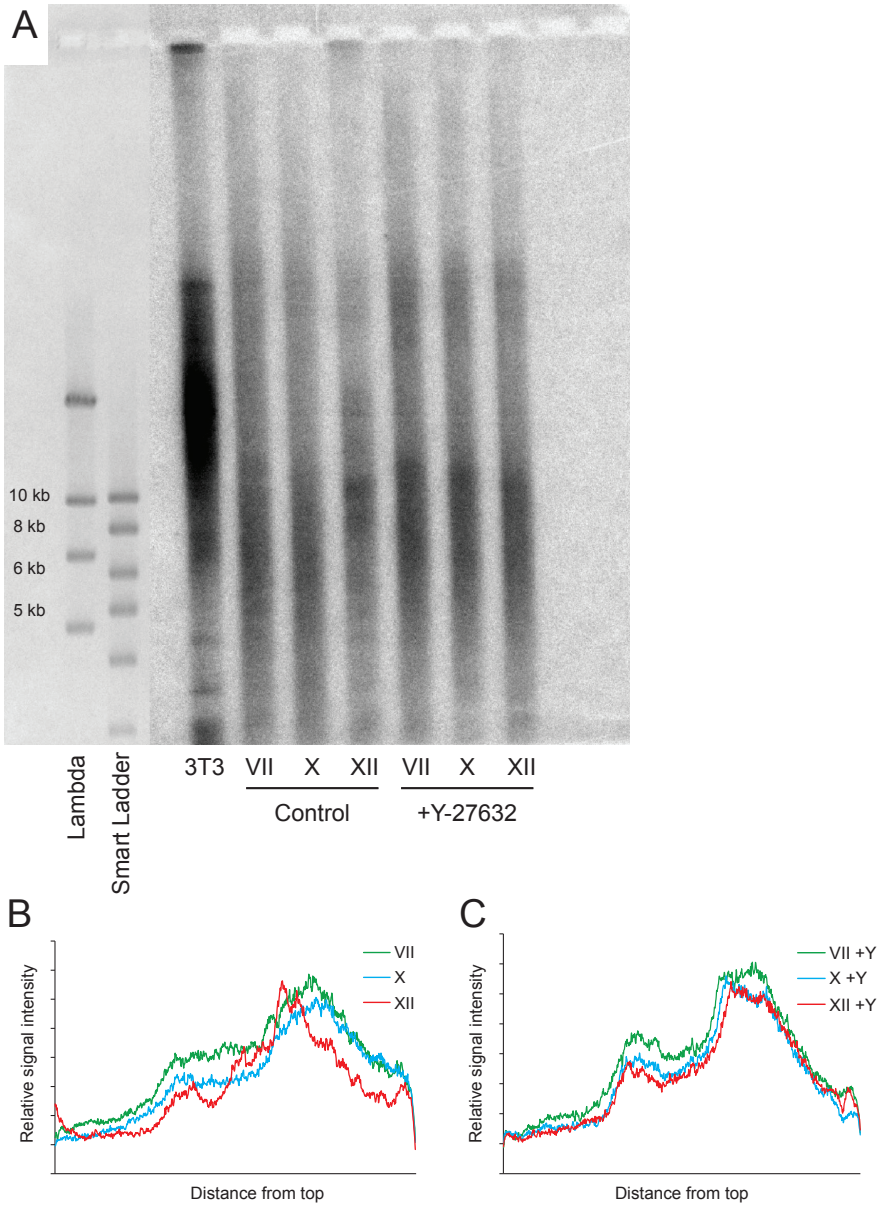

Figure 4.20 – Telomere length analysis of human keratinocytes *in vitro*. YF29 were cultured in presence or absence of Y-27632 for several passages (from passage number VII to passage number XII, equivalent to 35 cell doublings on average). Shortening of telomeres was analyzed by Southern blot (A). The analysis revelead that telomere did shorten in both conditions (B and C).

4.2.3 ROCK inhibition does not increase the expression of ITGA6 or CTNNB1

Suprynowicz and colleagues (2012) suggested that ROCK inhibition could reprogram epithelial cells to a stem-like phenotype instead of promoting cellular immortalization. In their 2012 paper, they showed that Y-27632 treatment results in an increase expression of ITGA6 and CTNNB1 in human normal ectocervical cells. To assess if their observations could be reproduced in human keratinocytes, we analysed the expression of ITGA6 and CTNNB1 (β catenin) with or without Y-27632 treatment.

YF29 cells were cultured with feeder cells in presence or absence of Y-27632. For CTNNB1 expression, subcellular protein fractionation was performed. Western blot analysis revealed that there was no difference in CTNNB1 expression between the two conditions (Fig. 4.21). To assess if Y-27632 treatment did impact ITGA6 expression, we performed flow cytometry analysis on cells cultured with or without Y-27632. Again, Y-27632 treatment did not result in an increased ITGA6 expression (Fig. 4.22).

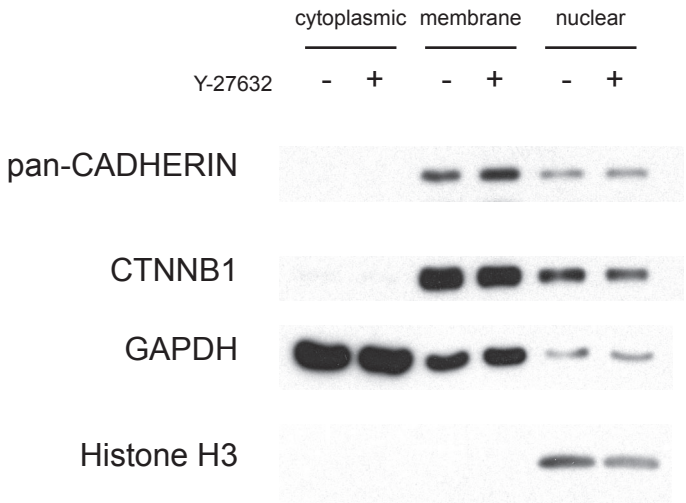

Figure 4.21 – Western blot analysis of CTNNB1 (β-catenin) expression. Human keratinocytes (YF29) were cultivated in presence or absence of Y-27632. There was no difference in the expression of CTNNB1 in all subcellular compartments.

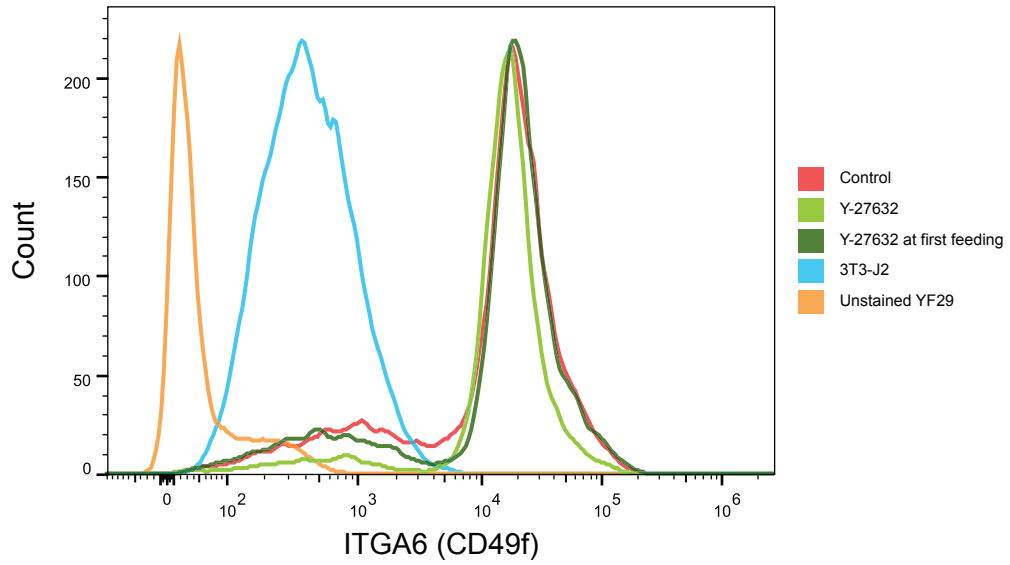

Figure 4.22 – Flow cytometry analysis of ITGA6 (CD49f) expression. Human keratinocytes were cultured in presence or absence of Y-27632. In one condition, Y-27632 was added at the first feeding (3 days of culture). There were no difference in the pattern of expression of ITGA6.

4.2.4 ROCK inhibition increases the colony forming efficiency of ITGA6 positive cells

To investigate which cell populations were impacted by Y-27632 treatment, we decided to sort human primary keratinocytes based on their ITGA6 expression and perform colony forming efficiencies in presence or absence of Y-27632. We decided to sort on the basis of ITGA6 expression as it marks clonogenic epidermal basal cells (Li et al., 1998).

Skin biopsies were obtained from adult patients undergoing mastectomy. The biopsies were processed to obtain a single cell suspension of epidermal cells. Cells were then incubated with a conjugated antibody targeting ITGA6 and processed by FACS. Two populations could be identified based on ITGA6 expression. After the sort, ITGA6^{high} and ITGA6^{low} cells were seeded at low density on 60 mm petri dishes in presence or absences or Y-27632. After 14 days, the plates were fixed and stained with rhodamine B to count the number of colonies. First, we confirmed that ITGA6 expression levels correlated positively with the number of colony forming keratinocytes (Fig. 4.23 and Table 4.5). However, there was a strong variation between the repeats as the biopsies were from different patients. Nevertheless, we found that Y-27632

treatment improved the colony forming efficiencies of all fractions (Table 4.5). However, it never allowed the ITGA6^{low} fraction to reach the ITGA6^{high} fraction's performance. Again, these results confirmed the impact of ROCK inhibition on primary keratinocytes procurement, regardless of their age or genotype. Y-27632 is known to improve keratinocyte adhesion and to inhibit keratinocyte differentiation (McMullan et al., 2003; Gorostidi, 2012). Both properties are fundamental for the preparation of CEA.

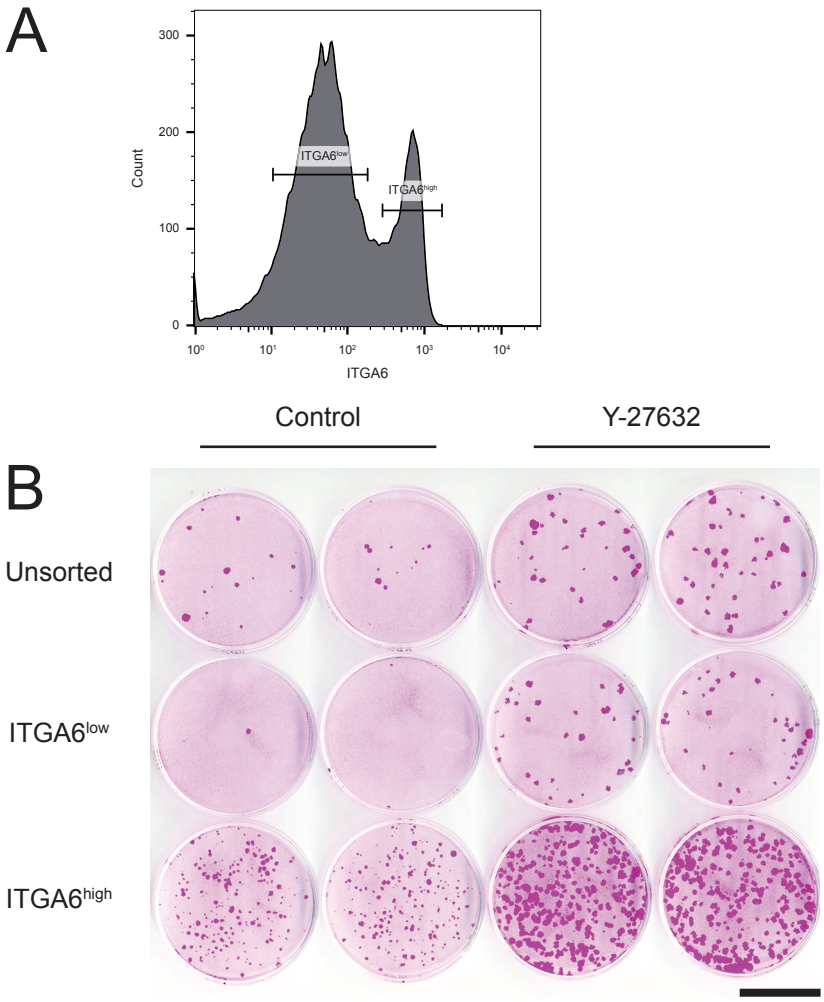

Figure 4.23 – Colony forming efficiency of freshly isolated keratincoytes. Epidermal cells were sorted based on their expression of ITGA6 (A) and cultured for 14 days. 1000 cells were seeded in each 60 mm petrid dish (B). Scale bar: 3 mm.

Table 4.5 – Colony forming efficiency of freshly isolated keratincoytes. Epidermal cells were sorted based on their expression of ITGA6 (CD49f) and cultured for 14 days. 1000 cells were seeded in each 60 mm petrid dish. The table indicates the number of colonies for all 3 patients.

		Control				Y-27632			
Patient	Sample	1	2	3	mean	1	2	3	mean
1	Unsorted	21	16	x	19	42	50	x	46
	$ITGA6^{low}$	2	2	x	2	41	42	x	42
	$ITGA6^{high}$	241	231	x	236	368	328	x	348
2	Unsorted	0	1	3	1	18	20	12	17
	$ITGA6^{low}$	0	0	0	0	5	6	7	6
	$ITGA6^{high}$	2	6	1	3	48	28	39	38
3	Unsorted	14	14	22	17	93	91	110	98
	$ITGA6^{low}$	1	3	1	2	24	38	30	31
	$ITGA6^{high}$	10	19	13	14	108	133	136	126

4.2.5 ROCK inhibition does not impact YAP subcellular localization in human keratinocytes

The Hippo pathway is one of the signaling pathway that is modulated by the activity of RHO/ROCK signaling. We chose this signaling pathway because it has been demonstrated that the Hippo pathway integrates different signals, such as integrin signaling, local stiffness and cellular crowding, into proliferative decisions (Halder et al., 2012). Briefly, the molecular effectors of the Hippo pathway, YAP/TAZ, can translocate from the cytoplasm to the nucleus when phosphorylated and act as co-transcription factors with other molecules. ROCK inhibition can influence the subcellular localization of YAP in various cell lines (Dupont et al., 2011). Moreover, overexpression of mutant YAP (S127A, a mutation that leads to enhanced nuclear localization) in mouse epidermis results in hyperproliferation of the basal layer *in vivo* (Zhang et al., 2011; Schlegelmilch et al., 2011). Therefore, we hypothesized that Y-27632 would also produce similar changes in YAP/TAZ subcellular localization in human keratinocytes.

YF29 cells were grown in presence or absence of Y-27632. Subcellular protein extraction was performed 30 minutes or 1 day after the last feeding with EGF. YF29 cells were also cultivated on coverslips for immunofluorescence analysis of YAP subcellular localization. Both Western blot and immunofluorescence analysis did not reveal a change in YAP localization (Fig. 4.24 & Fig. 4.25). In both experimental conditions, similar levels of YAP were detected in all cellular

compartments. These results suggested that YAP subcellular localization was not impacted by ROCK inhibition in human keratinocytes cultivated with 3T3-J2 feeder cells.

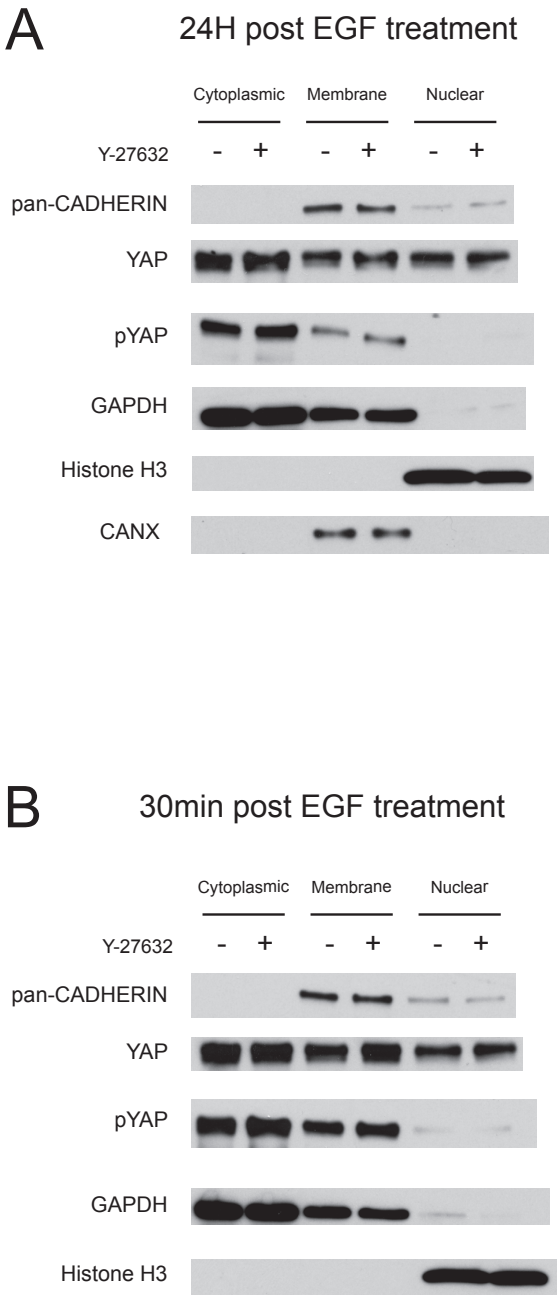

Figure 4.24 – Western blot analysis of YAP expression. Keratinocytes were cultivated in presence or absence of Y-27632. Protein fractions were extracted 24h after the last feeding (A) or after 30 minutes (B). There was no difference in the expression of YAP & pYAP.

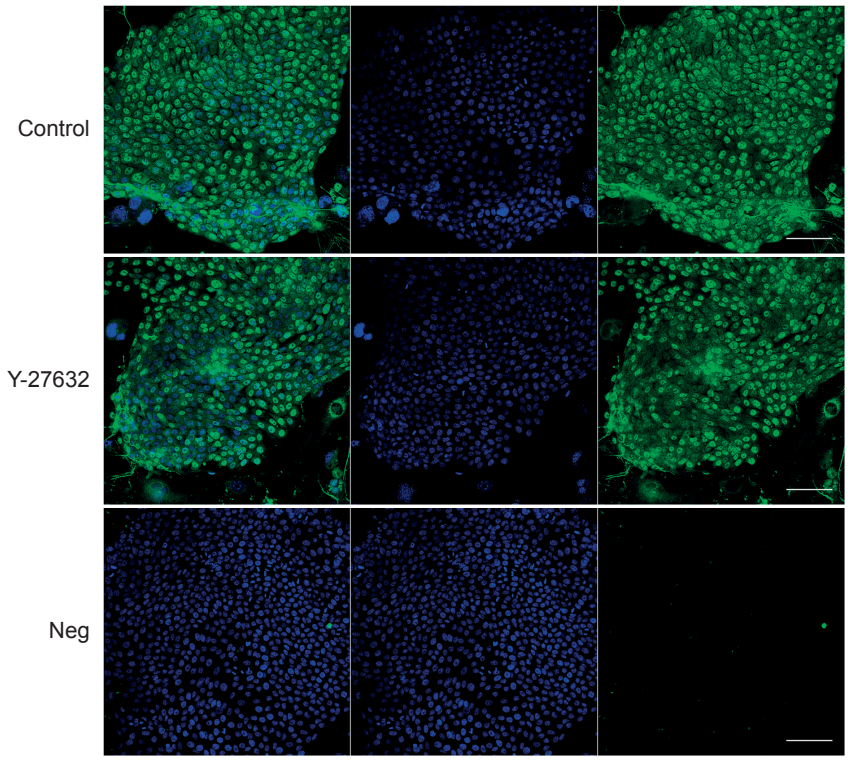

Figure 4.25 – Immunofluorescence analysis of YAP localization. There was no evident difference in the expression and localization of YAP in human keratinocytes. Scale bars: 100 μm.

5 Discussion & Perspectives

5.1 Deconvolution of the 3T3-J2 culture system

High-throughput RNAi is a powerful tool to study molecular and cellular processes (Berns et al., 2004; Hasson et al., 2013; Gonzales et al., 2015). Here, we described how we implemented this technology to investigate the culture system developped by Rheinwald and Green. The 3T3-J2 cells are essential for promoting the growth and self-renewal of human keratinocyte stem cells *in vitro*. However, the molecular effectors are unknown. Our strategy to dissect the system was very simple. We designed an assay to specifically target the 3T3-J2 cells with siRNAs and then measure the impact on human keratinocytes using the rhodamine assay.

Using this approach, we only identified a small number of genes. When these genes were down-regulated in 3T3-J2 cells, it resulted in a reduced signal intensity of rhodamine B total fluorescence. Surprisingly, all of the hits were unexpected. However, when we screened the literature, we found several studies that linked these genes to epidermal homeostasis and wound repair. For example, *IL20*, *FURIN* and *MMP1B* are linked with epidermal wound repair (Ouyang et al., 2011; Ovaere et al., 2009; Rohani et al., 2014). *IL20* is also linked to psoriasis (Wolk et al., 2009), a hyperproliferative disorder of the epidermis. *EFNA1*, an ephrin ligand, is also expressed in the basal layer of the epidermis and it is overexpressed in psoriasis (Gordon et al., 2013). In addition, we also identified *SMC3* (*Bamacan*), the gene that encodes the major proteoglycan of the epidermal basement membrane. BAMACAN is also one of the major proteoglycan present in the protein mixture produced by Engelbreth-Holm-Swarm

(EHS) mouse sarcoma cells, also known as Matrigel® (Couchman et al., 1996). Matrigel is the key component of the culture system used by Sato and colleagues for the growth of intestinal epithelial stem cells (Sato et al., 2009). Proteoglycans, such as BAMACAN, might have a fundamental role in shaping the signaling landscape of the extracellular matrix, both *in vivo* and *in vitro*. We tried to produce recombinant BAMACAN (SMC3) with the protein expression core facility, but it did not work. It would be of interest to clone the *SMC3* cDNA in a plasmid containing an additional signal peptide for protein secretion and retry the production. Nevertheless, the identification of *Bamacan* and other putative "feeder" genes suggest that the 3T3-J2 cells produce an artificial wound-like microenvironment to sustain the *in vitro* expansion of human keratinocytes.

To further confirm the results of the screen, we decided to use the CRISPR-Cas9 technology to generate a *Furin* knockout 3T3 cell line. This approach is based on the CRISPR/CAS adaptive immune system found in some prokaryotic organisms (Westra et al., 2014). Cas9 is very convenient as it can be programmed to specifically cut DNA at a specific location using a single chimeric guide RNA (Jinek et al., 2012). A mistake in the DNA repair process will result in a mutation that can lead to a loss of function. We decided to use this technology to confirm the results of RNAi for the *Furin* gene in 3T3-J2 cells. Using the plasmid gifted by Prof. Constam and Dr. S. Bessonnard, we generated multiple 3T3 clones and selected them by Western blot analysis for FURIN expression. Then, we co-cultured human keratinocytes with these new 3T3 cell lines. When co-cultured with a *Furin* KO 3T3 clone (clone 52), human keratinocytes attached to the dish but produced smaller colonies. We thought that IGF1, a known growth factor produced by the 3T3-J2 cells and a FURIN substrate, could compensate for the loss of *Furin*. Barreca and colleagues had previously demonstrated that IGF1 could enhance keratinocyte proliferation *in vitro* (Barreca et al., 1992). In our experimental conditions, recombinant human IGF1 did not result in a higher rhodamine signal. The results of Barreca and colleagues were obtained in insulin-, EGF- and serum-free media. This could explain why, in normal cFAD (EGF-free) medium, IGF1's effect is not noticeable. The presence of serum and recombinant insulin could act redundantly of IGF1. Other FURIN substrates could also account for the decreased rhodamine signal and therefore would require additional work for their identification. However, using an alternative approach to study the role of FURIN in the

culture system, we found a very striking and unexpected phenotype.

In parallel to the CRISPR experiments, we also investigated the effect of a cell permeable FURIN inhibitor (PC inhibitor or I24) on the culture system. In this case, both cell types (feeder cells and human keratinocytes) are targeted by the inhibitor. When human keratinocytes were treated with the FURIN inhibitor, the signal of the rhodamine assay also decreased. At first, it confirmed what we expected as both *Furin* RNAi and CRISPR targeting in 3T3-J2 cells also did result in a decreased rhodamine B signal intensity. However, FURIN inhibition resulted in an increase in both cell confluency and number of KI67 positive keratinocytes. These results suggested that treatment with the inhibitor increased keratinocyte proliferation. Moreover, RT-qPCR analysis revealed different mRNA levels of *KRT1* and *IVL* in human keratinocytes. Together with the rhodamine B signal intensity, these results suggested that FURIN inhibition had an effect on keratinocyte differentiation. Although the results of FURIN inhibition are in opposition with the results of *Furin* RNAi and CRISPR targeting in 3T3-J2 cells, they are likely due to the fact that the inhibitor targets both human keratinocytes and feeder cells. Moreover, the phenotype that we observed with FURIN inhibition is consistent with the fact that several FURIN substrates (TGFβ and NOTCH1) are positive regulators of keratinocytes differentiation *in vitro* and *in vivo* (Pietenpol et al., 1990; Shipley et al., 1986; Rangarajan et al., 2001; Nicolas et al., 2003). Interestingly, ITGA6 (a basal cell marker) also harbours a proteolytic cleavage site that can be recognized and cleaved by FURIN *in vitro*. However, uncleaved pro-ITGA6 can still form a complex with ITGB4 at the cell surface in some cell lines (Lehmann et al., 1996). This suggests that FURIN inhibition might not be deleterious for ITGA6 mediated signaling and cell adhesion. Both flow cytometry analysis of ITGA6 expression and cell adhesion assays with neutralizing antibodies for ITGA6 could assess if FURIN inhibition does impact ITGA6 molecular function. Moreover, A. Amici demonstrated that FURIN activity is modulated by LEKTI (Amici, 2011). His results showed that a strong FURIN activity was deleterious for *LEKTI* knockout keratinocytes, which further support the idea that FURIN inhibition could improve keratinocyte *in vitro* expansion.

FURIN is not the only proprotein convertase that is expressed in the epidermis. PACE4 and PC5/6 are also expressed by epidermal keratinocytes (Pearton et al., 2001). The FURIN inhibitor, or I24, can also inhibit these two PCs (Becker et al., 2012). It is known that PACE4

and FURIN can have redundant activities in the epidermis (Pearton et al., 2001). Currently, we don't know if the phenotype that we observed with the inhibitor is solely the consequence of FURIN inhibition. It is likely that broad proprotease inhibition in keratinocytes might be responsible for the effect in our culture system.

Recently, Mou and colleagues demonstrated that dual inhibition of TGBβ and BMP signaling (with A-83-01 and DMH1 respectively) could inhibit keratinocyte differentiation *in vitro* and enable their long term growth without feeder cells. To achieve this result, they also had to supplement the culture medium with the ROCK inhibitor (Y-27632) (Mou et al., 2016). TGFβ and BMP4 are processed by FURIN and both promote keratinocyte differentiation *in vitro* (Constam, 2014; Constam and Robertson, 1999; Mou et al., 2016). It suggests that the phenotype of FURIN inhibition could result from reduced TGFβ and BMP signaling. To confirm this hypothesis, we could first investigate the level of active TGFβ and BMP4 in the culture supernatant when we supplement the culture medium with the PC inhibitor. Then, we could also investigate the pattern of SMAD phosphorylation in keratinocytes treated with the PC inhibitor.

In the context of CEA based therapies, the starting material is often small. Large burn patients have only few cm^2 left of intact skin that can be used for CEA production. Thus, several rounds of culture passages are required to produce enough therapeutic cell sheets. As mentioned before, the current system relies on the use of animal feeder cells and animal serum, which raises strong concerns from the regulatory affairs and clinicians. The system is also unable to prevent the progressive loss of growth potential of cultured cells. Mou and colleagues suggest that these issues could be addressed by combining both TGFβ/BMP/SMAD signaling inhibition and ROCK inhibition (Mou et al., 2016). To some extent, this approach can alleviate the use of feeder cells and enable long term growth of cultured basal epithelial cells. However, we don't know if this new implementation of the culture system is compatible with CEA production. Two important questions will have to be addressed. First, does it work with small starting cell densities? High starting cell densities can alleviate the use of feeder cells, but large burn patients don't have that luxury. Second, we don't know if TGFβ/SMAD/ROCK inhibition allows long-term growth of human keratinocytes *in vitro*. These two questions are also valid for PCs inhibition. Our results suggest that FURIN inhibition or PCs inhibition could

also benefit the culture system. We are going to investigate if dual inhibition of proprotein convertases and ROCK can also benefit the culture of human keratinocytes.

Last year, two studies published different high-throughput strategies to investigate the 3T3 co-culture system (Ligaba et al., 2015; Burleigh et al., 2015). In the first study, Ligaba and colleagues used siRNA to reverse transfect 3T3 cells alone and stored the conditioned medium for further testing. Then, they assessed the ability of the conditioned medium to enhance keratinocyte growth in normal culture condition (with non-transfected 3T3 cells). In the second study, Burleigh and colleagues also used RNAi to investigate the genes required for mammary epithelial cell proliferation *in vitro*. Their study differed completely as they targeted an immortalized mammary epithelial cell line that was co-cultured with 3T3 cells. They did not target the feeders with siRNA. Both studies identified different genes from the ones we identified. This difference might be explained by the fact that we all used a different approach. Nevertheless, Ligaba and colleagues identified the *Fstl3*, *Lefty1* and *Lefty2* genes which are known antagonists of TGFβ (Ulloa and Tabibzadeh, 2001). It is interesting that multiple studies landed on TGFβ signaling. Together they further support the idea that inhibition of TGFβ signaling would be required for feeder-free culture of keratinocytes.

Although we did not confirm the biological function of all our hits, the literature suggests that they all would be strong candidates for further studies. It would be of interest to confirm their expression and biological activity. Then, it will also be important to study the putative candidates' impact on keratinocyte stem cells both *in vivo* and *in vitro*. The required work is out of this thesis, but it will likely provide the foundation for the development of a defined *in vitro* microenvironment for epithelial stem cells.

Our RNAi strategy successfully identified several genes influencing human keratinocyte *in vitro* expansion. But, we could not identify genes with redundant function or genes that were not covered by the siRNA library. Nevertheless, we and others have demonstrated that the Rheinwald & Green culture system is compatible with a high-throughput setting. We believe that the same procedure could be used to screen either siRNAs or small molecules to answer other biological questions using the same culture system. In the context of this thesis, we only had one measurement, the rhodamine signal. But, the same approach could

be applied in high content screening by looking at the expression of various markers such as cell morphology, cell cycle dynamics or keratinocyte differentiation.

5.2 The impact of ROCK inhibition on cultured keratinocytes

In parallel to the screening project, we investigated the impact of ROCK inhibition on cultured keratinocytes. Indeed, similarly to rapamycin, Y-27632 is one of the first molecule that showed a potential to improve the culture system developed by Rheinwald & Green. However, the literature has been very cautious about the phenomenal effect of ROCK inhibition on the culture of normal human epithelial cells. In this work, we showed that ROCK inhibition could be compatible with the current system for CEA production.

Previous studies argued that Y-27632 could trigger cellular immortalization of normal keratinocytes (Chapman et al., 2010; Palechor-Ceron et al., 2013b). Some of the observations to support this idea were the increased maximum number of cell doublings and the increased expression of *TERT* mRNA in presence of Y-27632. To test their hypothesis, we decided to investigate the impact of ROCK inhibition on telomeres maintenance. We found that telomere shorten in both culture conditions. It is known that telomere shortening can induce cellular senescence when telomeres reach a critical length. Indeed, the limit in cell doublings of normal diploid fibroblasts theorised by L. Hayflick (HAYFLICK, 1965) was later correlated with telomere shortening. Overexpression of TERT can allow normal diploid cells to grow indefinitely. Therefore, TERT expression and increased cell doublings *in vitro* have been interpreted as cellular immortalization. However, there are multiple evidences which suggest that it is more complex than that. First, the number of cell doublings *in vitro* is dependant on both the cellular type and the culture system. For example, the holoclone (epidermal stem cell) can undergo 180 doublings *in vitro*, which is more than twice than the limit (40-60) proposed by L. Hayflick (Rochat et al., 1994; Claudinot et al., 2005). Moreover, the maximum number of keratinocyte doublings is dependant on the addition of EGF to the culture medium (Rheinwald and Green, 1977). Second, the role of TERT in cellular immortalization does not require telomere length maintenance (Stewart et al., 2002). While, it is known that TERT can have other non-canonical functions, its precise role in cellular immortalization is still elusive (Martínez and Blasco, 2011; Park et al., 2009). In addition, the role of TERT is poorly

characterized in adult stem cells. Unfortunately, we could not verify that cultured keratinocytes express TERT by Western blot analysis (lack of working primary antibody for TERT). Telomere length analysis and telomeric repeat amplification protocol (TRAP) assays are both able to assess the activity of TERT. However, we could not perform the TRAP assay as 3T3-J2 cells also express TERT. In addition to telomere length analysis, we also demonstrated that the two phosphorylated forms of RB are present in keratinocytes cultured with or without Y-27632. Immortalized keratinocyte cell lines only display the hyperphosphorylated form of RB, which allows E2F to be constantly active and promote DNA synthesis (Lathion Droz-Georget et al., 2015). Altogether, the telomere length analysis and Western blot analysis of pRB support the idea that Y-27632 did not induce the immortalization of human keratinocyts.

We also investigated if Y-27632 treatment did increase the expression of ITGA6 and CTNNB1. Suprynowicz and colleagues suggested, based on the increased expression of these two markers, that Y-27632 could reprogram epithelial cells to a stem-like state (Suprynowicz et al., 2012). Unexpectedly, we were unable to reproduce their results. Indeed, we found similar expression levels of both ITGA6 and CTNNB1 in human keratinocytes treated with or without Y-26732. We think that the discrepancy between our observations and their results could be explained by different control conditions for human keratinocytes culture.

ROCK inhibition improves the colony forming efficiency of freshly isolated keratinocytes (Terunuma et al., 2010). Keratinocytes colonies are also larger. F. Gorostidi also demonstrated that the cell cycle of epidermal cells is shorter in presence of Y-27632 *in vitro* (Gorostidi, 2012). In this work, we confirmed the previous observation that Y-27632 increased the number of colony forming cells (CFC) in freshly isolated human keratinocytes. In addition, we demonstrated that it also increased the number of CFC in the ITGA6^{high} fraction. Epidermal stem cells are located within the basal layer of the epidermis, which is marked by the expression of ITGA6. With the results discussed previously, our data suggested that there might be more stem cells in the basal layer of the epidermis than what was previously thought. In addition, recent lineage tracing studies in mouse epidermis suggest that all basal cells contribute to epidermal homeostasis (Clayton et al., 2007; Jones et al., 2007; Doupé et al., 2010; Mascré et al., 2012). In this context, our data would support a similar model for human epidermal homeostasis.

We know that epidermal stem cells can self-renew *in vitro*. Otherwise, the development of CEA would not have been possible. However, these cells display a behavior that is different from what can be observed *in vivo*. Keratinocytes express both basal keratins (KRT5 and KRT14) and wound induced keratins (KRT17) *in vitro* (Lindberg and Rheinwald, 1990). The results of the screen are in agreement with the opinion that the 3T3-J2 cells promote keratinocyte proliferation through a wound-like microenvironment. But, this surrogate microenvironment is suboptimal. After few divisions or serial cultivation, the growth potential of clonogenic keratinocytes is lost through clonal conversion. When keratinocyte colonies reach a certain size (after 7 days), they stop to grow exponentially (Barrandon and Green, 1987a). However, EGF or TGFα can sustain exponential grow by stimulating keratinocytes migration *in vitro* (Barrandon and Green, 1987a). Recently, it has been shown by Roshan and colleagues that Y-27632, similarly to EGF and TGFα, could also delay this process (Roshan et al., 2016). The molecular mechanism underlying the effect of Y-27632 on both keratinocyte adhesion and proliferation is still unknown. In our culture conditions, we could not link ROCK inhibition to the Hippo pathway. It is possible that ROCK inhibition impacts other signaling pathways. It is well known that ROCK is a key regulator of actin filament organization (Schober et al., 2007). Actin filament dynamics can modulate the response to EGF in human keratinocytes and affect stem cell maintenance (Nanba et al., 2013). Moreover, Nanba and colleagues demonstrated that keratinocyte migration *in vitro* is a good indicator of epidermal stemness (Nanba et al., 2015). Together, these results suggest that the phenotype of ROCK inhibition could act through its impact on actin filament dynamics and keratinocyte migration *in vitro*. It could be possible that Y-27632 promotes a constant cellular migration, allowing keratinocytes to maintain an active growth phase while also improving their adhesion to the culture dish.

In this work, we have identified several genes that might be used one day as recombinant products to replace the 3T3-J2 cells. In addition, we also provided multiple evidences to support the use of Y-27632 for the culture of epidermal cells. Together, these two complementary approaches provide several ideas for the development of a new culture system for CEA production and basic stem cell research. Our work opens the door to complementary projects to pursue our goal.

A Abbreviations

3T3	Mouse embryonic fibroblasts 3T3 cell line
3T3-J2	3T3 clone J2
BMP	Bone morphogenetic protein
BS	Bovine serum
BSA	Bovine serum albumin
CANX	Calnexin
Cas9	Crispr associated protein 9
CEA	Cultured epidermal autografts
cFAD	Complemented F12 Adenine DMEM
COL4	Collagen type IV
CRISPR	Clustered regularly interspaced short palindromic repeats
CTNNB1	Catenin beta 1
DACT1	Dishevelled-binding antagonist of Beta-Catenin 1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco-Vogt Modification of Eagle's Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDA	Ectodysplasin A
EDTA	Ethylenediaminetetraacetid acid
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPFL	Ecole Polytechnique Fédérale de Lausanne
EPU	Epidermal proliferative unit
ES cells	Embryonic stem cells
F10	Coagulation factor 10
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FGF2	Fibroblast growth factor 2
FN1	Fibronectin

Appendix A. Abbreviations

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
H3	Histone H3
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOPX	Homeodomain only protein X
HTS	High throughput screening
ICC	Immunocytochemistry
ICM	Inner cell mass
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL1R	Interleukin 1 receptor
IL-20	Interleukin 20
IPS	Induced pluripotent stem cells
ITGA6	Integrin alpha 6
IVL	Involucrin
KEGG	Kyoto encyclopedia of genes and genomes
KGF	Keratinocyte growth factor
KRT	Keratin
LEKTI	Lympho-epithelial kazal-type inhibitor
LIF	Leukemia inhibitory factor
LRC	Label-retaining cell
LRIG1	Leucine-rich repeats and immunoglobulin-like domains protein 1
MEF	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase/ERK kinase
miRNA	Micro RNA
MMP	Matrix metalloproteinase
MMP1	Matrix metallopeptidase 1
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
P53	Tumor protein 53
P63	Tumor protein 63
PACE	Paired basic amino acid cleaving enzyme
PBS	Phosphate-buffered saline
PC	Proprotein convertase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Plk1	Polo-like kinase 1
qPCR	Quantitative polymerase chain reaction
RB	Retinoblastoma
RHO	Rho GTPase
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference

ROCK	Rho associated kinases
S1P	Sphingosine-1-phosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SMC3	Structural maintenance of chromosome 3
TACE	Tumor necrosis factor alpha converting enzyme
TBP	Tata-box binding protein
TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TFRC	Transferin receptor
TUBB	Tubulin Beta Class I
WNT	Wingless-related integration site
YAP	Yes associated protein 1

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WORK EXPERIENCE

09/11 – 09/16 **Ecole Polytechnique Fédérale de Lausanne**
PhD assistant - Laboratory of Stem Cells Dynamics
- Developed a high throughput screening strategy that led to the identification of new genes responsible for the growth of skin stem cells
- Fostered a strong collaboration with the Biomolecular Screening Facility
- Presented the research in multiple conferences (talks & posters)
- Mentored several successful Master and Bachelor students

02/10 – 06/10 **Ecole Polytechnique Fédérale de Lausanne**
Laboratory Assistant – Laboratory of Systems Biology and Genetics
- Produced the reagents for a high throughput study

EDUCATION

09/10 – 06/11 **Kyoto Prefectural University of Medicine**
Master thesis, supervised by Prof. T. Nakamura – Department of Ophthalmology
- Characterized the functional role of a protein in cornea homeostasis

2006 – 2011 **Ecole Polytechnique Fédérale de Lausanne**
Bachelor & Master in Life Sciences & Technology
- "Mention d'excellence"

2002 – 2005 **Gymnase de Nyon**

EXTRACURRICULAR ACTIVITIES

09/08 – 08/09 **Association SV-Travel 2009**
Vice president
- In charge of the fundraising activities which allowed 50+ Bachelor students to visit Singapore (budget: 100'000 CHF)

LANGUAGES

French – mother tongue
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COMPUTER SKILLS

Word / Excel / Powerpoint / Matlab / R / Illustrator

INTERESTS

Volleyball, Badminton, Crafts (Ceramics)

PUBLICATIONS

1. Nakamura T, Hamuro J, Takaishi M, Simmons S, Maruyama K, **Zaffalon A**, Bentley AJ, Kawasaki S, Nagata-Takaoka M, Fullwood NJ, Itami S, Sano S, Ishii M, Barrandon Y & Kinoshita S (2014) LRIG1 inhibits STAT3-dependent inflammation to maintain corneal homeostasis. J. Clin. Invest. 124: 385–97

2. Barrandon Y, Grasset N, **Zaffalon A**, Gorostidi F, Claudinot S, Droz-Georget SL, Nanba D & Rochat A (2012) Capturing epidermal stemness for regenerative medicine. Semin. Cell Dev. Biol. 23: 937–44

3. Lathion Droz-Georget S, Rochat A, Knott G, Recchia A, Martinet D, Benmohammed S, Grasset N, **Zaffalon** A, Besuchet Schmutz N, Savioz-Dayer E, Samuel Beckmann J, Rougemont J, Mavilio F and Barrandon Y. A single epidermal stem cell strategy for ex vivo gene therapy. EMBO Mol. Med. 7, 380-393 (2015). doi:10.15252/emmm.201404353