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II. ABSTRACT

The majority of tissues and especially those with high turnover, such corneal and skin epithelia are maintained and repaired by a small population of resident stem cells. These tissues provide a powerful experimental system for studying stemness and homeostasis. Autologous keratinocyte stem cells (KSC) derived from epidermis and cultured on murine feeder layer are used to produce grafts that regenerate an epidermis over a full-thickness wound or like skin ulcer. Cell therapy relies several criteria for the quality of the cultures; in particular, large-scale grafting success depends on adequate number of the stem cells essential for long-term epidermal renewal. The lack of univocal KSC markers is the major limit in this field, since it prevents selection of naive cells. In this study, we compared the gene expression profiles of three different cell populations: KSCs-enriched (EnSC) and Transient Amplifying (TAC) cells, both sorted on the basis of α6-integrin expression in basal layer, and post-mitotic differentiating cell (PMDC) arisen from the primary cell culture serial passaging. The three populations were characterized on the basis of growth potential, clonogenic capacity and p63-bright cell fraction, parameters linked to the presence of stem cells. In an effort to reveal genes involved in stemness maintenance and early commitment we carried out validation of selected genes in Holoclone, Early and Late Meroclone cell progenies. The analysis showed that candidate stem-cell related genes decrease their expression level during clonal conversion.

1. BACKGROUND

1.1 THE EPIDERMIS AND ITS APPENDAGES

The skin is the first barrier of our body that protects us from infectious agents, hazardous substance, UV radiation, and mechanical stress. Human skin is a stratified epithelia and the epidermis represents the major part of epithelium covering the body. The epidermis is derived primarily from ectoderm germ layer, like so its appendages. The other components of the skin are the dermis that gives rise from the mesoderm germ layer recovering a crucial role into promote epidermal development and hair placodes formation during embryogenesis.

The dermis is tightly connected to the epidermis by a basement membrane. Structural components of the dermis are collagen, elastic fibers, and extrafibrillar matrix. On the other side, the dermis is connected with hypodermis preserving the body from stress and strain. The dermis structure is divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region. In itself many mechanoreceptor/nerve endings are located that provide the sense of touch and heat. The dermis also contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. The blood vessels provide nourishment and waste removal from the dermis and epidermis. It shows a crucial role during early stage of epidermal keratinocytes development releasing inductive signals to promote ectodermal progenitors to specify epidermal commitment. Epithelial tissues in the human body undergo constant renewal; the skin epithelium is regenerated continuously through the periodic proliferation of resident stem cells and an intricate balance between growth and differentiation. In human, it is estimated that the epidermis turns over every 40-56 days [1-3]. This constant turnover of the epidermis is mediated by keratinocyte stem cells (KSCs), which reside in the basal layer. Each one generates columns of epidermal proliferative units composed by Transient Amplified cells (TACs) [4]. The TACs reside in the basal layer and they undergo a few rounds of cell division before initiate an asymmetric cells division to lead terminal differentiation [5, 6]. As cells exit from the basal layer and begin their journey towards the skin surface, they switch from the expression of keratins K14 and K5 to K1 and K10 [7]. After few rounds dividing cells in the basal compartment of the epidermis continually execute a program of terminal differentiation. This switch was discovered more than 25 years ago, and remains the most reliable indication that an epidermal cell has undergone a commitment to terminally differentiate. The first suprabasal cells are known as spinous cells, reflecting their cytoskeleton of keratins K1/K10 filament bundles connected to robust cell–cell junctions known as desmosomes. These connections provide a cohesive, integrated mechanical framework across and within stacks of epithelial sheets. K6, K16 and K17 are also expressed suprabasally, but only in hyperproliferative situations such as wound healing. This keratin network not only remodels the cytoskeleton for migration but also regulates cell growth through binding to the adaptor protein 14-3-3σ and stimulating Akt/mTOR signaling [8]. These connections provide a cohesive, integrated mechanical structure across and within stacks of epithelial sheets. Keratin6, Keratin16 and Keratin17 (K6; K16 and K17) are also expressed suprabasally, but only in hyperproliferative condition such as wound healing. This keratin network not only remodels the cytoskeleton organization for migration during wound healing but also regulates cell growth through binding to the adaptor protein 14-3-3σ and stimulating Akt/mTOR signaling [8-10]. As spinous cells progress to the granular layer, they produce electrondense keratohyalin granules packed with the protein profilaggrin which, when processed, wrap up keratin intermediate filaments even more to generate large macrofibrillar structures. In addition, cornified envelope proteins (Lce), which are rich in glutamine and lysine residues, are synthesized and deposited under the plasma membrane of the granular cells. As response to the increased permeability to the calcium, the cells activate transglutaminase, generating γ-glutamyl ε-lysine crosslinks to create an indestructible barrier (FIG.1A).

A **B**

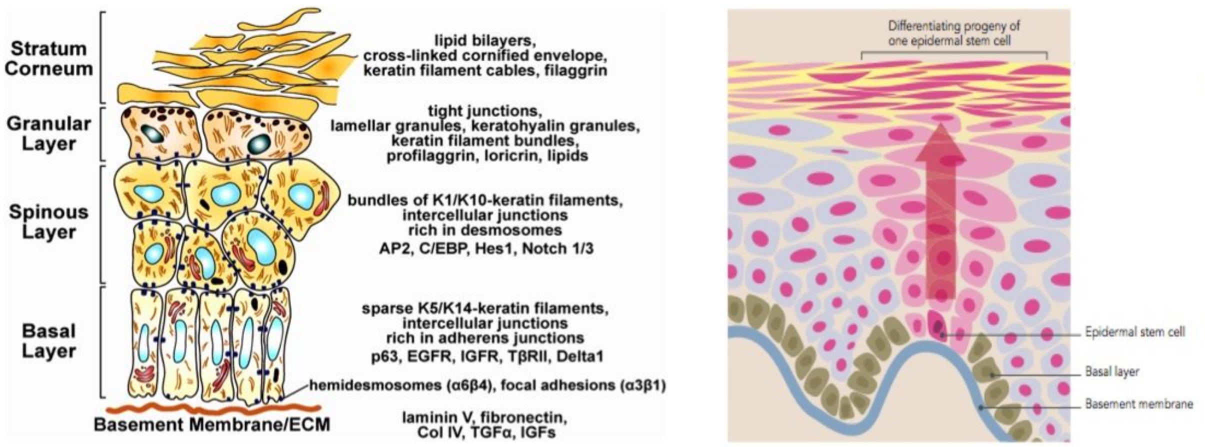


Figure 1. Schematic representation of epidermis and EPU structure. (A) A skin cartoon: cells of the basal layer attach to an underlying basement membrane. Basal cells are mitotically active but they lose this potential when they detach from the basement membrane and embark on the outward trek toward the skin surface. (B) Diagram of the epidermal proliferation unit (EPU). A putative slow-cycling basal keratinocyte stem cell occasionally divides giving rise to a stem cell daughter and a transient amplifying daughter. The latter divides two to four times and its progeny leaves the basal layer by moving towards the suprabasal strata and starts to differentiate.

The final steps of terminal differentiation involve the destruction of cellular organelles including the nucleus, and the extrusion of lipid bilayer packaged in lamellar granules, onto the scaffold of the cornified envelope. The dead stratum corneum cells create an impenetrable layer that is continually replenished as inner layer cells move outwards and are sloughed from the skin surface [11]. The hair follicle is a component of the skin and shows a more complex structure, composed at least of seven distinct cell types organized in three compartments: an outer root sheath (ORS), an inner root sheath (IRS) and hair shaft [12]. In the epidermis two different populations of stem cells have been found the interfollicular stem cells and hair follicle stem cells. The hair stem cells reside in the bulge region, amorphologically distinct area of the hair follicle located in the mid portion of the follicle [13]. One gene that is expressed at high levels in the bulge region is Keratin15 (K15). K15 expression is also detected in the interfollicular epidermis, although at lower levels [14]. During physiological growth of the skin, interfollicular and hair follicle stem cells contribute to their respective compartment of origin (FIG.2).

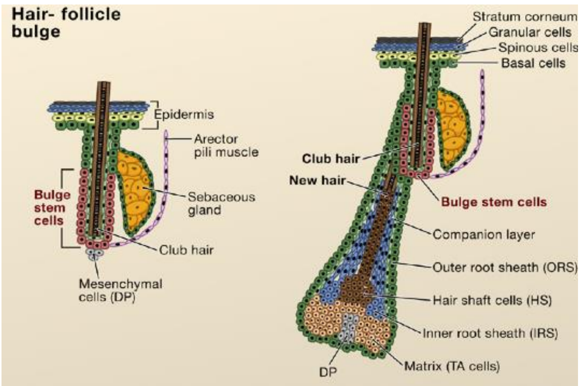


FIG. 2 . **Hair follicle development.** Label-retaining stem cells of the hair follicle reside below the sebaceous gland in a region known as the bulge, which is connected to the arector pili muscle. During periods of rest, bulge stem cells form the base of the follicle, which is adjacent to the specialized mesenchymal cells (dermal papillae). At the start of each hair cycle, stem cells at the bulge base become activated to form the highly proliferative new hair germ. As the germ grows, a proliferative compartment of TA cells (matrix cells) engulfs the dermal papilla at the base. These cells progress to differentiate to form seven concentric shells of discrete cell lineages, which are from outer to inner: the companion layer, the three layers of the inner root sheath, and the three layers of the hair shaft. These differentiated layers are surrounded by the outer root sheath, which extends below the bulge and is thought to contain stem cells that continue to migrate down to the follicle base during the growth phase of the hair cycle .

During physiological growth of the skin, interfollicular and hair follicle stem cells contribute to their respective compartment of origin. However, mouse transplantation experiments and lineage tracing experiments have demonstrated that, in response to injury, firstly bulge-derived keratinocytes participate into repair of the interfollicular epidermis [15, 16]. In the murine model, upon severe injury, the interfollicular Stem Cells (IFE) can even regenerate hair follicle stem cells (HFs). Thus, at least in the mouse, epidermal cells have also multipotent characteristics [17]. Recent studies with lineage tracing of single basal cells in rat tail skin, suggests that, while the majority of labeled cells are lost within 3 months, some survive and clonally expand in size over time. This behavior seems indicate that exist a discrete epidermal proliferating unit composed of one stem cell surrounded by a steady-state pool of ~10 transit-amplifying progeny that subsequently exit the niche and terminally differentiate [5]. The exact identity and location of interfollicular epidermal stem cells remains unknown. Recently, a population of murine HF stem cells was identified and these populations are enriched for Lrg1 and Blimp1. These cells give rise to IFE and Sebaceous Glands cells (SGs) when stimulated by retinoic acid *in vivo* [18]. It has been suggested that these cells might be the elusive IFE stem cells. However, three different lineage tracing studies using Cre-recombinase driven by either *Shh*, *Sox9*, or cytochrome *P450* promoters each document that mouse IFE harbors its own resident progenitors, which can sustain long-term epidermal homeostasis [19-21]. It is widely shown that, in addition to stem cells, the epidermis contains a second population of proliferating cells, known as transit amplifying cells (TACs) [22]. TACs are the stem cell progeny that are destined to undergo terminal differentiation; however, prior to cell-cycle withdrawal, they are believed to undergo a limited number of rounds of division. TACs are further hypothesized to have an internal ‘‘memory,’’ which specifies a set number of cell divisions prior to the onset of terminal differentiation. Because TACs divisions amplify the number of differentiated daughters resulting from each stem cell division, the stem cells need to divide relatively infrequently to maintain epidermal homeostasis. Nevertheless, in this SC/TAC model, continual slow cycling of stem cells is required to maintain the short-lived TAC population.

By far the most influential model of the role of stem and TACs in epidermal homeostasis is that of the ‘‘epidermal proliferative unit’’ (EPU). This is based on the observation that in some regions of the mouse IFE cells are arranged in columns, with a single cornified cell at the top. Cornified cells, which have reached the final stage of the terminal differentiation process, are hexagonal in shape, and thus the columns are packed next to one another in a regular array. If one assumes that cells only migrate vertically when they leave the basal layer, it follows that each stack of cornified layer cells will be descended from the cells in the basal layer that lie directly beneath it, forming an EPU (FIG. 1B).

1.2 CELLURAR BEHAVIORS IN EPIDERMAL HOMEOSTASIS

a) Signaling pathways in epidermal development

In mouse model, after gastrulation, the embryo surface emerges as a single layer of ectoderm and specific signals determinate the commitment of neuroectodermal cells to neural or epidermal fate [23]. One of the most important signaling required for this process is the Wnt signaling that plays a crucial role in early step of ectodermal commitment. Wnt signal blocks the ability of ectoderm to respond to fibroblast growth factors (FGFs). In the absence of FGF signaling the cells are prone to respond to bone morphogenetic proteins (BMPs), initiating the epidermal development. Conversely, the acquisitions of neural fate depend on the absence of a Wnt signal. In this condition the ectoderm is able to receive and translate activating signal mediated by FGFs to induce neurogenesis. Loss of Wnt signaling induces the expression of specific inhibitors of BMP signaling (Follistatin, Chordin or Noggin) that induces the neural fate [24] (FIG. 3).

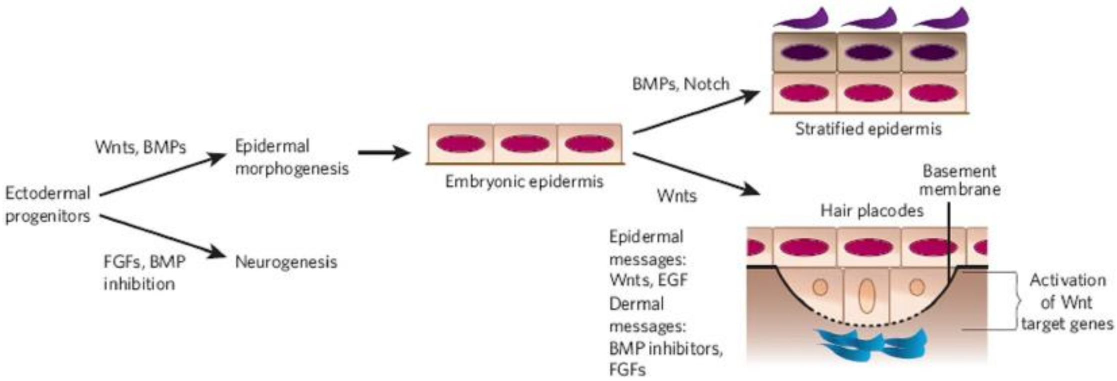


FIG. 3 . Signaling pathways in early mouse embryogenesis. In early stages of embryogenesis the Wnt signaling plays an important role. In the absence of Wnt signal, ectodermal progenitors respond to FGFs, downregulate BMP signaling and progress towards neurogenesis. On the other hand the Wnt signaling blocks the ability of early ectodermal cells to respond to FGFs, allowing them to respond to BMP signaling to drive and epidermal fate. As development progress, a single-layer expressing Wnt signaling is formed. Some cells fail into respond to Wnts signal, and these become fated to become epidermal cells trough BMP, EGF, and Notch signaling. The cells that do respond to Wnt signaling also receive underlying FGF and BMP inhibitory signals from mesenchymal compartment and, together, these signals the cells to make an appendage (hair follicle and sebaceous glands).

The embryonic epidermis that results from this process consists of a single layer of multipotent epithelial cells expressing the ectodermal markers Keratin 8 and keratin 18 (K8 and K8) [25, 26]. When the commitment to stratification occurs, K8 and K18 expression is down regulated with concomitant induction of K5 and K14 expression (E13.5). In the adult K5 and K14 are only expressed in stratified epithelia and never expressed in single-layered epithelia representing a specific markers of stratified epithelia [27]. One gene that is essential for this switch and for the commitment to stratification is the transcription factor p63, a p53 family member [28]. In the primordial epidermis a transient protective layer known as periderm recovers the whole embryo. This layer is characterized by K17 expression [29]. During late stages of skin development the periderm is lost when the epidermal cells begin to stratification and differentiate becoming impermeable. As described before, epidermal development is due to a complex signaling network in early stage of embryogenesis where the BMP signaling shows a key role as determinant of ectodermal specification in low vertebrates [30]. In mammals a putative BMP function in regulating epidermal fate or specific gene expression has not been demonstrated consistent with a possible redundant function among the BMP family members or with other signaling pathway. How it has been demonstrated, the BMP signaling during late stage of embryogenesis have a crucial role in the hair follicle morphogenesis, it is active in the interfollicular epidermis in which represents as much an epidermal promoting signal as it is a follicle inhibitory signal [31, 32] (FIG. 3).

b) The holoclone: behind adult stem cell self-renewal

Considerable progress in understanding the biology of skin stem cells has been accomplished in the mouse and there are excellent recent reviews that describe these advances [33, 34]. Lineage tracing experiments and functional skin reconstitution assays in the mouse have unambiguously demonstrated that the interfollicular epidermis[35-37], the upper constant region of pelage hair follicles and the ducts of sweat glands of the foot pad contain cells with stem cells properties [38-41]. These stem cells are multipotent and clonogenic and , slow-cycling and , or express cell surface proteins like Lgr5 and Lgr6 , CD34 , Plet1 (MTS24) and Lrig1 [42-48]. Similarly, multipotent stem cells have been identified in whisker follicles of the mouse and the rat by clonal analysis and serial transplantation [49, 50]. Furthermore, the classical scheme of epidermal renewal based on a hierarchy of slow-cycling stem cells and rapidly cycling transient amplifying cells is a matter of discussion with opposing opinions [35-37, 51]. First stem cell markers described in the mouse do not apply to human, second label retaining, lineage tracing or genetic manipulation experiments cannot be performed in human for obvious ethical reasons and third clonogenic assays remains the sole reliable read-out to capture stemness in human epidermis or epidermal appendages[52-55]. Ultimately a human keratinocyte is considered a stem cell if it forms a colony that can be serially subcultured and that can generate an epidermis when transplanted onto immunodeficient mice [52, 54, 55]. However none of the cell surface proteins described so far (see the section above: *The proliferative compartment*) is stem cell specific and the quest for a reliable marker of human keratinocyte stem cells remains. For many years, cell size has been the most reliable and efficient means to enrich in clonogenic keratinocytes either as single cells or after elutriation [56-58]. Finding these stem cells and studying their properties presents a more difficult challenge, because such cultures contain a mixed population of cells [59]. In the 1970s, Rheinwald and Green's pioneering works largely revulotionated human keratinocytes cultivation using a feeder layer of lethally irradiated mouse 3T3-J2 cells, introducing an irreplaceable condition for epithelial regeneration [60]. Indeed, cultures of autologous keratinocytes have been used so far to prepare grafts that can permanently restore severe epithelia defects, such as skin and ocular burns, massive and genetic skin disorders [61, 62]. Human keratinocytes are usually characterized in relationship to their clonogenic capacity and growth potential by clonal analysis. The former indicates the capacity of a basal cell to generate a colony, the latter deals with the self-renewal potential, hence the 'stem-ness' of that cell. Epidermal basal layer epithelial cell when cultured under particular conditions can give rises three clonal types: holoclone, meroclone and paraclone (FIG.4) [63].

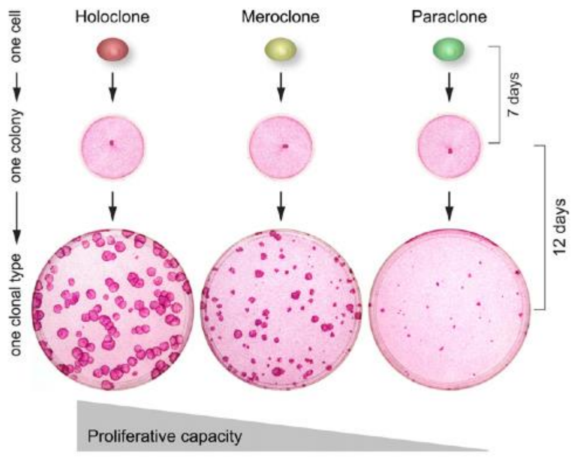


FIG. 4 . Clonal analysis. A holoclone (red) generates a progeny that forms large progressively growing colonies and almost no terminal colonies (less than 5%). A meroclone (yellow) forms both large progressively growing and terminal colonies. A paraclone (green) generates only terminal colonies. [20M]

Ideally, single keratinocytes are isolated under an inverted microscope and individually cultured. Typically cloning efficiency ranges between 40 and 70% if cultured keratinocytes are selected on small size [56]. After 7 days of cultivation, each clone is identified under an inverted microscope and subcultured onto indicator dishes containing an irradiated feeder layer. After 12 days, indicator dishes are fixed and stained with Rhodamine B. The size and the number of terminal colonies containing squame-like cells expressing markers of skin terminal differentiation (involucrin, filaggrin, LEKTI) define the clonal type [55]. Holoclones generate large progressively growing colonies and almost no terminal colonies. They have a tremendous growth capacity up to 180 divisions and can theoretically generate enough cultured epidermal graft to cover the entire body of an adult human [52, 54, 55]. It is now widely accepted that holoclones are the phenotype of human keratinocyte stem cells in culture [64]. Indeed, holoclones have all the hallmarks of stem cells, including self-renewal capacity, telomerase activity and long telomeres, and an impressive proliferative potential [15, 65-67]. A single holoclone can indeed generate the entire epidermis or the entire corneal epithelium of a human being [68]. Holoclone-forming cells generate all the epithelial lineages of the tissue of origin permanently restore massive epithelial defects [61, 69-71] and can be retrieved from human epidermis regenerated from cultured keratinocytes years after grafting [72]. Meroclones derive from holoclones and generate a mixture of progressively growing colonies and terminal colonies. Paraclones are transient amplifying cells committed to a small number of divisions (from 1 to 15 divisions – 2 cells to 32,000 cells respectively) that generate only terminal aborted colonies containing large differentiated squame like-cells on indicator dishes. Paraclones are mostly generated by meroclones. The conversion of holoclones to meroclones and paraclones is termed clonal conversion and is an irreversible phenomenon under normal circumstances [55].

c) The proliferative compartment resides in the basal layer

The epidermis (FIG. 5 A) maintains homeostasis by proliferation of a single inner (basal) layer of dividing progeny of stem cells (KSCs). As they withdraw from the cell cycle, the transiently amplifying cells (TACs) commit to terminally differentiate, detaching from the basement membrane and initiating a trektoward the skin surface.

This process is sustained by nutrients from blood vessels in the underlying dermis. The epidermis and dermis are separated by a basement membrane that is composed of extracellular matrix proteins, including collagen IV, fibronectin and laminin 5. Both the epidermis and the dermis contribute to the synthesis of basement membrane components, but the basal layer of the epidermis seems to be the sole manufacturer of collagen IV and laminin 5. The basal layer of epidermal cells synthesizes these components and adheres to them, and also polymerizes and/or organizes them into the basement membrane. Clusters of alpha3- or beta1-integrins, transmembrane proteins, act as laminin 5 receptors, and are required for the assembly of the basement membrane and for the adherence of epidermal cells to it. In the epidermis, beta1-integrin also partners with alpha2-integrin to make a receptor and organizer for collagen, and with alpha5-integrin to make a receptor and organizer for fibronectin. So, in the integrin knockout mice, the severity of basement membrane synthesis or assembly defects and of the skin blistering due to subsequent epidermal detachment is much greater when beta1-integrin is missing than when alpha3-integrin is absent [73-75].

The alpha6beta4-integrin also binds laminin 5 and is even more crucial for epidermal attachment to the basement membrane than is alpha3beta1-integrin [76-78]. Instead, alpha6beta4-integrin forms the core of the hemidesmosome junction, which is morphologically but not biochemically analogous to a half-desmosome. Hemidesmosomes associate with proteins that link to the keratin intermediate filament cytoskeleton (FIG. 5 B). These features provide robust mechanical strength to the base of the skin epidermis, as well as strong anchorage to the basement membrane [79]. By contrast, alpha3beta1-integrins associate with proteins that link them to the actin cytoskeleton.

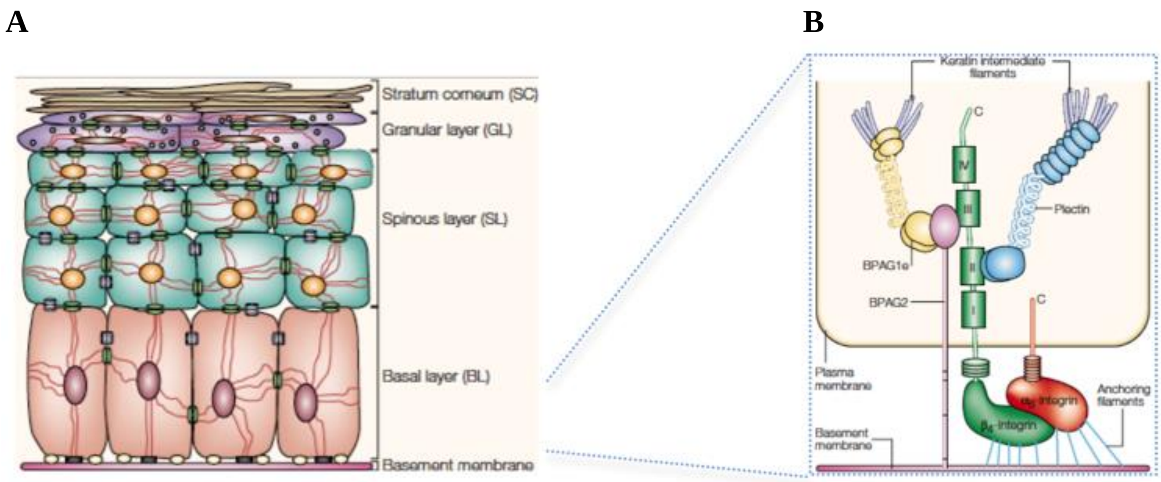


FIG. 5. The epidermal layers and a hemidesmosome junction. **(A)** The epidermis is stratified squamous epithelium that is composed of several cell layers. Resting on the basement membrane is the basal layer (BL), consisting of proliferating, KSCs and TACs (see text). **(B)** This structure is based on biochemical and molecular evidence of protein–protein interactions in the hemidesmosome. alpha6beta4-integrin heterodimers form the core of the hemidesmosome, along with BPAG2, a transmembrane protein with an extracellular domain similar to collagens. BPAG1e and plectin are two hemidesmosomal proteins that are members of the plakin family of coiled-coil proteins. These two proteins have intermediate (keratin) filament-binding domains on their non-helical carboxy-terminal (C) segments.

A combination of classical human genetics and gene-targeting technology has revealed the unfortunate consequences of mutations in the genes that encode the epidermal integrins and their associated proteins. For example, lesions in the genes that encode alpha6- and beta4-integrins, and an associated transmembrane protein, called BPAG2 (also known as COL17A1; collagen, type XVII, alpha 1) (Fig. 5), are all known to result in the loss of hemidesmosomes, which causes the epidermis and the underlying basement membrane to separate, as in junctional epidermolysis bullosa (JEB) [80-82].

Therefore, human basal keratinocytes express the β1 integrins α5β1 and α3β1 as well as the integrin α6β4. Important evidence for proliferative heterogeneity in human basal keratinocytes has been provided by recent work that used a fluorescence-activated cell sorting (FACS) approach, demonstrating that both cultured and primary human foreskin keratinocytes could be separated into cells with high levels of β1 integrin that had a high plating efficiency assayed after 2 weeks in culture compared with those keratinocytes with low levels of this integrin). Furthermore, keratinocytes expressing high levels of β1 integrin were shown to be capable of generating an epithelial sheet when grafted onto mice, suggesting that this fraction of the basal layer contain KSCs [19, 83]. *In vivo* studies suggest that epidermal stem cells constitute between 1 and 10% of the basal layer, depending on the methodology used [84, 85]. Because ≈40% of the basal layer in human foreskin exhibits high levels of β1 integrin in vivo [19, 83] and this antigen is expressed in suprabasal layer as well, it is highly likely that keratinocytes with this phenotype contain both the KSC population and a significant number of TACs. On the other hand, α6β4 represents only basal layer cell population. This integrin is expressed on both the KSC and TACs, but with different antigen expression levels. In vitro, KSCs presents high levels of α6β4, TACs low expression levels of the this integrin [86, 87]. In the work bench, a third type of cell population is described with lower α6β4 expression levels and higher expression levels of differentiation marker (involucrin and K10) than previous KSCs and TACs. This kind of cell population is called post-mitotic differentiating cell (PMDC) and shows very reduced capability to proliferate in vitro when sorted using fluorescence-activated cell sorting (FACS) approach. In vivo, the precise localization of PMDCs is undefined, but there is evidence of a barely α6β4 signal during BL to SC layer cell transition. Instead, PMDCs corrisponds to abortive colony containing involucrin positive cells (FIG, 4. See the section before: *Behind adult stem cell self-renewal*) [88]. Several cell surface proteins like CD71 (transferring receptor), CD49f (alpha 6 integrin), CD49a (alpha 1 integrin) CD49b (alpha 2 integrin), CD49c (alpha 3 integrin), CD104 (beta 4 integrin), CD29 (beta 1 integrin) Lrig1 or ABCG2 are proposed to capture stemness [89-93]. However none of these cell surface proteins is stem cell specific and the quest for a reliable marker of human keratinocyte stem cells remains.

1.3 p63 IS A MAIN FACTOR IN EPITHELIAL MORPHOGENESIS

a) p63 belongs to p53 family

The p63 gene is a member, together with p73, of the p53 family. All three proteins show high amino acid homology and share three functional domains commonly found in transcription factor: an N-terminal transactivation domain which shares 25% homology with N-terminal part of p53, a central DNA binding domain which shares 65% of homology with the corresponding p53 domain and C-terminal tetramerization domain, which shares 35% of homology with the oligomerization domain of p53 (FIG. 6 A). The p63 and p73 genes are transcribed at least in six different isoforms due to two alternative promoter at the N-terminus of the gene. The two alternative promoters give rise to TA and ΔNp63 transcript. In addition to use two different promoters, the p63 and p73 genes increase their complexity by alternative splicing at the C-terminus, which gives rise to three different carboxyl terminal isoforms named α, β, γ and seven isoforms (α, β, γ, δ, ε, ζ, η) respectively for p63 and p73 [94, 95](FIG. 6 A).

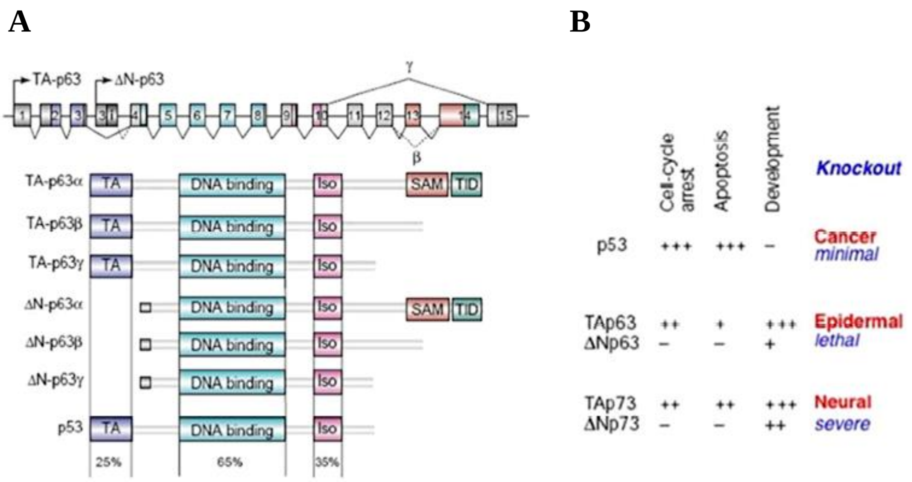


FIG. 6 . p53 family. **A)** The p63 gene encodes a tetrameric transcription factor that can be expressed in at least six isoforms with widely different transactivation potential that share an identical DNA binding domain. Alternative transcription start sites give rise to transactivation (TA) isoforms, encoding proteins with a canonical transactivation domain similar to p53. **B)** A very high degree of homology is seen in the different domains of p53, p63 and p73. All three members of the family are able to regulate cell cycle arrest, apoptosis and in parallel show developmental effects. However, the knockout mice reveal striking differences, with p63 showing mainly an epidermal phenotype, and p73 a neuronal phenotype, whereas no developmental defects were observed in p53 null mice.

TAp63 isoforms contain an N-terminal transactivation domain, whereas ΔNp63 isoforms lack this domain and show an alternative transactivation domain [96]. In addition, the p63α isoforms (TA and ΔN) contain a sterile alpha motif (SAM) domain at C-terminal of the gene, which is absent in p53 [97, 98]. This domain is a protein-protein interaction domain. It is an evolutionary conserved domain commonly found in proteins controlling developmental processes such as several Eph receptor tyrosine kinase [99]. Although the SAM domains are known to be involved in protein-protein interactions, in vitro

studies demonstrated that SAM domains of p63 are able to bind to RNA or lipids [100-102]. Another unique domain of p63alpha isoforms is the post-SAM domain Transcriptional Inhibitor Domain (TID) that has been shown to have an inhibitory function [103, 104]. The transactivation inhibitory domain of p63 binds to the N-terminal TA domain masking residues that are important for transactivation. In fact, p63 isoforms that contain the γ and β C-termini are associated with higher transactivation competency that the ones with α terminus protein [105]. The lack of TA domain in ΔNp63α isoforms suggests that they are transcriptionally incompetent. Since ΔNp63 isoforms retain the oligomerization and DNA binding domains, it is plausible that they act as dominant negative inhibitors of p53 and TA containing p53 family members [95, 106]. Indeed, numerous studies show that co-expression of ΔNp63α with either TAp63, TAp73, or p53 has inhibitory effect on TAp63-mediated transcription. A plausible mechanism is due to the formation of transcriptionally inactive ΔN-TA heterotypic or homotypic tetramers (composed of either all-TA or all-ΔN monomers) that compete for the same DNA binding sites. Despite the well-documented role of ΔNp63 as a dominant negative transcriptional repressor, several studies have shown that ΔNp63 isoforms directly transactivated a set of genes including Hsp70 and p57Kip [107, 108].

The expression pattern of p63 in early stage of embryogenesis at E9.5 is restricted within oral ectoderm, in the ectodermal part of limb buds (Apical Ectodermal Region, AER) and tail bud region, whereas in later stages of embryogenesis the expression of p63 is restricted to interfollicular epidermis and in outer root sheath of hair follicle. The essential role of p63 in skin development was revealed by two independent studies of p63-null mice generated trough two different knockout gene strategies. These mice lack stratified epidermis, producing a disorganized single layered surface epithelium that is negative for epidermal markers such as K5 and K14 [109, 110]. Both two groups showed that the p63 null mice lack the epidermis and its appendages, associated to limb truncation, craniofacial abnormality. Although, the mutant phenotype in these two studies is similar, the interpretation of the biological function of p63 remains controversial. Nonetheless, these mouse models have been showed a unique role played by p63 during development, in contrast to the p53 null mice that it doesn't show development defects (FIG. 6 B)

b) p63 is required for cell proliferative potential

p63 gene and in more detail the ΔNp63α isoforms is highly expressed in the basal layer of epidermis and in the highly proliferative compartment of stratified epithelia as the cervix, urogenital tract, prostate and breast tissues [95, 111].The knockout mouse model generated by McKeon's group is born alive but has striking developmental defects. Their limbs are absent or truncated, defects caused by a failure of the apical ectodermal ridge (AER) to differentiate. This region is characterized by p63 expression from E9.5 during embryogenesis. The lack of a proper AER limb buds in p63 null mice results from a failure of the ectoderm to undergo growth and differentiation that give rise to this stratified epithelium. At birth, p63-deficient mice have striking and visible skin defects; in fact, they die within few hours from the birth for dehydration. Structures dependent upon epidermal mesenchymal interactions during embryonic development, such as hair follicles, teeth and mammary glands, are also absent in p63 deficient mice (FIG. 7). The skin lacks expression of the basal layer markers as K5 and K14 and also spinous layer markers K1 and K10, although the isolated patch of the epidermis showed the expression of late differentiation markers such as Loricrin, Involucrin, Filaggrin.

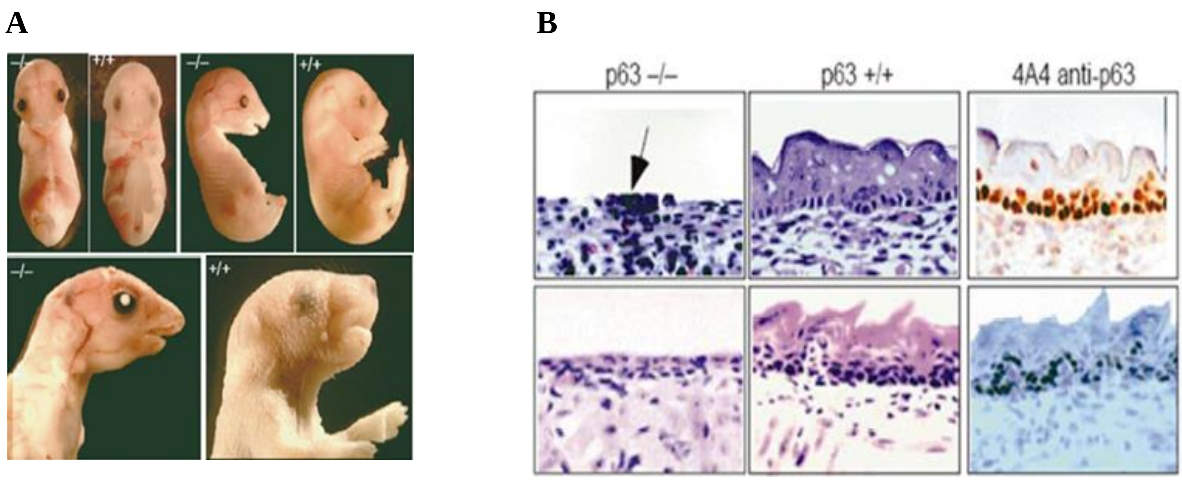


FIG. 7 . The phenotype of p63 knockout mice. **A)** The p63 null mice at birth show defects in limb formation craniofacial defects associated with skin and appendages aberrant development for lack of stratification and differentiation. **B)** Defects in stratified epithelial differentiation in p63-deficient mice. H&E staining of the epidermis at E17, p63-/- mice lacking squamous stratification in the epidermis (top) and tongue epithelium. Middle, wilde- type H&E control mice showing extensive stratification. In the right the basal staining with anti-p63 antibody to show the endogenus expression of p63 in the epidermis.

The authors argue that p63 is required for the maintenance of epidermal stem cell, because the p63 null mice showed in late stage of embryogenesis and in newborn mice isolated patch of disorganized epithelial cells expressing the late differentiation markers. The epidermis appears as disorganized epithelia. The cells showed highly pyknotic nuclei which positive for TUNEL indicating apoptosis processes. This study concluded that p63 is required for the initial development and continued regeneration of the epidermis and that the loss of p63 in the tissues failed to maintain the proliferative potential of stem cells but not in differentiation processes . In addition, to reinforce this hypothesis the same authors in another study strongly showed that p63 is dispensable to maintain the proliferative potential of epithelia stem cells of the thymus and epidermis. The thymus has an epithelial component derived to ectoderm germ layers. In p63 null mice this region exhibited a hypoplastic phenotype compared to wild type. The p63 null thymus showed the typical expression markers for epithelia development and in this case was fully competent to support the maturation of developing T cells. However, the hypoplastic phenotype of the thymus is due to a reduction in proliferative potential of stem cells in thymus compartment as well as in epidermis. They showed by colony- forming assay that p63 is involved in proliferative potential of epidermal keratinocytes. Clones lacking p63 had a reduction in cell size for decrease of proliferative potential of stem cells. These data, supported the hypothesis that p63 is involved in maintenance of proliferative potential of stem cells in stratified epithelia [112]. Other studies reported the main role of ΔNp63α in controls differentiation processes. ΔNp63α represses the expression of p21 and 14-3-3σ, two genes induced during terminal differentiation of epidermal cells. The expressions of these genes are required for cell cycle progression including cyclin B2 and cdc2 [113]. These genes are directly inhibited by p63 in the basal layer where the cells are undifferentiated. Previous works showed that, the balance between un-differentiation and differentiation state is regulated by cross talk between p63 and Notch [114]. Indeed, in primary mouse keratinocytes, p63 expression counteracts the ability of Notch1 to restrict growth and promote differentiation in keratinocytes; effect mediated by repression of Hes1 an activator of Notch. In these way the cells in the basal layer proliferates whereas, when the cells escape to highly proliferative state show a reduction of ΔNp63α and the induction of Notch activity inducing cell differentiation. These studies showed that p63 and in more details the TAp63 isoforms in involved in early stage of embryogenesis to drive epidermal specification whereas the ΔNp63α is important for maintenance of proliferative state of epidermal cells in the basal layer inhibiting terminal differentiation.

c) p63 is required for cell adhesion

In the epidermis and in all epithelia, cell adhesion has a fundamental role into support epithelia formation and structure. Three different types of junction complexes mediate cell-cell adhesion in epithelia: tight junction, adherents junction and desmosomes junction. While the tight junction perform a role in establishment of barrier formation and in para-cellular transport, the adherents junction and desmosome are crucial in promoting cell adhesion.The importance of these junctions for epidermal integrity is highlighted by the autoimmune or genetic blistering diseases in humans caused by dysfunctional desmosome components [61, 77]. Similarly, mouse model lacking desmosomal components are prone to epithelial blistering resembling the phenotype of p63 null mice [115]. Cell adhesion process, as attachment to the extracellular matrix via integrins is an important step not only for structural organization of tissues but also for epithelial cell proliferation, migration, differentiation and survival. Therefore, physical detachment from the extracellular matrix for defects in cells adhesion results in induction of apoptosis or anoikis in a wide range of epithelial cell types [116]. Loss of p63 causes the reduction in gene expression of adhesion molecule with consequent cell death. The ability of p63 to regulate key matrix adhesion proteins could play an important role in maintenance of tissue integrity since the attachment of cells to underlying basement membrane is crucial for epithelial tissue integrity and homeostasis. Carrol et al in 2007 showed the reduction of multiple adhesion molecules in p63 null mice [117, 118]. They found that p63 directly controls the gene expression of Perp a desmosomes junction. Perp is highly express in stratified epithelia and it is a component of desmosomes junction. Interestingly, Perp null mice showed lethally associated with the presence of epithelia blistering in the skin and in oral epithelium, some phenotype that resembled p63 null mice defects [119]. Moreover, these two studies showed also that p63 directly control the expression of other adhesion molecules as integrin alpha 3, 4, 5 and 6 and laminin-2. Thus, genes encoding many classes of proteins are involved in multiple aspect of cell adhesion were regulated by p63, including extracellular matrix component, integrins, adherents and desmosomal junction. Accordingly with these data, the down regulation of p63 in breast cancer cells (MCF10A) or in mouse primary epithelia cell, induced cell detachment and subsequent apoptosis. Interestingly, this affects are specific of down-regulation of ΔNp63 isoforms, whereas the reduction of TAp63 isoforms had a little phenotype. Conversely, the induction of ΔNp63α and TAp63γ isoforms protected the cells from death induced by detachment of the cells from matrix. These finding suggest that the ΔNp63 isoforms are essential for adhesion process and survival in epithelial cells in vitro [117, 118].

d) p63 identifies adult epithelial stem cells

The discover of an epithelial morphogenesis factor led developmental biologists to investigate the role of p63 in the neoplastic tissue. Even though it is not yet clear the relationship among p53 family members during the tumor event [94, 120], immunostaining of p63 revealed in noticeable detail the high expression level of p63 in the basal or progenitor cells of many of the tissues that sustain a majority of cancers and other neoplasias including skin, esophagus, and urothelia, as well as secretory epithelial tissues including lacrymal glands, mammary glands, and prostate glands [95]. Although it is clear that p63 expression is not limited to "stem" cells of the epithelial tissues that express this gene, a number of studies contend that the stem cells in fact express the highest levels of p63 [121]. Notably, the undifferentiated ectoderm in the mouse that gives rise to cells of the stratified epidermis at embryonic day 14 (E14) expresses high levels of p63 as early as E8.5, far in advance of any obvious decision towards commitment (FIG. 8). Thus signals from underlying mesoderm appear necessary to trigger discrete functions of p63 in the ectoderm toward commitment, differentiation, and regeneration [122, 123]. There is general agreement that the progenitor cell layers of skin, breast, and prostate express high levels of the nontransactivating DNp63 isoforms to the near exclusion of the transactivating isoforms [106, 124-127].

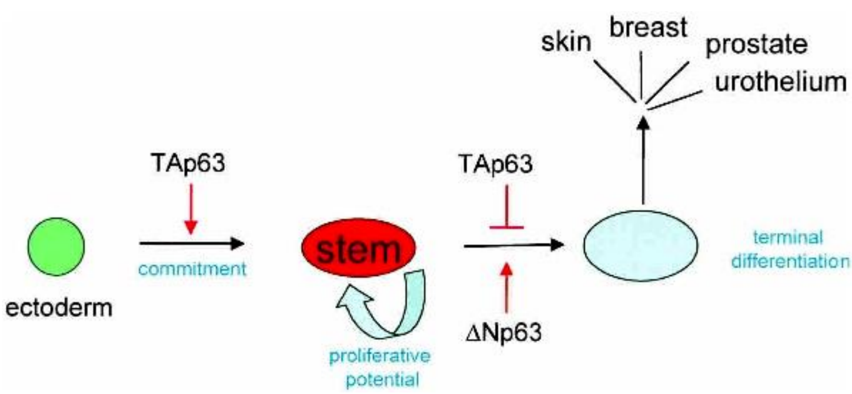


FIG. 8. Assignment of p63 isoform functions in epithelial morphogenesis. Schematic of epithelial morphogenesis depicting the roles ascribed to TAp63 and DNp63 isoforms (Koster et al. 2004). In particular, TAp63 is seen to both promote commitment of the ectoderm to epithelial lineages and to suppress the differentiation of such committed progenitor cells. DNp63, on the other hand, acts to promote terminal differentiation via its competition and suppression of TAp63 actions in these cells.

The lack of TAp63 isoforms suggested that DNp63 must be shouldering much of the function of p63 in epithelial progenitor cells. Although good evidence exists that DNp63 can, in fact, engage in transcriptional activation processes [128], it has generally been assumed that DNp63 is a repressor molecule against TAp63 and p53, or simply functions to silence a set of genes in order to establish the correct genetic environment in epithelial progenitor cells (FIG.8, see the section *1.3.a : p63 belongs to p53 family*). In fact, the only work to cite anti-TAp63 antibodies saw TAp63 expressed in suprabasal layers that generally lack DNp63 [126]. In 2010, Pellegrini introduced the p63-bright cell concept [62]. This definition linked p63-high expression level to cell size, according to the notion that basal keratinocytes with a cell diameter between 10 and 12 μm show the highest clonogenicity and growth potential compared to the cell in the same origin epithelial tissue [129]. This parameter provides an amelioration method to determine the number of stem cells in culture. Infact, the determination of the number of holoclones in a culture requires some experimentation [63], and would be cumbersome as a standard test for the quality of cultured grafts. Fortunately, the number of aborted colonies (those that have stopped multiplication), is inversely related to the number of holoclones, and is easier to score [130]. The identification of holoclones by immunodetection of p63, and especially the α isoform, is an important and simple means of determining the presence of an adequate number of stem cells in a cultured keratinocyte graft. This criterion has been studied in detail in cultured grafts of ocular limbus, the site of stem cell precursors of the corneal epithelium [127]. p63 is present in basal cells of the limbus, but not in those of the central corneal epithelium [131-134]. Immunocytology for p63 was performed on subconfluent primary limbal cultures derived from 84 patients. The frequency of p63+ cells was evaluated by computerized analysis of the intensity of staining of single cells, as assessed by automated quantitative immunocytochemistry, using as a reference a limbal strain that contained a known percentage (14%) of holoclones.

Age of donor (years)	Number of donors	Holoclones* (%)	Meroclones‡ (%)	Paraclones§ (%)	Age of donor (years)	Number of donors	Cells with p63 (%)
A. By clonal analysis					*B. By frequency of p63+ cells*		
19–25	7	**11.0**	58.0	31.0	16–29	15	**7.9**
					30–40	19	**6.0**
41–61	6	**7.8**	58.0	34.2	41–60	35	**5.4**
					61–76	15	**3.8**
76–86	3	**2.0**	26.5	71.5			

*Less than 5% of colonies formed are terminal;
‡From 5 to 95% of colonies formed are terminal;
§All colonies formed are terminal.

TAB. 1. Correlation of p63+ cells with the number of holoclones (KSCs) in cell culture.

Considering that the frequency of p63+ cells (Table 1) was not determined on the same patients as the frequency of holoclones, the two values correlated well and show the same gradual decline with age of patient.

Primary cultures possessing less than 3% of cells with high levels of p63 are not generally used to prepare grafts, but exceptions may be made for elderly patients [72]. In view of the recent demonstration that ΔNp63α is the most specific isoform for holoclone identification [132], future evaluation of stem cell content in epithelial grafts should be carried out using that isoform (Fig. 9A).

In the cohort study by Rama et al. 2010, of the total of 116 patients with limbal deficiency resulting from chemical burns and treated by grafting of limbal cultures, there were 36 failures .The main causes can be attributed to the lack of quality control criteria for culture conditions: inappropriate cell culture conditions and the inadequacy of the preparation of the wound bed and/or surgical procedures and / or substrates used for cultures. The low success rate described in the literature has been useful to understand the contributions of these variables to the clinical performances of the cultures. One of them is the presence of an adequate number of stem cells (FIG. 9 B) [62], which can be determined by the expression rate of the transcription factor p63, a master regulator of epithelial morphogenesis.

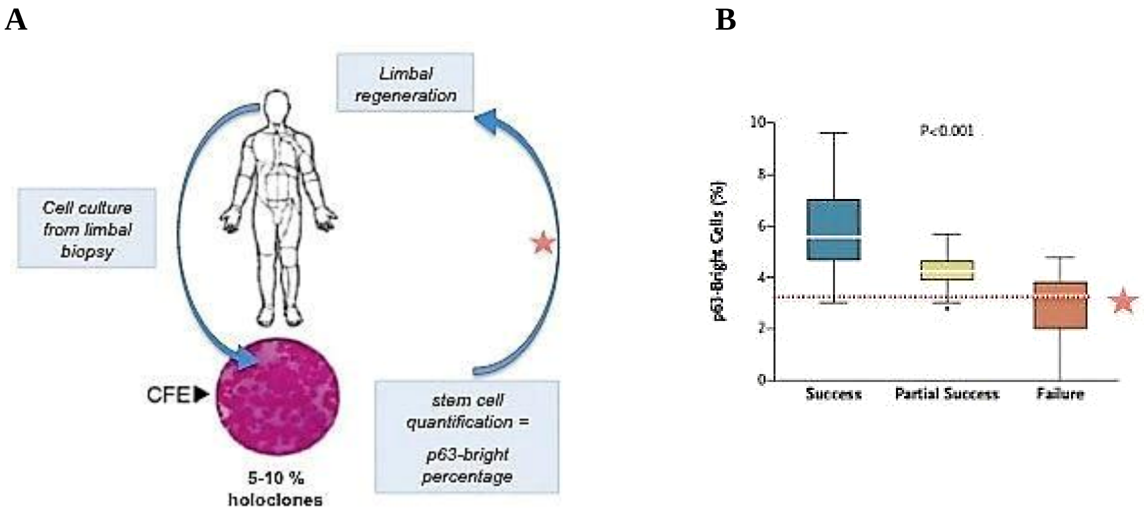


FIG. 9. Clinical outcome in relationship to p63-bright cell fraction. A) Primary cultures possessing less than 3% of cells with high levels of p63 are not generally used to prepare grafts, but exceptions may be made for elderly patients. B) Clinical failure is linked to a minimal stem cell amount in the culture used for regeneration of the epithelial graft. This threshold is defined to 3% (red star).

1.4 AIMS OF THE THESIS

The possibility of cultivating and grafting different types of epithelial cells, as well as the possibility of their genetic modification, opens new interesting perspectives in epithelial gene and cell therapy. Although impressive ameliorations rate in these fields, like the introduction of new matrices for the regeneration of graft, the lack of univocal keratinocyte stem cell marker is the major limit in the isolation of a pure population of naïve cells.

In view of functional data demonstrating the role of integrin α6β4 in mediating adhesion of basal keratinocytes to the basement membrane via hemi-desmosomes, we reasoned that this integrin may provide a suitable marker for the isolation of proliferative keratinocytes because these cells are permanently anchored to the basement membrane. In this way, we designed a method based on magnetic microbeads integrin selection to achieve a cell population enriched in stem cell (EnSCs). Given the method to separate EnSC from Transit Amplifying cell (TAC) population, we sought to determine the comparative tissue regeneration ability of these keratinocyte progenitors. We demonstrate that the ability to regenerate a fully stratified epidermis with appropriate spatial and temporal expression of proliferation and differentiation markers in a short-term in vitro organotypic culture system is an intrinsic characteristic of both EnSCs and TACs, although only the enriched stem cell population is endowed of holoclones as shown by clonal analysis. Obtaining a third kind of sample, the post-mitotic differentiating cell population (PMDC) we were looking to answer on various aspects of cellular processes the repertoire of transcripts of a specific cell state (the transcriptome). Therefore, gene signature of EnSC compared with TACs and PMDCs revealed genes involved in early and late commitment. According to the notion that clonal analysis is the only method to isolate a pure population of holoclones, we compare the trascriptome of different clone progeny types. These data, together with analysis of fractioned cell population sorted using α6β4, provided a list of genes differentially expressed in the high progenitor and late committed cell populations. After the bioinformatic analysis and the screening of output genes, we confirmed the decrease expression level of selected genes during clonal conversion both in mRNA and protein points of view, providing KSC candidate markers with a possible main role in maintaining tissue homeostasis.

2. MATERIALS AND METHODS

2.1 Cell cultures

Human primary keratinocytes were obtained from five abdomen biopsies of healthy adult donors (20-52 years old) of and expanded by cultivation onto lethally irradiated 3T3-J2 cells (a gentle gift from H. Green's lab) in growth KNO medium, a DMEM and Ham's F12 media mixture (2:1) containing FBS (10%), penicillin-streptomycin (1%), glutamine (2%), insulin, adenine, hydrocortisone, cholera toxin, triiodotyronine. After 3 days KNO medium was replaced and KC medium (KNO medium containing 10ng/ml EGF) was added to the culture. Keratinocytes were trypsinized at sub-confluence and replated onto a new feeder-layer. Mouse NIH/3T3 fibroblast cell line was maintained in Dulbecco's Modified Eagle's medium (Euroclone), supplemented with 10% fetal calf serum. For preparation of PMDC population, keratinocytes were cultivated for several passages with feeder layer in KC.

2.2 Isolation of progenitor cell fractions (EnSC and TAC)

In order to avoid any interference of the enzymatic treatment with the expression of different markers in keratinocytes, we chose a well-established protocol to obtain a single cell suspension. Tissues were rinsed with phosphate-buffered saline (PBS), the epidermis was removed from the connective tissue and cut into smaller pieces. To obtain viable single keratinocyte cells, the epithelial sheets were treated with 0.05% trypsin for 3 h at 37°C. The cells were resuspended in KNO medium and plated in a 60 mm dish containing yet feeder layer up to 3-4 days of cultivation. After that, the same human abdominal keratinocytes in culture were used to isolate EnSC and TAC fractions. We provided a new method based on magnetic microbeads consisting in 2 separation steps: at first, the negative selection for feeder layer depletion and, then, the positive selection for the proliferative compartment selection.
A negative selection is mandatory prior to expression profiling experiments cause it will be beneficial in order to guarantee reproducibe results. Indeed, murine and human genome show high homology percentage, up to 92% of identity (89M). To obtain a pure keratinocyte cell population, the cell culture was trypsinized for the detachment. The single cell suspension, made up of Keratinocytes and 3T3-fibroblasts, is magnetically labeled with Mouse-Feeder Removal Microbeads (Miltenyi Biotec Inc cat. no. 130-095-531), according to productor datasheet. The EasySep *Do-It-Yourself* Selection Kit (Stem Cell Tech., cat. no. 18098) contains the necessary components to create a specific positive selection cocktail, mixing with your own mouse IgG1 monoclonal α6β4 integrin [450-30A] (Thermoscientific) antibody.

This selection cocktail, along with the magnetic nanoparticles and EasySep magnet, was then used to positively select cells with the cell surface antigen recognized by α6β4 monoclonal antibody to obtain the EnSCs. The unsorted cells realesed by the magnet representes the α6β4-dim (TACs) fraction, while the magnetically labeled cells, representing the α6β4-positive fraction, were retained in the column. After the two magnetic separations the two fractioned cell population, α6β4 bright and dim cells, are investigated using CFE assay, p63-bright fraction estimation, clonal analysis and molecular markers quantification to define their progenitor level identity

2.3 Clonal analysis

Skyn biopsy was subjected to standard trypnization protocol (see before). Cell suspension was serially diluited until to obtain a final concentration of 1 cell/ 0,1 ml. Single cells were isolated using limiting dilution (0,5 cell / well) into a multi 96 well plate already containing irradiated 3T3 cells and KNO medium. After 3 days in culture, cells were feeded with KC supplemental medium. Three donors are processed and each one was tested for 5 plates (240 cells). At the 7th day of cultivation, clones are identified under an inverted microscope. Each clone was photographed and then it was dissociated with trypsin. After centrifugation, a colony quarter (1/4 cell suspension in the falcon) is plated in a 100 mm dish containing lethally irradiated 3T3 cells (indicator dishes) and the remaining colony (3/4 cell suspension in the falcon) in a T-25 flask containing feeder layer (clone progeny flasks). For the indicator dishes, the cells were cultivated for 12 days before the cultures were fixed and stained with rodamine B. The clonal type was determined by the percentage of aborted colonies (scored as in Barrandon and Green, 1987) formed by the progeny of the founding cell. When 0–5% of colonies were terminal the clone was scored as holoclone. When all colonies formed were terminal (or when no colonies formed), the clone was classified as paraclone. When > 5% but < 100% of the colonies were terminal, the clone was classified as a meroclone (Barrandon and Green, 1987). Selected clones were serially propagated to determine the number of cell generations. The cells in the T25 were cultured for 4-7 days checking day by day under an inverted microscope that the colonies did not interact each other to promote clonal conversion. At 30 % of confluence, the colonies were detatched by trypsin and were subjected to feeder depletion step (see the text before). At this point, Keratinocytes are freezed (-80°C) and harvested for molecular characterization. The entire procedure of cloning and subcultivation was done under strict timing conditions identical for each clone.

2.4 Determination of N° of Cell Doublings (cell generations)

The number of cell generation was calculated using the following formula: x=3,322 log N/No. The number of cells (No) was the actual number of colony-forming cells plated, since they were the only cells capable of dividing. CFE data, determined separately at the time of cultures were passaged, were used for the calculation. N was the total number of cells obtained at passage.

2.5 p63-bright cell fraction quantification

Single cell suspension from each step of negative and positive selection is used to prepare cytospin methanol fixed. The frequency of p63+ cells was evaluated by computerized analysis of the inensity of staining of single cells (500 cells are counting for each sample). The estimation rate of p63-high cell was assessed by automated quantitative immunochemestry, using as a reference a limbal strain that contained know percentage (14%) of holoclones. Positive cells selected are then estimeted for thei cell diameter. Only the p63-high cells with a diameter under 13 μm are considered p63-bright cells.

2.6 Quantitative Real-Time PCR

Total cellular RNA was extracted from 2-5 x 10^5 EnSC, TAC, PMDC populations and total keratinocytes cells, using the miRNeasy Plus Mini kit (QIAGEN). Total cellular RNA was extracted from 1-3 x 10^5 Holoclone, Early and Late Meroclone progenies using Nucleo Spin miRNA (Machery Nagel GMBH & CO KG). This kit provide the possibility to isolate sample total proteins in the same extraction procedure. For all the samples, 100 ng of extracted RNA were loaded on a denaturing 1% agarose gel to check for RNA integrity. The RNA samples were used to set up the retrotranscription reaction (SuperScript III kit, Invitrogen) using Oligo dTs or random examers as primers. Samples with no RT enzyme were processed in parallel with real samples to control for residual DNA contamination. One tenth of the RT reactions were then subjected to PCR, either semi-quantitative or quantitative Real-Time PCR (qRT-PCR). QRT-PCR was performed on an ABI 7900 machine using SYBR green detection chemistry (Applied Biosystem). GAPDH primers were used as an internal control in both PCR approaches. QRT-PCR data were analysed using both 2-ΔCT and 2-ΔΔCT methods.

a) Oligo sequences for gene expression analysis

GAPDH
GTATGACTCCACTCACGGCAAA
TTCCCATTCTCGGCCTTG

ITGA6
ATTCTCATGCGAGCCTTCAT
GGAAACACAGTCACTCGAACC

p63α
GACCACCATCTATCAGATTGAGCAT
TGCTCAGGGATTTTCAGACTTG

p63-TOT
GGCTGTTCCCCTCTACTCGAA
CATGAGCTGAGCCGTGAATTC

INV
CCATCAGGAGCAAATGAAACAG
AGCTCGACAGGCACCTTCTG

KRT10
GCAAATACTACAAAACCATCGATGA
AGCAGGATGTTGGCATTATCAGT

PTGER2
GCAGTCTCCCTGCTCTTCTG
CACCGAGACAATGAGAAGCA

ING5
CATCCTGGCTGCAGAGTACA
AATCACTGCCCTCCATCTTG

2.7 Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% deoxycolate, 1% Triton X-100, 0,1% SDS, 0,2% sodium azide, pH 7,5 containing protease inhibitors) for 30' on ice, and protein amount determined by Bradford assay (Thermoscientific). Cell lysate samples (7-20 μg) were run on a 6-10% SDS-PAGE (100 V, 1-2 hrs) gel, transferred onto nitrocellulose membrane (Biorad) and immunoblotted (100 mA at 4°C, overnight) using mouse monoclonal antibody against either the p63 protein (clone 4A4, 1:200, or Santa Cruz Biotechnology), or p63α protein (custom 1:50.000, Primm) or against Involucrin protein (clone SY5, 1:500, Leica), or against Integrin-β4 protein (1:500, Santa Cruz Biotechnology), or against Keratin-10 protein (Covance), or against ING5 (1:250, AbCAM) , or against PTGER2 (1:250, AbCAM). Protein detection was carried out using a chemiluminescent labelling detection reagent (ECL, Thermoscientific).

2.8 Gene expression profiling

The expression profile of EnSC, TAC and PMDC populations and of the clone progenies was determined by microarray analysis using Affymetrix HG-U133 Plus 2.0 GeneChip arrays. RNA was isolated using miRNeasy Plus Mini kit (Quiagen) and reverse transcribed by GeneChip 3'IVT Express kit. Affymetrix HG-U133 Plus 2.0 GeneChip arrays hybridization, staining and scanning, were performed using Affymetrix standard protocols. Fluorescence signals were recorded by an Affymetrix scanner 3000 and image analysis performed with the GeneChip Operating Software (GCOS) software. All data analyses were performed in R using Bioconductor libraries and R statistical packages. Probe level signals have been converted to expression values using the robust multi-array average procedure (RMA) or Affymetrix MAS5.0 algorithm Specifically, intensity levels have been background adjusted, normalized using quantile normalization, and log2 expression values calculated using median polish summarization and the custom definition files (CDF) for Human Gene U133 Plus 2.0 arrays and the Gene Annot CDF based on Entrez (18,185 Entrez genes). (version 12; http://brainarray.mbni.med.umich.edu/Brainarray/default.asp. The 1st data set was composed of 15 samples generating 3 datasets: 5 samples defining the EnSC dataset, 5 samples defining the TAC dataset and 5 samples defining the PMDC dataset. The 2nd data set was composed of 15 samples generating 3 datasets: 5 samples defining the Holoclone dataset, 5 samples defining the Early Meroclone dataset and 5 samples defining the Late Meroclone dataset. Differentially expressed genes have been identified using Affymetrix HG-U133A probesets, normalized by ANOVA and paired T-test, we selected transcripts whose level of differential expression in the 2 datasets was statistically significant ($p < 0.01$).

2.9 Functional clustering analysis

Functional cluster analysis of genes targeted by retroviral integrations and from control sequences was performed using the DAVID 2.1 Functional Annotation Tool (http://david.abcc.ncifcrf.gov). In the DAVID annotation system, a Fisher exact test corrected for multiple comparisons (DAVID's EASE score) is adopted to measure the level of gene-enrichment in Gene Ontology (GO) annotation terms with respect to a background population, and GO categories considered over-represented when yielding an EASE score < 0.05. Genes were also analyzed by the network-based Ingenuity Pathways Analysis tool (Ingenuityn Systems, www.ingenuity.com), to search for the most relevant molecular interactions, functions and pathways linking them. Gene identifiers were uploaded into the application, and mapped to their corresponding Focus Gene in the Ingenuity Pathways Knowledge Base, a structured and context-rich knowledge base manually compiled from scientific literature. Gene networks were algorithmically generated based on the direct or indirect interaction between Focus Genes. The Functional Analysis of each network identified the biological functions and/or diseases that were most significant to the genes in the network (Fischer's exact test).

2.10 Fibrin matrix and organotypic cell culture

The fibrin sealant. The fibrin sealant (Tissucol) was purchased from Baxter-Immuno (Wien, Austria). Tissucol is composed of two frozen fibrinogen and thrombin stock solutions. The fibrin gels were prepared as follows: solution A was obtained by diluting the original thrombin stock solution to a final concentration of 3 IU/ml thrombin in 1.1% NaCl and 1 mM CaCl2. Solution B was prepared by mixing 5 ml of the original fibrinogen stock solution with 5.8 ml of a saline solution (final concentrations: 1.1% NaCl; 1 mM CaCl2). The fibrin gels were then prepared in 144-cm2 plates not treated for cell culture (Greiner, Stuttgart, Germany). To obtain a 100-μm thick fibrin gel, 3.6 ml of solution A and 3.6 ml of solution B were uniformly mixed in each plate; the plates were left at room temperature for 10-15 min (until a complete substrate polymerization was obtained) and stored overnight at 4°C.

Generation of skin equivalent. Keratinocytes were cultured on a feeder layer of irradiated 3T3 cells seeded on fibrin matrices (experimental cultured epithelia). Keratinocytes ($2x10^5$ cells) were added to a 3,8-cm^2 culture Petri dish (Greiner). Fibrinoysis of the matrix by the growing keratinocytes was inhibited by adding bovine aprotinin (150 kUI/ml, Antagosan, Hoechst, Puteaux, France) to the culture medium for the experimental grafts. Keratinocytes were then cultured to confluence. At this time, the fibrim start to be degradated and the co-colture is passaged on human derma sheet (Banca del pelle. Cesena, ITA). The culture is moved on a metal support to permit the exposure of the surface to the air. The airlift is continued for a week. After 14 days in culture, keratinocytes were able to regenerate a multilayer squamous epithelium. Skin equivalent was paraffin embedded or detached with Dispase II (Roche), rinsed several times with serum-free DMEM. Experimental cultured epithelia grown in the presence of a fibrin matrix were carried out twice times with a6-bright (EnSCs) and a6-dim cell (TACs) fractions.

2.11 Immunohistochemistry

Frozen sections of human neonatal foreskin (4 μm) were fixed with a buffered paraformaldehyde solution (3%) containing sodium cacodylate (0.1 M) and sucrose (0.1 M), for 10 min at RT. The sections were blocked with 2% Bovine serum albumin (BSA) in PBS for 30 min before incubation with α6β4 (Thermoscientific, 1:100 dilution in PBS/ 2%BSA) or p63α (Custom Rabbit Polyclonal Antibody, Prime. Dilution 1:5000) for 60 min at room temperature. The sections were washed three times with PBS containing 0.5% BSA and then incubated with secondary anti-mouse Alexa-488 or Alexa-568 antibody (Invitrogen) at 1:2000 dilution for 1 h at RT. Sections were washed as above and then counter-stained with 1:40.0000 of DAPI solution (Dako) per ml for 5 min.

2.12 Fluorescence-activated cell sorting (FACS) staining and assay procedure

Keratinocytes were processed for single (α6-FITC) and double (α6-FITC/ Feeder-PE) staining along with the appropriate negative controls and single-color positive controls to establish compensation settings on the flow cytometer. Primary antibody: α6-FITC (clone 4F10, Millipore), anti-feeder-PE (Miltenyi Biotec.) Cells were incubated first with the primary antibodies and then with secondary antibodies (DAKO) for 30 min each at +4°C. Between each antibody layer, cells were washed twice with DMEM containing 5% fetal bovine serum. At the end of the staining procedure, cells were resuspended at 2–3 × 10^5 cells per ml, and analazyed using FACStarPlus (Becton Dickinson). For flow cytometric analyses a minimum of 10,000 cells per experiment were analyzed after gating, to ensure statistical significance.

3. RESULTS

3.1 CORRELATION BETWEEN p63-BRIGHT CELL FRACTION AND HOLOCLONES (KERATINOCYTE STEM CELLS) IN EPIDERMAL CELL CULTURE.

In 2001, Pellegrini and collaborators identified p63 as new putative limbal keratinocyte stem cell marker. This is provided by the correlation between the level of p63 and the percentage of holoclone in the cell culture. Subsequently, the same authors with different collaborators, showed that the isoform DNp63a has a main role in controlling limbal regeneration, the ocular surface stem cell niche, and that the indicated isoform is absent in the central cornea. Basal layer progenitor cells, consisting in KSCs and TACs, are positive for p63 but only KSC presents the highest DNp63a expression value. This knowledge and the notion about stem cell size led an approved method to measure stem cell (p63-bright cell fraction) in limbal culture. To shed light on the same role of p63 in defining epidermal stem cells in the skin, we performed the clonal analysis after estimation of the p63-bright cell fraction. First of all, we defined the localization of p63a protein in skin epithelial tissue. As expected, p63a expression level is not exclusive of basal layer. In addition, there are different levels of positivity to the antigen (Fig. 10), a high and a low protein state (Fig. 10 magnification).

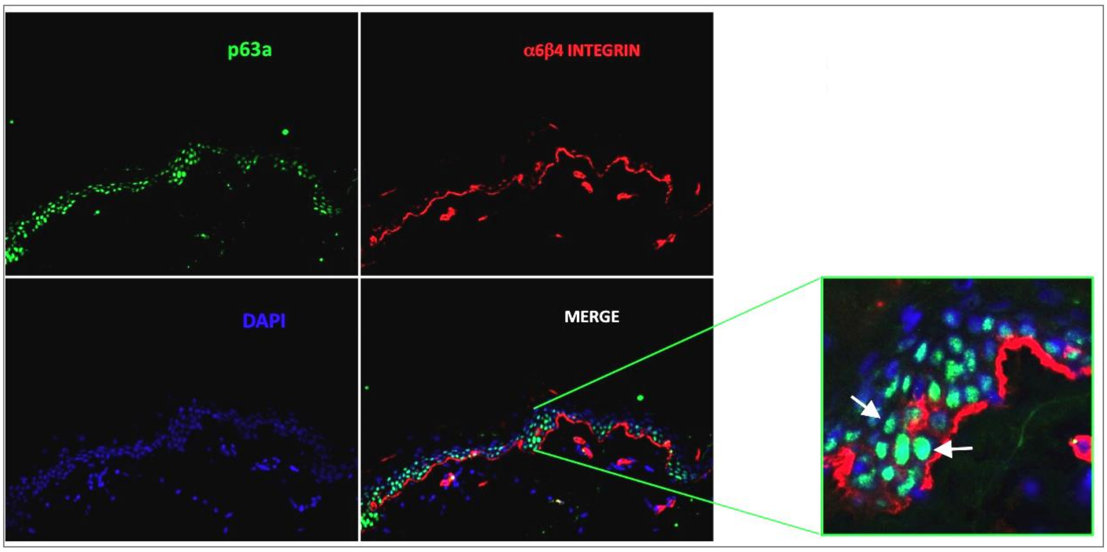


FIG. 10. Assignment of p63a isoform localization in epidermal tissue. A paraffin-embedded resting skin. p63a isoform is expressed in different layers of epidermis. In particular, we showed the antigen a high expression level in the basal layer and a faint detection in the suprabasal layer. Even though p63a antibody potentially detects both DN and TA isoforms, there is no evidence of TAp63a expression in the resting skin. Therefore this assay, permit to identify different expression levels of the same isoform, DNp63a, as shown by white arrows in the figure enlargement.

Keratinocyte clonal analysis data collected in the past years allow us to define a threshold for the minimum expression level of p63a isoform in epithelial tissue. Cells that show positivity upper this threshold are named p63-high cells (Fig 11). Referring to the finding that the highest clonogenicity and growth potential is linked to the cell size included between 12 and 13 um, we define a scale range for the cells that are p63-bright cell (Fig 11). A p63-bright-cell surely is p63-high-cell as well, but not vice versa (formula).

Formula: ***p63-bright*** ***p63-high***

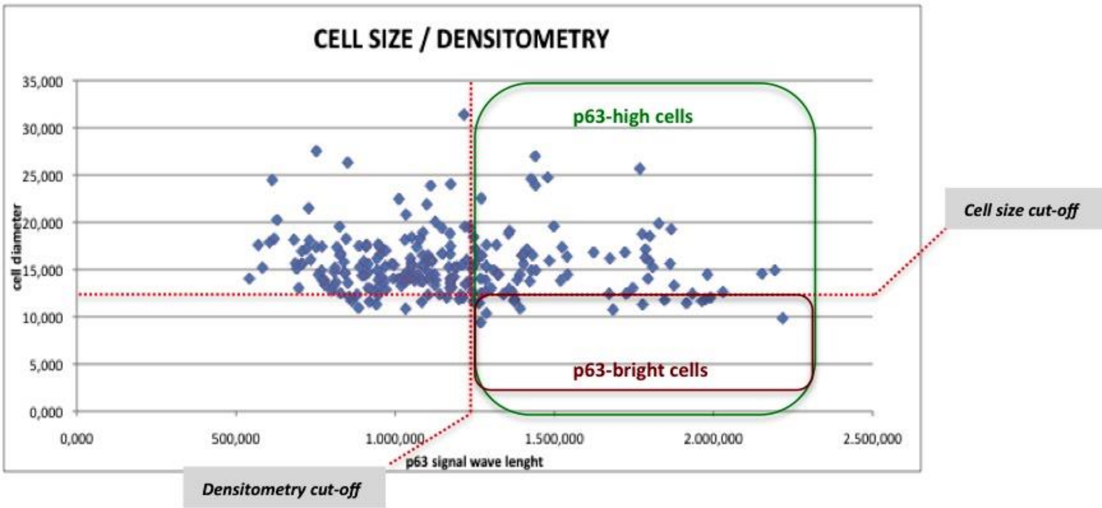


FIG. 11. Plotting values to define the p63-bright cell in epidermal cell culture. Estimation of cut-off values for defining of p63-high and p63-bright cells was experimentally determined. This notion elucidates the possibility that not all the p63a positive cells are stem cells (formula). Indeed, only some of them are p63-bright, putative stem cells in epidermal cell culture. Red-axes represent experimental cut-off values.

The direct estimation of the amount of stem cells in epidermal cell culture was obtained by clonal analysis of subconfluent primary keratinocytes.

Human keratinocytes plated onto a 3T3 feeder layer form colonies, each colony being the progeny of a single cell . This allows the precise evaluation of the basal keratinocyte clonogenic ability (CFE), which can be defined as the percentage of cells able to generate colonies. The clonogenic ability and the growth potential of basal keratinocytes are, however, two very different concepts. The former indicates the capacity of a basal cell to found a colony, the latter deals with its capacity of producing cell generations, hence it deals with its self-renewal potential (see also in the introduction above). Aborted colonies, for example, are generated by cells with very limited growth potential (terminal cells), and their relative percentage is inversely related to the overall residual growth potential of a keratinocyte strain.

The percentage of abortives and the rate of clonogenicity define three clonal types: holoclone, meroclone and paraclone.

Primary cell culture keratinocytes plated at clonal density can give rise to these clonal types. We performed 3 clonal analysis from different donors using limiting dilution assay and obtain holoclone range between 8 and 15 %. Here we report the case in which we obtain the highest holoclone value (Fig. 12, see Materials and Methods).

A **B**

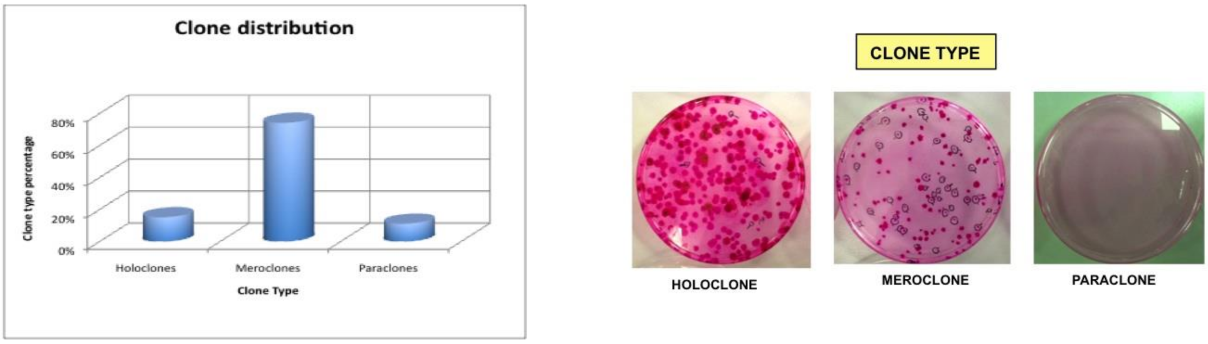


FIG. 12. Clone types distribution in primary epidermal cell culture. A) Basal layer keratinocyte plated at clonal density on 3T3 feeder layer give raises different clonal types: holoclone, merclone and paraclone. B) These clonal types are different for clonogenicity (as shown in the CFE) and growth potential. Holoclones are widely considered KSCs.

According to the data that fulfill the criteria for holoclones arising from stem cells, we identified a correlation between the number of holoclones (KSCs) and p63-bright cell percentage . Starting from the same single cell suspension used in the previous clonal analysis, we carried out immunostaining detection of p63a on cytospin methanol fixed. Bidimensional assay allow us to define p63-bright cell fraction in a value equal to 9.2% (Tab. 2, see Materials and Methods).

Holoclones	Meroclones	Paraclones	p63-Bright cell %
15%	74%	11%	9.2%

TAB. 2. Holoclones (KSCs) can be prospectively identified using p63-bright assay. Primary epidermal cell culture was processed using standard protocol (see above) until to obtain a single cell suspension. This sample was then used to measure the p63-bright cell fraction by bidimensional assay and the number of holoclone by clonal analysis. We obtain an evidence of a correlation between the number of holoclone and the percentage of p63-bright cells.

Taken together, these data demonstrate that p63-bright assay represents a suitable method to estimate the number of human keratinocyte stem cells. This evidence, could question about the quality of an epidermal cell culture destined to autologous transplantation in full-thickness wounds or, in this thesis, can validate the experimental sample destined to comparison.

3.2 PURE-POPULATIONS OF KERATINOCYTE PROGENITORS FROM EPIDERMAL CELL CULTURE

The first part of the project is designed to define the genes involved in the maintenance of stemness and early-commitment genes during differentiation of human epithelial progenitor cells, through the use of high-throughput screening assay. Epidermal stem cells, hence referred as keratinocyte stem cells (KSCs), represent only 3-10% of the whole pool of epidermal keratinocytes. A number of molecular markers have been described to be preferentially expressed by putative KSCs; the differential expression of these molecules, anyway, does not represent a distinctive feature of stem cells. Therefore, selecting for these surface markers does not allow to isolate KSCs but, more simply, to enrich for the fraction of cells with highest proliferative potential within a mixed population. Human keratinocytes are normally cultivated onto a derma-like substrate, like a murine fibroblast feeder layer, in order to preserve their long-term culture ability, their physiologic morphology and functions. Although keratinocytes can be easily detached from the underlying murine feeder layer and isolated for genomic DNA extraction, the purification procedure cannot completely avoid a relevant contamination from murine cells.

Here we show a novel approach based on α6-integrin (ITGA6) expression using magnetic micorbeads. The method, named *"two-step separation"*, primarily require a single cell suspension to deplete the murine feeder from mix cell population and then a second step guarantee the recovery of 2 side cell populations: a6-integrin-bright and a6-integrin-dim cells, with high and low affinity for the antigen respectively (Fig. 13).

a) Colony-forming efficiency (CFE)

The colony-forming ability of the starting cell MASS, ITGA6 positive selected and ITGA6 unselected cells was determined after 12 day in culture, in five separate experiments. Here we report a representative case: the results showed that ITGA6 positive cells consistently gave rise to higher colony numbers than ITGA6 un-selected fraction (52.4% col. and 7.6% Ab. vs. 25% col. and 9% Ab respectively) and than the MASS cell population (52.4% col. and 7.6%

Ab vs. 22.8% col and 12.3% Ab respectively) (Fig. 13). These results suggest that the majority of proliferating cells were in the ITGA6 positive cell fraction (Fig. 13 A). Indeed, this positive cell fraction consistently gave rise to higher colony numbers than the other fractions, indicating that ITGA6 was enriched 2.3 fold in colony-forming cells compared to the MASS cell population.

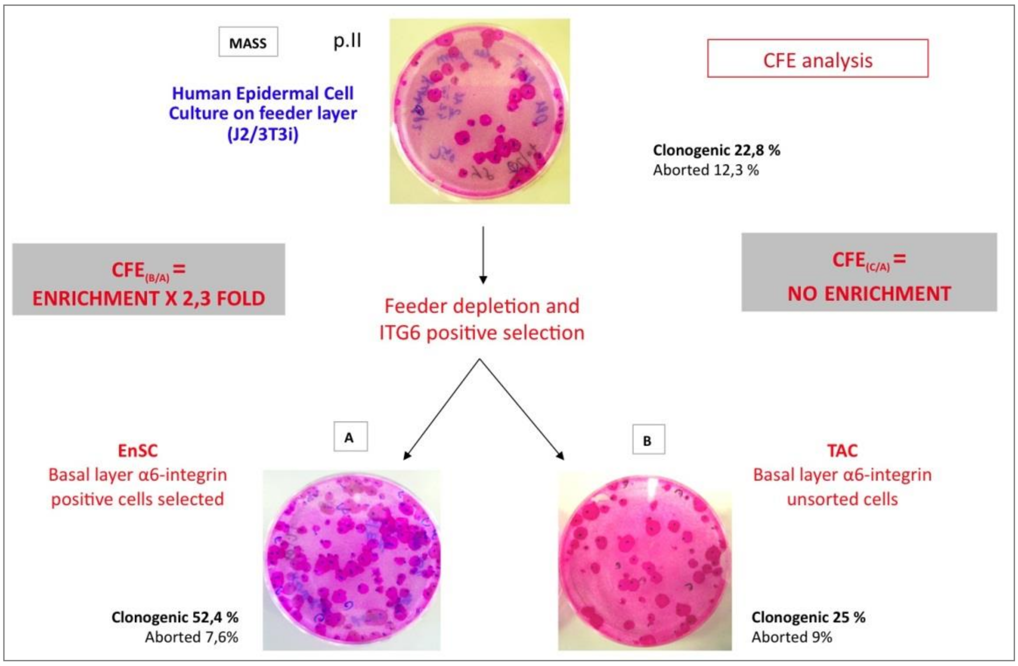


FIG. 13 . CFE after Two-step separation method based on α6-integrin magnetic sorting. A single cell suspension from the secondary epidermal culture is magnetically depleted form feeder contaminant and sorted on the basis of a6-integrin expression level. Here we report a representative sorting experiment of five used donors. A) positive cell fraction showed 2.3 fold increase of clonogenicity compared to the MASS. B) In the negative cell fraction the colony rate is comparable to the starting cell mass population.

b) p63-bright percentage in fractioned cell populations

Following magnetic separation based on α6β4 integrin expression and feeder depletion, the three cell population MASS, ITGA6-pos and ITGA6-neg were analyzed for their p63α expression level and cell size using cell-analysis software. In the case donor report (Fig 14), the percentage of stem cell measured by p63-bright cell is 2.3 fold increased in ITA6-pos cells if compared with starting cell mass. On other hand, ITGA6-neg cells had very low level of p63-bright cells indicating a depletion of stem cell in this cell fraction (Fig. 14). These results are correlated with colony-forming ability, indeed the cells showing an increase in colony numbers together with stem cell amount.

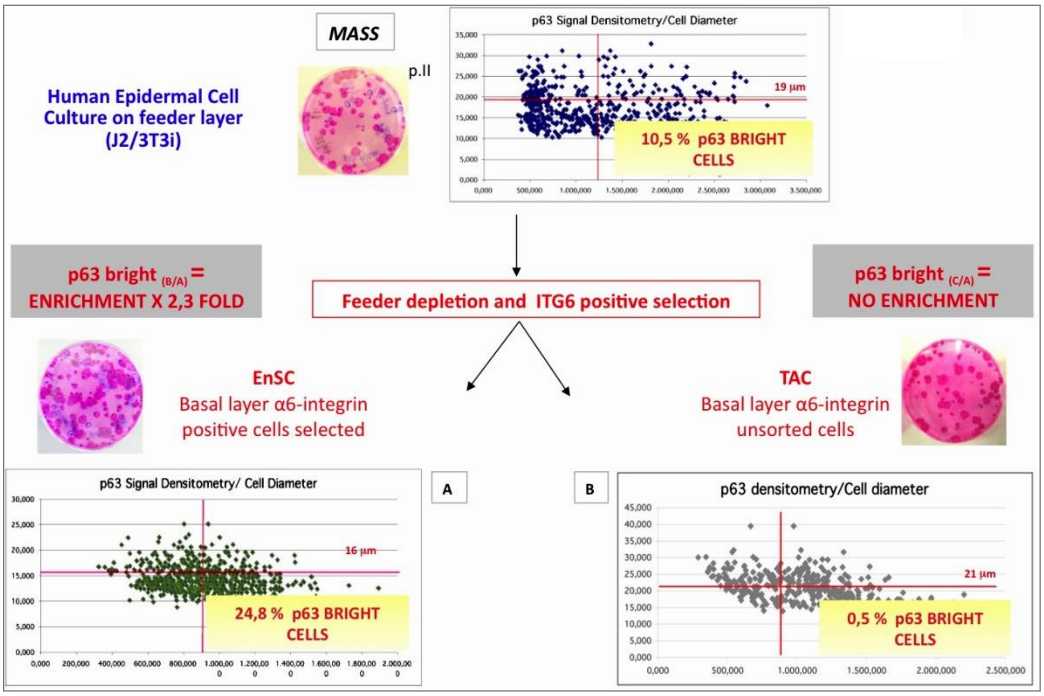
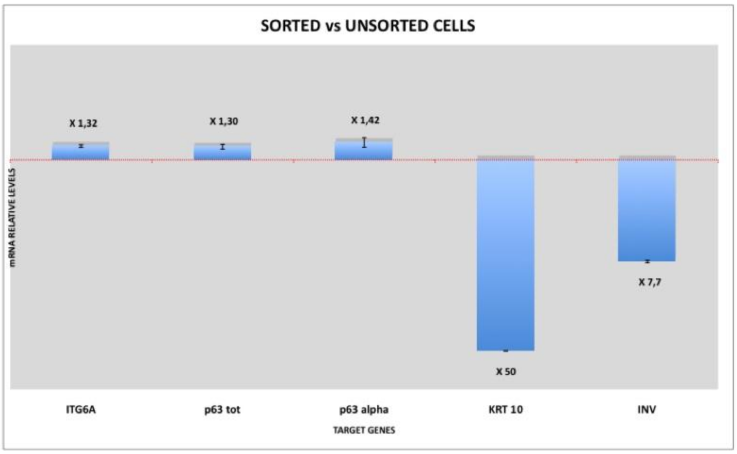


FIG. 14 . p63-bright assay after two-step separation method based on α6-integrin magnetic sorting. A single cell suspension from the secondary epidermal culture is magnetically depleted form feeder contaminant and sorted on the basis of a6-integrin expression level. A) positive cell fraction showed 2.3 fold increase of p63-bright cell amount compared to the MASS. B) In the negative cell fraction the p63-bright-cells reserve is almost empty. Red-axes represent cut-off mean values.

c) Expression of stem cell and differentiation markers

Following each magnetic separation, we examined, by immunostaining, the expression of keratinocyte stem cell markers p63 and α6β4, and of keratinocyte differentiation markers involucrin and keratin 10. Analysis of protein expression through SDS-PAGE demonstrated an increase of both p63 tot and p63a expression levels in the positive selected fraction compared to negative cells, while an inverse tendency is seen when looking at the early differentiation marker involucrin (Fig 15). The results were confirmed by qRT-PCR, which showed a 1,3 -fold increase of the two isoforms of p63 and of α6-integrin in the ITGA6 fraction compared to the unsorted cell fraction; conversely, the two differentiation markers keratin 10 and involucrin were visibly enriched in the ITGA6-neg fraction and strikingly under-represented in the EnSC fraction (Fig 15). As ITGA6 expressed all the investigated marker genes in the expected manner, and also exhibited a higher clonogenic potential compared with corresponding ITGA6-neg and MASS populations, I conclude that the proposed separation procedure was successful, allowing us to enrich for KSCs (EnSC).

A



B

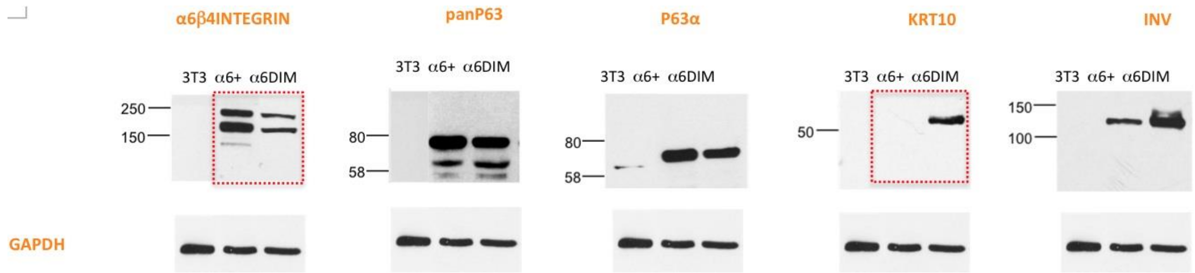


Fig 15. Two-step separation method allows to obtain enrichment of keratinocyte stem cells (EnSCs). The enrichment in stem cells of the ITG6-positive cell population was assessed also by transcript and protein expression through qRT-PCR and SDS-PAGE, respectively. **A)** qRT-PCR data were analysed by the $2^{-\Delta\Delta CT}$ method, using starting cell mass as referring sample. **B)** Cell lysate samples (20µg) were run on 4-8% SDS-PAGE gel and transferred onto nitrocellulose membrane. 3T3 cell lysate was used as negative control. Total proteins were normalized using GAPDH antibody. Antibodies used for immunoblotting are shown at the top of the panel. The migration positions of molecular mass markers are shown on the left.

d) EnSCs cell cycle analysis

Immediately after ITGA6 magnetic separation, the positive fraction contained more resting cells (G0/G1 phase) than the MASS cell population (64.5 ± 1.1 vs. 55.6 ± 0.6 respectively). Also, the percentage of actively cycling cells was significantly lower in ITGA6-pos cell fraction than in the negative fraction (for G2/M phase: 3.3 ± 1.4 vs. 10.3 ± 0.5, respectively (Fig. 16). These results are in agreement with previous findings relative to the presence of KSC in the positive fractioned cells.

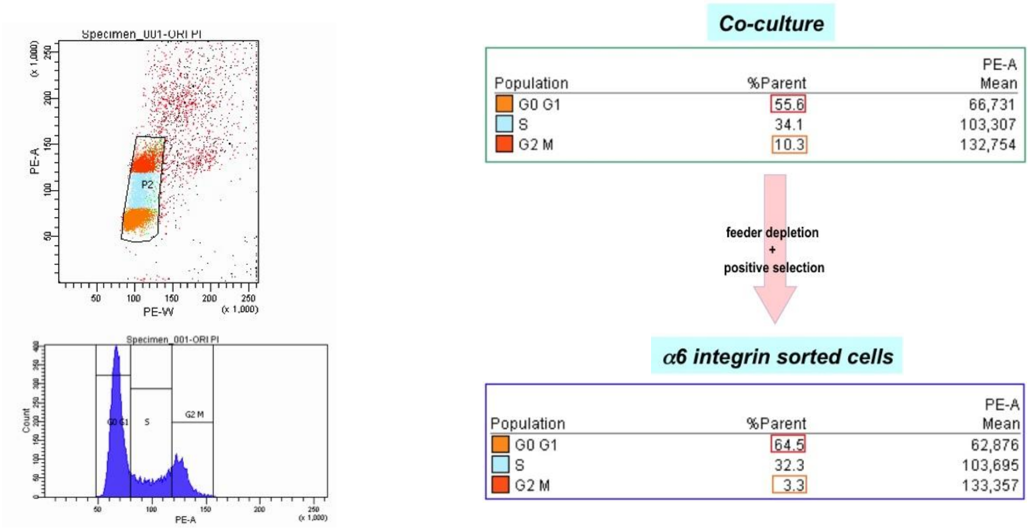


Fig 16. Two-step separation method allows to obtain enrichment of keratinocyte quiescent cells (EnSCs). The enrichment in stem cells of the ITG6-positive cell population was assessed also by cell cycle analysis. It is shown an increase of cells in Go/G1 phase after cell sorting based on (keratin 10 and involucrin, K10 and IVL). The difference in marker expression was assessed in three different donors.

e) Tissue reconstitution ability of fractionated cells

Progenitor cells should be able to regenerate the origin tissue form which they are sorted. Hence we provided a well approved method to produce graft starting from a single cell suspension (see material and methods). Given that tissue regeneration ability from small numbers of cells is considered to be a hallmark of KSCs, we titrated the skin reconstitution activity (Fig.17). Both ITG6-positive and negative cell populations were feeded in the same conditions. Using allogenic cadaveric derma, 3T3-feeder layer and fibrin matrix we tested the capability for each cell fraction to give rise a well stratified epithelium. In each case we provided an evidence of the ability to regenerate epidermis when compared to a normal tissue cryosection. In particular, we performed staining of a6-integrin and p63a (proliferative compartment and growth potential markers, respectively) in all the report cases and we observed a basal positive signal, confirming that both EnSC and TACs can be ideal progenitor to obtain a multilayer epidermis (Fig. 17).

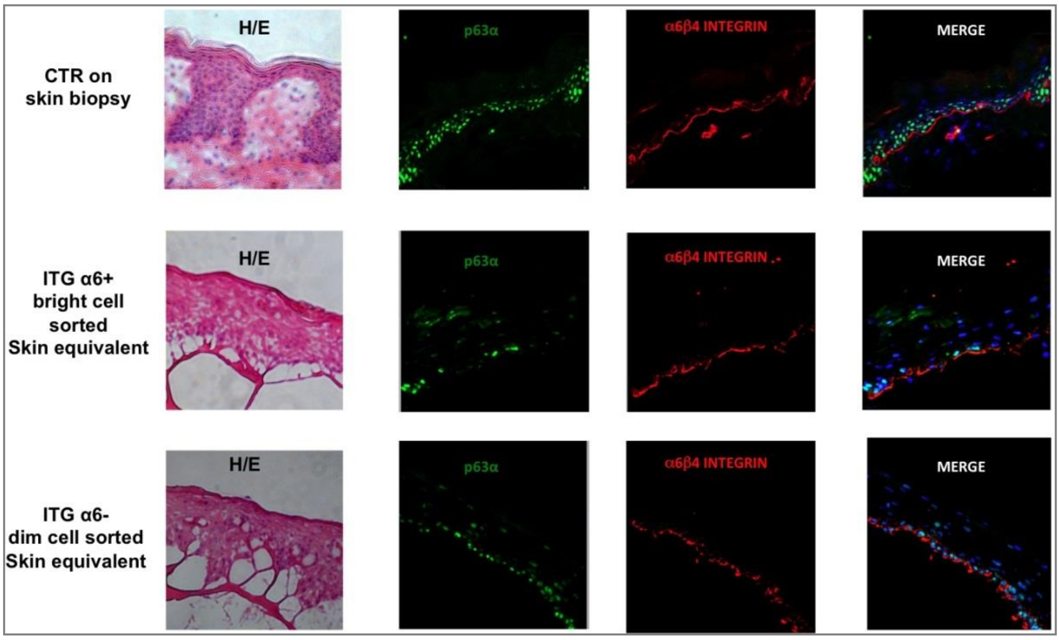


Fig 17. Tissue reconstitution ability. Both ITG6-positive and negative cell populations were feeded in the same conditions. Using the allogenic cadaveric derma, 3T3-feeder layer and fibrin matrix we tested the capability for each fraction to give rise a well stratified epithelium. Staining of a6-integrin and p63a (proliferative compartment and growth potential markers, respectively) were performed on normal and regenerated tissue cryosections.

f) Only EnSC population is endowed of holoclone

Although is clear that both EnSCs and TACs could be used in particular culture conditions to generate an epithelial graft, we wondered if both cell types possess long term ability to restore epithelium. An in vitro formal evidence to sorting out this goal consists in the demonstration of the presence of holoclone (KSCs) by clonal analysis. Therefore, after a limiting dilution for each cell fraction we are in the condition to detect different progenitor types. These clones are named after the CFE validation on the basis of the clone dimension (data unshown) and the presence of abortives (Fig. 18, CFE on the left). As we shown in the imagine (Fig.18 A-B), only the ITGA6 positive cell fraction can give rise holoclone, that is KSC. On other hand, ITGA6-dim is characterized mainly by meroclone types, which are TACs. Using p63-bright cell percentage measure we predicted the presence or the absence of KSCs in the sorted cell populations. Only EnSC population is endowed of holoclones. According to the restriction of KSCs to the basal compartment, this experiment is a validation of basal compartment cell enrichment (EnSC).

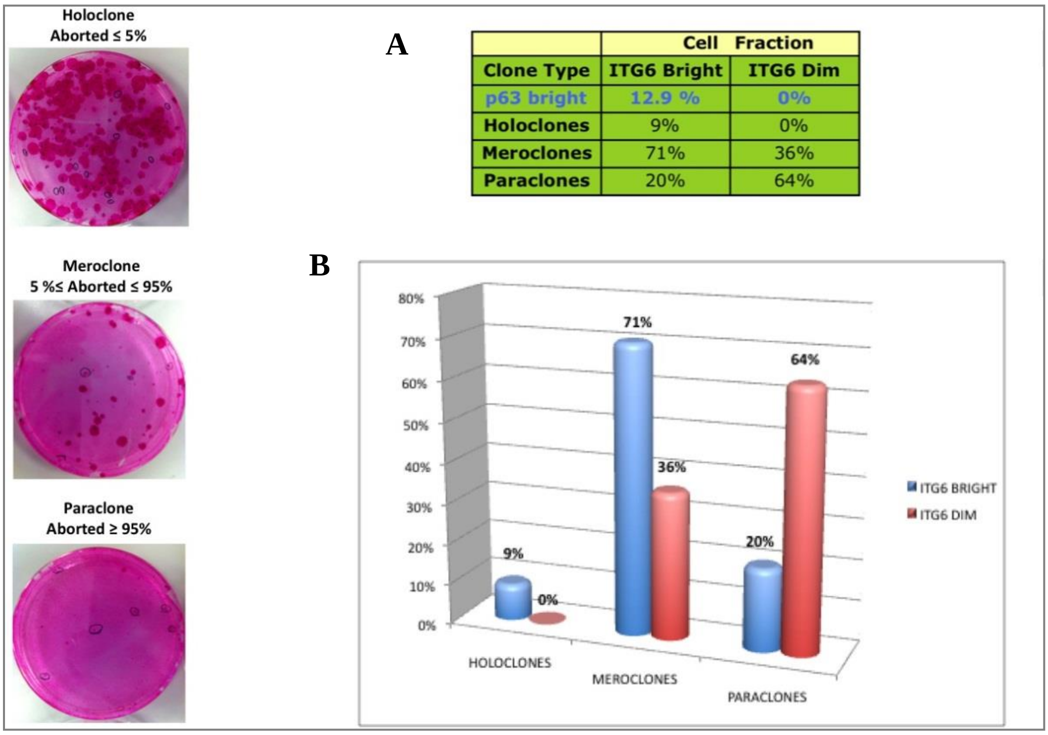


Fig 18. Basal compartment enrichment validation by clonal analysis. A) ITGA6-positive and negative cell populations single cell suspensions were feeded in the same conditions and used for separate limiting dilutions. As predicted by the p63-bright cell percentage values, we found holoclone only the positive ITGA6 cell sorted. **B)** Number clone types (shown on the left) were reported for each clonal analysis.

Altogether these data clearly supported the existence of different cell populations during in vitro expansion of epidermal progenitor cells, and the utility of p63-bidimensional assay to discriminate between human stem/progenitor cells with different regenerative potency (Fig 13 A-B). The comparison between EnSC and TAC is an introductive step that identifies them as ideal samples for Microarray assay.

3.3 RISING TO THE DIFFERENTIATION STATE FROM EPIDERMAL CELL CULTURE

After the hard task to define two-step separation cell sorting conditions, we were able to obtain two candidate cell populations, EnSC and TACs. Having these cells the ability to generate the epithelium, we considered these fractioned cell as progenitor cells. Therefore we have to obtain a third kind of cell population for the massive experiment to investigate stem maintenance and commitment stage signatures in epidermal development.

For the preparation of differentiated keratinocytes for Microarray assay, keratinocytes were cultured for seven passages (P7) on feeder layer at low density (700 Keratinocytes/cm^2, that is 1/10 of normal secondary culture density) supplemented with appropriate hormones as indicated in Material and Methods. The clonogenic ability of the culture was assessed through Colony-Forming Efficiency (CFE) assay, p63-bright fraction monitoring and total cell doublings counting at every single passage (Fig. 19 -20). The clonogenic ability of the keratinocyte culture diminished at P7, where only 2 % of cells were able to form productive colonies (CFE% = 2) and the majority of them gave rise to abortive colonies (Ab% = 55).

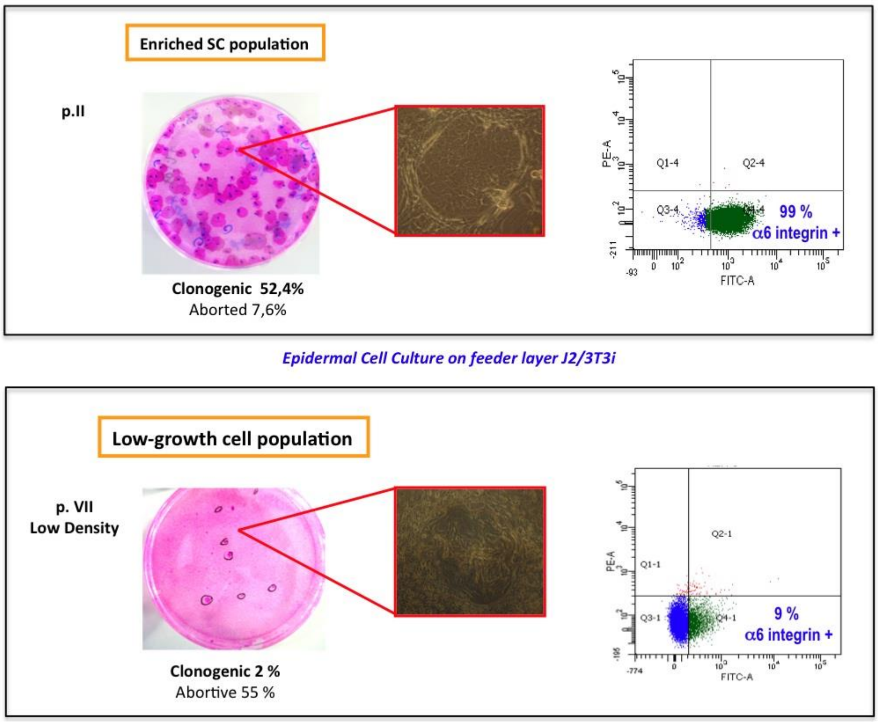


Fig 19. *In vitro* low-growth cell population. CFE and FACS assays determined a decrease for the colonogenic ability and basal compartment a6-intergrin marker expression level in the low-growth cells. EnSC and Differentiated cells showed morphological differences as well.

When expanded *in vitro*, keratinocyte progenitor cells use up clonogenic and growth potential to create daughter cell generation. Total number of cell doublings can represent a KSC cutoff measure during life-span. In this way, coupling p63-bright bidimensional assay to the cell doubling counting, we defined doublings threshould value. Over this value KSC pool is completely depleted (Fig. 20).

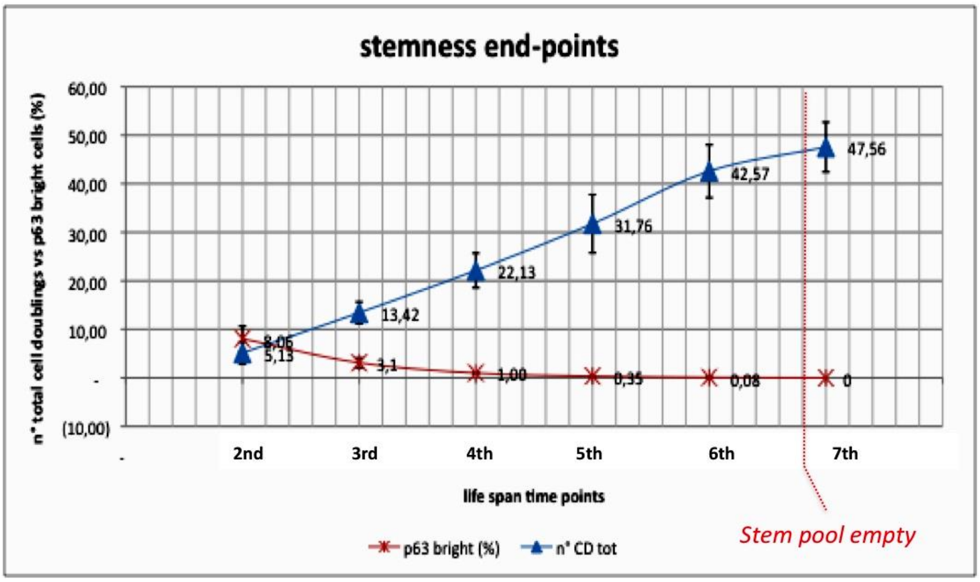


Fig 20. ***Stemness drop during human epidermal cell culture .*** p63-bright percentage measure confrmed a complete stemness reserve depletion at 7th life-span cell culture passage. This happened after 42.57 ± 5 total cell doublings. Graph is realized monitoring 5 different epidermal cell cultures. Mean values are shown.

Starting from the same five donors cited above, we provided an evidence of long term keratinocytes capability to generate up to 48 cell generations before complete depletion of the stemness reserve. Over this value, cell culture is sustained only by TACs and their daughter cells, that are post-mitotic cells (PMDCs, Fig. 20). In vitro, this type of cells are comparable to abortives colonies with a very irregular colony shape, containing involucrin positive cells and low level of growth potential markers. To confirm this data we carried out experimental procedure for understanding the level of differentiation state when compared to EnSC, a cell population sustained by KSC. Analysis of protein expression through SDS-PAGE demonstrated the highest p63 expression levels in the EnSC fraction when compared to differentiated cells, while an inverse tendency is seen when looking at the early differentiation marker involucrin (Fig 21). The results were confirmed by qRT-PCR, which showed an increase of p63 alpha isoform and of α6-integrin in the EnSC fraction compared to the differentaited cell fraction; conversely, the two differentiation markers keratin 10 and involucrin were visibly enriched in the low-density growth cell fraction and strikingly under-represented in the ITG6-bright cell fraction (Fig 21).

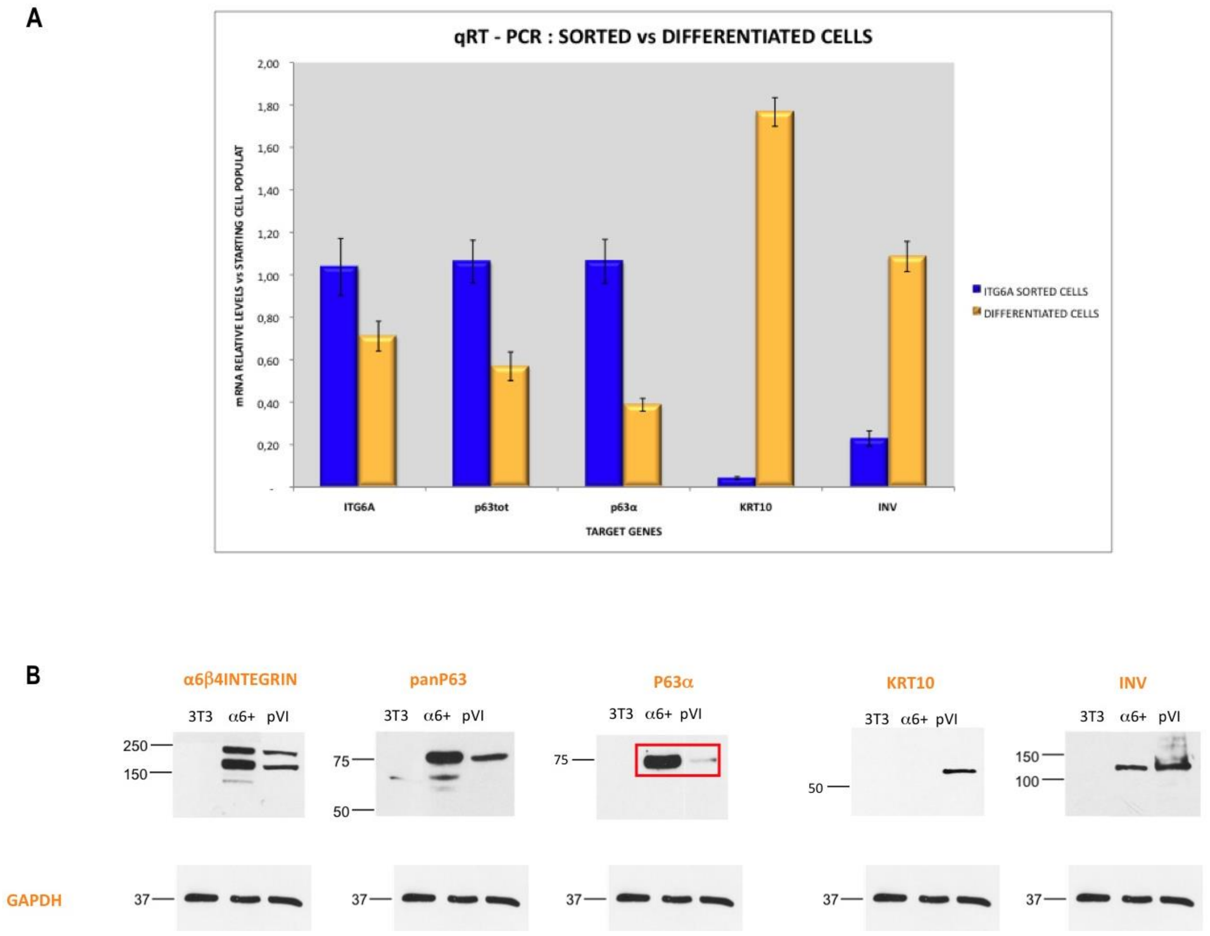


Fig 21. ***Differentaition state comparison between EnSCs and PMDC:*** **A)** qRT-PCR data were analysed by the $2^{-\Delta CT}$ method. **B)** Cell lysate samples (20µg) were run on 4-8% SDS-PAGE gel and transferred onto nitrocellulose membrane. 3T3 cell lysate was used as negative control. Total proteins were normalized using GAPDH antibody. Antibodies used for immunoblotting are shown at the top of the panel. The migration positions of molecular mass markers are shown on the left.

In this manner, experimental procedure provided a third cell population: Post-mitotic differentiated cells (PMDCs). After that a feeder layer deepletion is needed to carry out high throughput experiment in order to compare this kind of cell to other fractioned cell populations dscribed before. Indeed, to perform microarray analysis we collected 3 cell populations from five donors: EnSC showed high clonogenigity and is sustained by holoclone cell type, TAC population composed from committed cell expressing differentiation marker but with a discrete clonogenicity without stem cells and PMDC population with reduced growth potential and clonogenicity and ,overall, without stem cells (Tab.3).

Cell Culture Stain	Cell Fraction	p63 bright %	CFE %
K_1	ITG6A Brtght	24	52.4
	ITG6A Dim	0.5	25
	Differentiated	0	2
K_2	ITG6A Brtght	11,2	25
	ITG6A Dim	0	6,5
	Differentiated	0	1,6
K_3	ITG6A Brtght	11.5	67.5
	ITG6A Dim	2	36
	Differentiated	0	0,1
K_4	ITG6A Brtght	14.3	45
	ITG6A Dim	0	27
	Differentiated	0	1
K_5	ITG6A Brtght	12	56
	ITG6A Dim	2	23
	Differentiated	0	0,2

Tab. 3 Experimantal overview of the micorarray samples obtained from five donors. For each epidermal cell culture p63-bright cell fraction (linked to the presence of KSCs) and clonogenicity are shown.

3.4 TRANSCRIPTIONAL PROFILING OF PROGENITOR AND DIFFERENTIATED KERATINOCYTES THROUGH GENE EXPRESSION MICROARRAYS

In an effort to shed light on regulatory gene network that control the epithelial homeostasis, the transcriptome of farctioned cells must be characterized through the description of the repertoire of actually transcribed genes. Expression profiling by microarrays has been used so far as a very successful tool for the retrieval of important information about how the transcriptome is deployed in different cell types and how it varies during differentiation. We undertook global gene expression profiling of fractioned ITGA6 positive (EnSC), ITGA6-dim (TACs) and differentiated (PMDCs) populations, in order to identify genes enriched in each individual population and firmly establish their distinct identity. Thus, microarray analysis was performed on mRNA isolated from freshly cell fractioned keratinocytes and PMD cells using the Affymetrix Human Genome U133 Plus 2.0 array, containing 54675 probesets that indicate the expression of more than 18400 transcripts and variants. A microarray experiment produces observations of differential expression for thousands of genes across multiple conditions. It is often not clear whether a set of experiments are measuring fundamentally different gene expression states or are measuring similar states created through different mechanisms. It is useful, therefore, to define a core set of independent features for the expression states that allow them to be compared directly. Principal components analysis (PCA) is a statistical technique for determining the key variables in a multidimensional data set that explain the differences in the observations, and can be used to simplify the analysis and visualization of multidimensional data sets. We show that application of PCA to expression data (where the experimental conditions are different, and the gene expression measurements are the observations) allows us to summarize the ways in which gene responses vary under a determined culture conditions (Fig. 22).

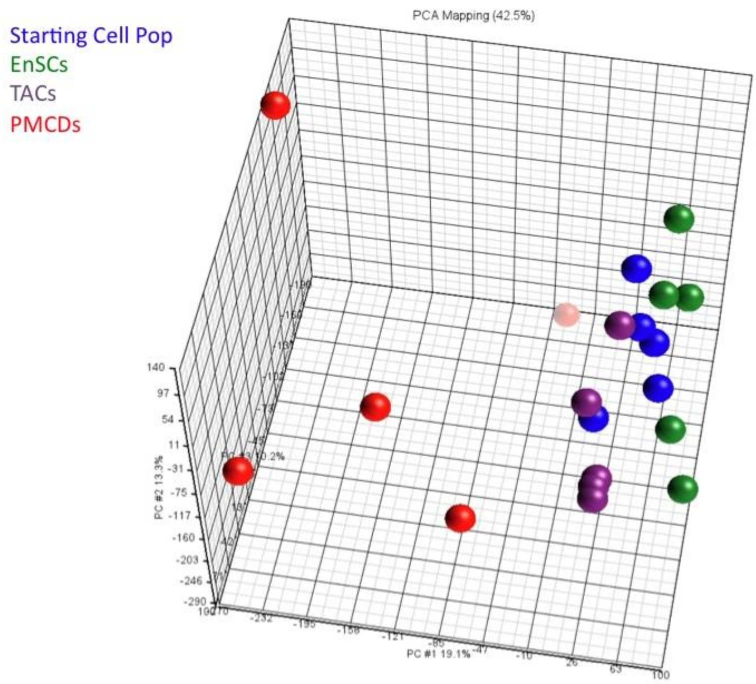


FIg. 22 Sample biological features in Principal Component Analysis (PCA). Unsupervised analysis of samples were analyzed for their biological status.

Unsupervised analysis of samples were analyzed for their biological status in the Principal Component Analysis (PCA). EnSCs and TACs are close to the MASS, indicating gene signatures overlap. On other hand, the first samples are far in the graph from the PMDCs. These seemed to be very different when compared to the other samples (Fig. 22). Probeset level data were generated and normalized using two different statistical algorithms, ANOVA and paired T-test, using Affymetrix HG-U133A probesets. Unsupervised hierarchical clustering of the quintuplicate arrays of each cell populations suggested that the three cell populations were indeed quite different from the gene expression point of view, since all the samples were correctly clustered in their own group (Fig. 23-A).

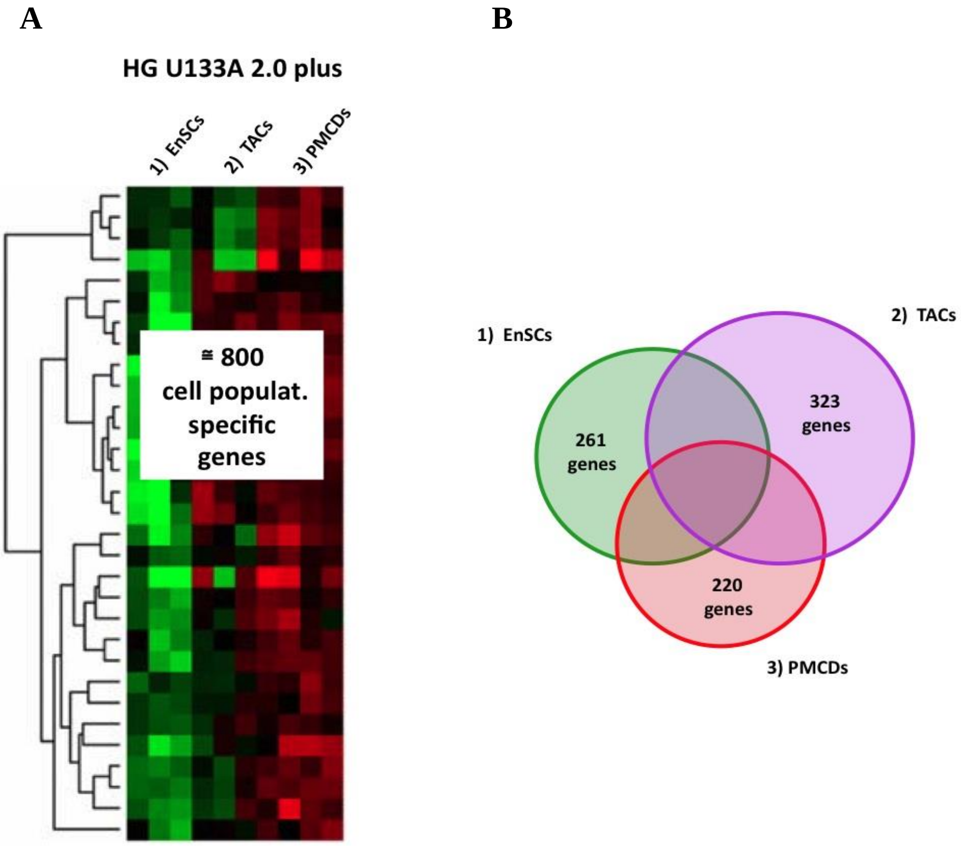


FIG. 23 Unsupervised and supervised analysis of the transcriptional profiles of Ensc versus TAC and versus PMDC populations. (A) Unsupervised hierarchical clustering analysis performed on arrays of EnSC, TAC, PMDC populations. Red patches represent gene up-regulated, while green patches represent genes down-regulated in one population or the other. (B) Supervised lists of differentially expressed genes (DEG) shows in the Venn diagram how many genes are esclusively expressed for each cell population. The total amount of DEG is equal to 800 genes about.

The supervised analysis was performed by a pair-wise comparison of two groups of arrays (1) EnSCs *versus* TACs and (2) EnSC *versus* PMDCs, estimating the number of differentially-expressed genes (DEGs) as those whose difference in log_2-based expression values exceeded a custom threshold of expression fold-change; the software calculates the absolute difference between the two group means and test whether the mean difference is equal to zero by the unpaired t-test, with a p-value threshold set to p< 0,01. Since tens of thousands of genes are compared, many genes can be false positives; False Discovery Rate (FDR) for multiple-comparison adjusted p-values, which can be estimated by performing random permutations of group labels. We performed the supervised analysis based on different criteria, in order to obtain a list of DEGs in a more/less stringent way; arrays were analysed with Affymetrix, using crescent fold-change thresholds (-2 > FC > 2) and stringent p-value thresholds, retaining only DEG lists with FDR < 10%. The striking huge number of DEGs obtained even at higher fold changes and stringent p-values confirmed what already assessed by the unsupervised clustering. We were able to find out 804 DEGs out of 54675 Affymetrix probesets, with a partition for each cell population indicated by the Venn diagram (Fig. 23-B). By looking at the list of genes found to determine each of the phenotype analysed, we could notice the presence of genes clearly associated to progenitor/differentiated states. In the PMDC up-regulated gene list we found genes linked to the differentiation signature, such as members of SPRR, S100 and SLC families, structural proteins of the stratum corneum such as FLG, DSC1 and DSG3, early differentiation markers as INV and LOR and members of differentiation-inducing signalling pathways, such as Notch and Myc ones. On the contrary, genes found up-regulated in the EnSC population were mostly progenitor-related markers, such as KRT14, TP63, ITGB1, ITGA6, MCSP-genes, as well as many genes involved in cell cycle regulation (cyclins), adhesion structure formation (among others, integrins) and negative regulators of differentiation pathways (DLL-1 and MAML2 as negative regulators of Notch pathway) or positive regulators of proliferation-dependent pathways (such as insulin, FGF, Jak/Stat pathways). In the Fig. 24, I reported two example grids about the partition of proliferation and differentiation gene pathways (Fig. 24).

A ***(NOTCH, TP63)*** **B** ***(SLC family)***

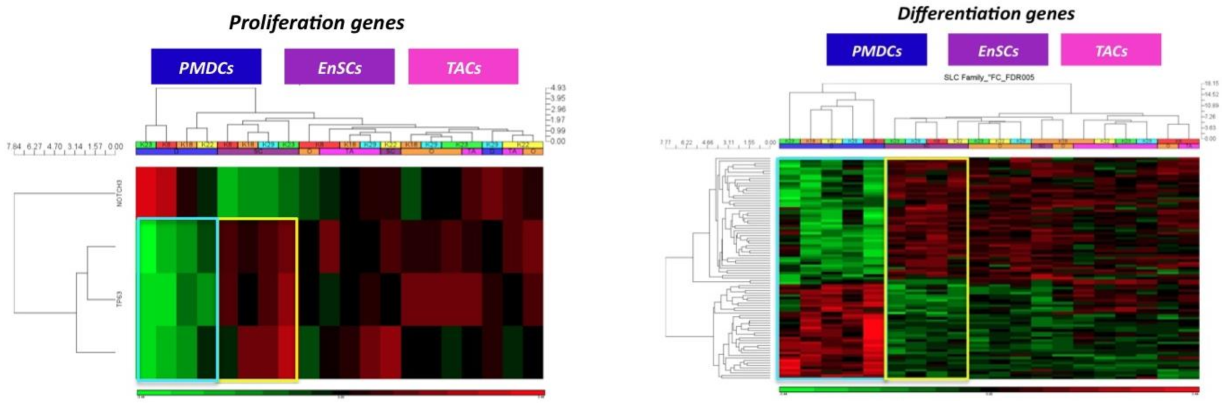


FIg. 24 Analysis of the transcriptional profiles are in agreement with cell hierarchy. Supervised hierarchical clustering analysis performed on arrays of EnSC, TAC, PMDC populations. Red patches represent NOTCH,TP63 (A) and SLC (B) gene up-regulated, while green patches represent the same genes down-regulated in one population or the other. As expected proliferation and differentiation gene marker were ascribed to Progenitor cells and PMDCs respectively.

3.5 GENES FOUND UP-REGULATED IN PROGENITOR AND DIFFERENTIATED KERATINOCYTES BELONGS TO SPECIFIC FUNCTIONAL CATEGORIES

To better define the differences in the transcriptional profiling of EnSCs, TACs and PMDCs and to determine which set of genes defines one's phenotype, we performed further analysis of gene enrichment and Gene Ontology functional annotation.

Using network-based Ingenuity Pathways Analysis (IPA) tool, the list of genes up-regulated in one population and the others are superimposed and defined by a prior microarray-based signal analysis (e.g. the DEGs defined by Affymetrix). The functional classification of 261 genes up-regulated in EnSCs (FC > 1.5 , p < 0,01 with Affymetrix HGU133 Plus 2.0 CDFs) showed statistically significant out-put score towards several gene categories (FIG 25). In particular, genes involved in the positive regulation of transcription, gene expression and metabolic processes, in cancer processes and molecules included in tight junction signaling were significantly more represented in the collection of genes up-regulated in EnSCs compared to TACs, with their expected frequency in the human genome (with Bonferroni correction for multiple testing).

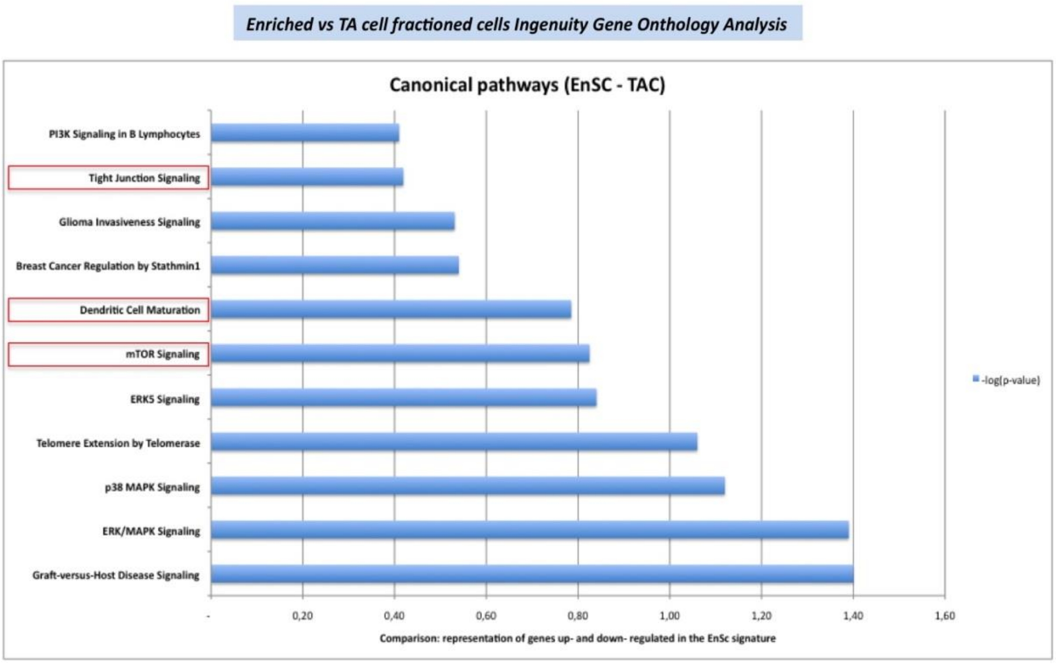


FIG. 25 EnSCs vs TACs comparison analysis: genes up-regulated in EnSC belongs to specific functional categories. Gene Ontology analysis of up-regulated genes in EnSC population. Functional categories are derived from IPA Function classifications. Blue bars indicate the number of up-regulated genes for each list annotated within the given category out of n genes eligible for each analysis . The output score is calculated using the formula "-log(p-value)". Squared clusters are

In addition, we performed further analysis on the EnSC vs PMDC comparison. In this case, the genes found up-regulated in EnSC keratinocytes were particularly enriched in functions referable keratinocyte and tissue progenitors, to regulation of cancer and cell cycle G1 phase, with a striking correlation with the phenotype which emerged frm the first comparison (FIG.26).

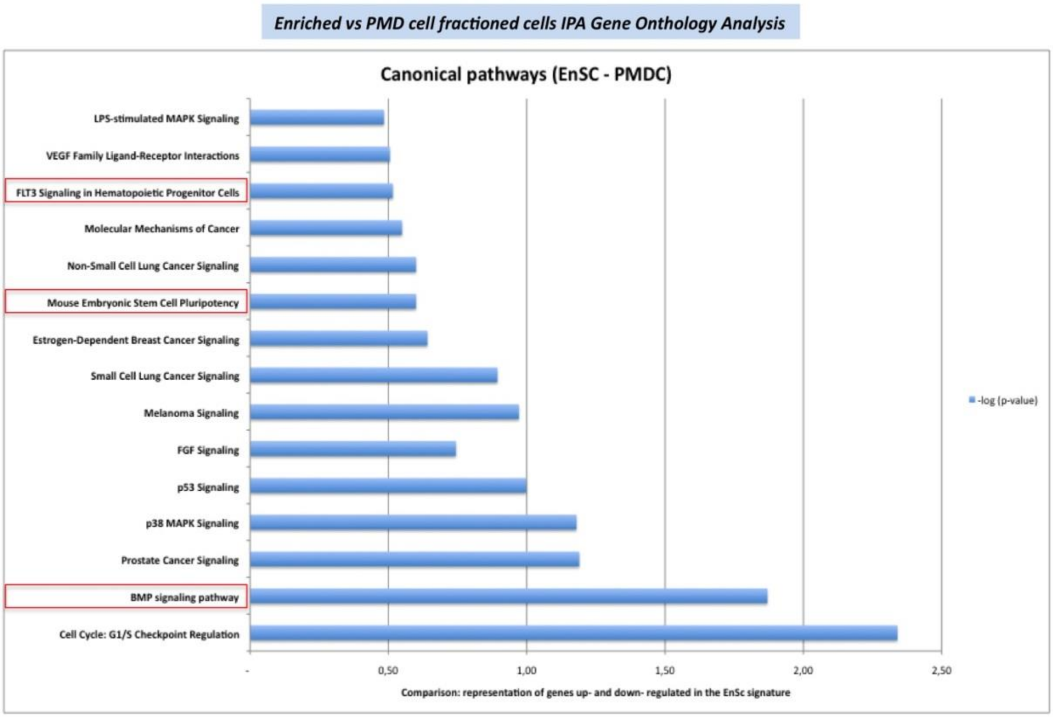


FIG. 26. EnSCs vs PMDC comparison analysis: genes up-regulated in EnSC belongs to specific functional categories. Gene Ontology analysis of up-regulated genes in EnSC population. Functional categories are derived from IPA Function classifications. Blue bars indicate the number of up-regulated genes for each list annotated within the given category out of n genes eligible for each analysis . The output score is calculated using the formula "-log(p-value)".

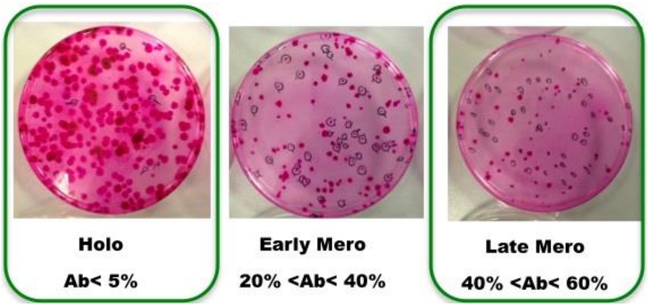
Finally, when performing a functional annotation of up-regulated genes by the network-based Ingenuity Pathways Analysis (IPA) tool, similar results were obtained when the analysis was performed on different pair-combined samples. In PMDCs no molecular and functional categories were found particularly represented in the list of up-regulated genes, with the exception of cellular development category ($p < 0.01$). Therefore, performing the IPA network analysis on the list of genes up-regulated in EnSCs, a significant number of those genes resulted functionally linked in molecular networks involved in cellular growth and proliferation, cellular development and cell cycle. Functional enrichment in top networks was less evident for PMDCs target genes, even if in the top gene network inherent to dermatological diseases genes were linked in keratinocyte differentiation pathways.

Altogether these results firmly demonstrate that EnSC and PMDC populations represent two distinct cell phenotypes, with distinct transcriptional identity and different functions; the whole core analysis showed that PMDCs are indeed representative of a late stage of keratinocyte differentiation, while EnSCs show precise feature similar and common to stem cells of other human tissues.

3.6 GENE SIGNATURE DURING CLONAL CONVERSION

Apart from the exclusive fractioned cell profile of the epidermal populations described above, important evidences about the differences in the transcriptome during clonal conversion can be extrapolated by looking at gene differentially expressed (DEG) by holoclone (H), early (EM) and late meroclones (LM). So far, the univocal method to define the clone type is provided by CFE analysis (FIG. 27-A), counting abortive colonies. Otherwise, this is not the best manner to define the biological behaviour of the clone type as reported in PCA graph (FIG. 27-B).

A



B

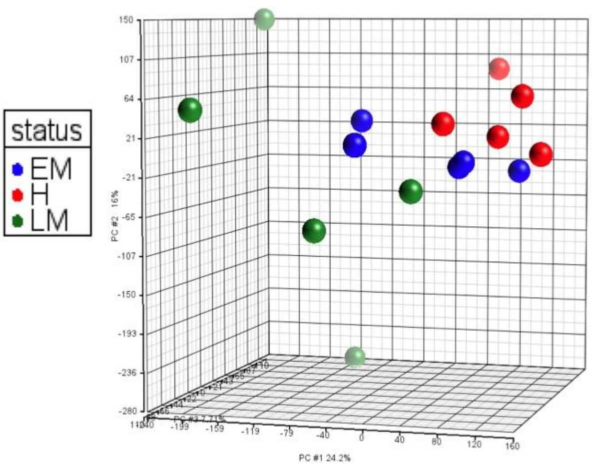


Fig. 27. CFE and PCA in clone types. A) Basal layer keratinocyte plated at clonal density on 3T3 feeder layer give raises different clonal types: holoclone, early and late merclones are shown. B) Principal Component Analysis (PCA) shows how the biological samples are more or less similar from gene signature point of views.

As defined in unsupervised analysis, H and EM types shared common features that did not allow a biological distinction as occurs when these samples are classified using CFE. Probeset level data were generated and normalized using two different statistical algorithms, ANOVA and paired T-test, using Affymetrix HG-U133A probesets. Unsupervised hierarchical clustering of the quintuplicate arrays of each clone type suggested that H and LM samples were quite different from the gene expression point of view, since all the samples were correctly clustered in their own group. Unsupervised analysis provides 1038 probes that showed how many genes are esclusively expressed when we compare H vs LM. On other hand, the method was not able to define a DEG set when we compare H vs EM types (Fig. 28).

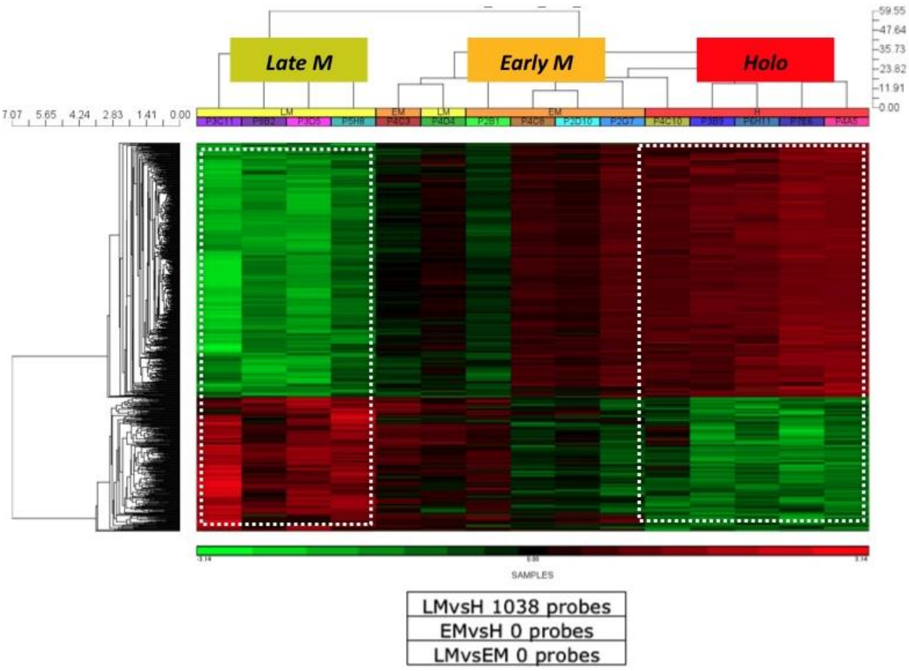


FIG. 28 Unsupervised analysis of the transcriptional profiles of clone types. A) Unsupervised hierarchical clustering analysis performed on arrays of Holoclone, Early Meroclone and Late Meroclone. Red patches represent gene up-regulated, while green patches represent genes down-regulated in one population or the other. Unsupervised analysis provides a lists of probes that showed how many genes are esclusively expressed when we compare H vs LM (white dotted line).

a) Holoclone differentially expressed genes belongs to specific functional categories

Gene Ontology functional annotation is an helpful tool to define the differences in the transcriptional profiling of Holoclone and Late Meroclone. In this way, using network-based Ingenuity Pathways Analysis (IPA), the list of genes which describe one'phenotype and the other are superimposed and defined by a prior microarray-based signal analysis (e.g. the DEGs defined by Affymetrix). The functional classification of genes up-regulated in

Holoclone (FC > 1.5 , p < 0,01 with Affymetrix HGU133 Plus 2.0 CDFs) showed statistically significant out-put score towards several gene categories (FIG 29). In particular, genes involved in the positive regulation of transcription, metabolic processes, in cancer processes and genes coding for molecules implicated in tight junction signaling were significantly more represented in the collection of genes up-regulated in H compared to LM.

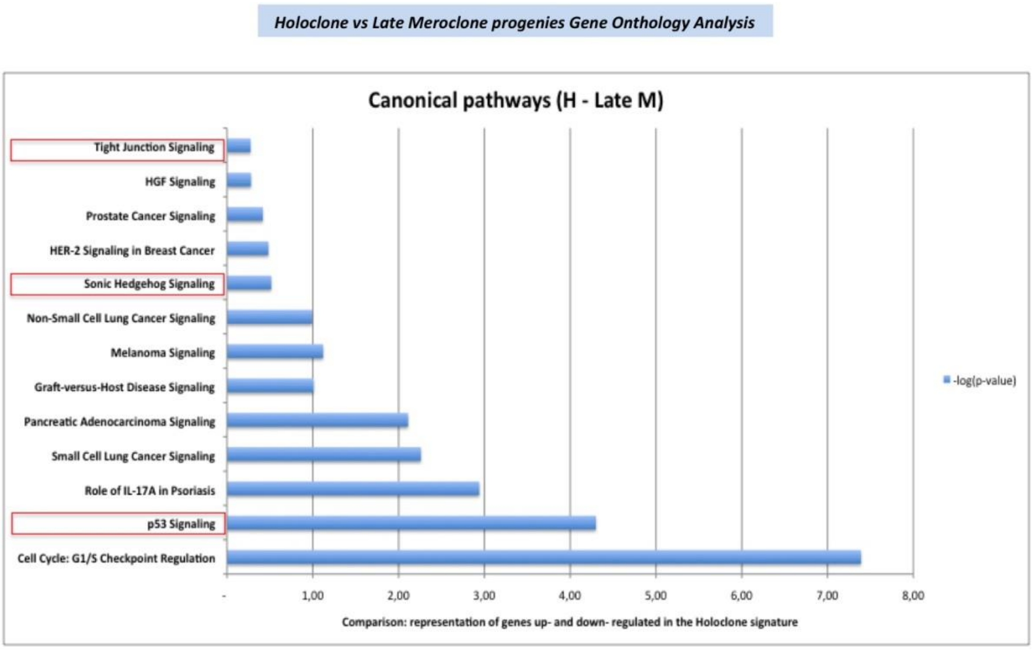


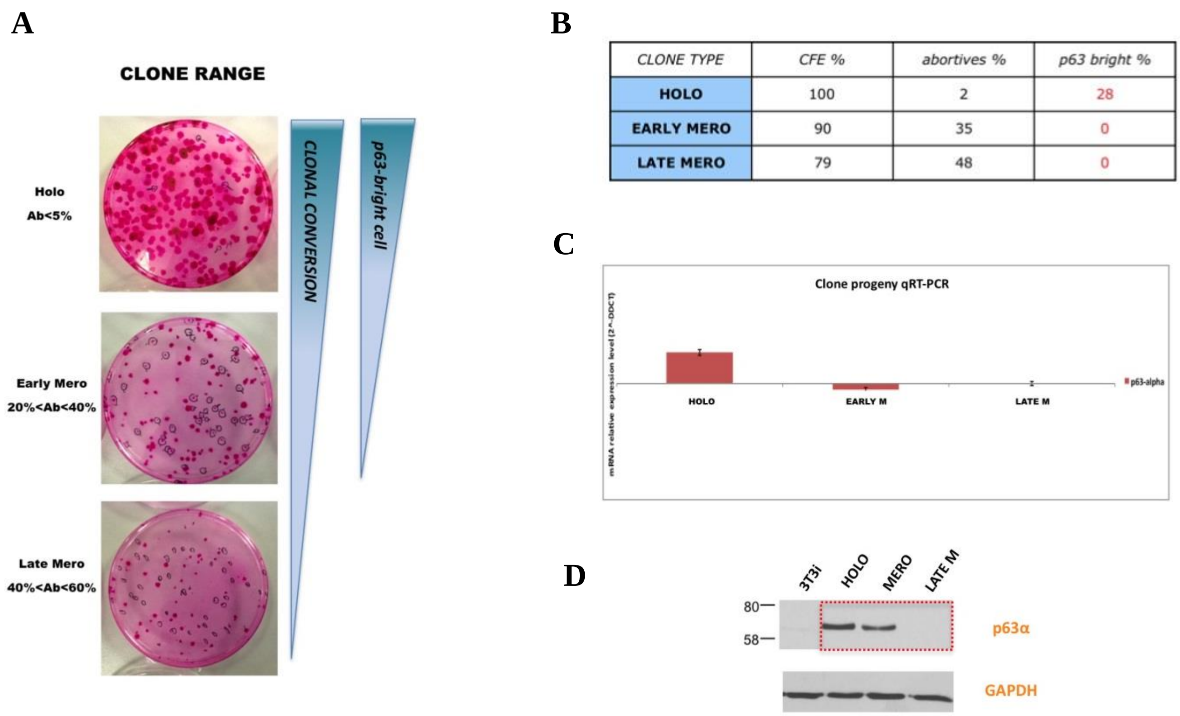
FIG. 29. Holoclone vs Late Meroclone comparison analysis: H up-regulated genes belongs to specific functional categories. Gene Ontology analysis of up-regulated genes in Holoclone type. Functional categories are derived from IPA Function classifications. Blue bars indicate the number of up-regulated genes for each list annotated within the given category out of n genes eligible for each analysis . The output score is calculated using the formula "-log(p-value)".

Holoclone differentially expressed genes belong to specific functional categories that were already found in the first data set analysis consisting in EnSC, TAC and PMDC populations.

By merging the distribution of up-regulated genes in the EnSC and in the Holoclone-type, we observed that a discrete number of differentially expressed genes was shared both in H and in EnSC, indicating a common transcriptional program wich is not different between the same cell types at distinct level of stem cell enrichment.

3.7 SELECTED GENES DECREASE DURING CLONAL CONVERSION

After having associated high progenitor-gene signature, we sought to identify univocal holoclone genes, as KSC markers. We considered as putative targets for further analyses all the overlapping genes identified by Affymetrix found in both EnSC and Holoclone; moreover, among this group we considered only associations in which the FC expression value was in higher in the cell type took in account. By looking at the evidence reported in the scientific literature concerning functional categories previous found in progenitor and holoclone populations, we selected 60 overlapping genes in EnSC/Holoclone. Using p63-bright percentage assay, we validate our samples consisting in different clone types. As predictable, this gene showed a defined expression level trend during clonal conversion (FIG. 30, A-D).



CLONE TYPE	CFE %	abortives %	p63 bright %
HOLO	100	2	28
EARLY MERO	90	35	0
LATE MERO	79	48	0

Fig. 30. p63 is coupled to clonal conversion. A-B) Clonal conversion is coupled with a decrease in p63-bright fraction. C) qRT–PCR mean value = n.2 cell progenies for each clone type. D) Cell lysate samples (10µg), corresponding to each clone type, were run on 4-12% SDS-PAGE gel and transferred onto nitrocellulose membrane. 3T3 cell lysate was used as negative control. Antibodies used for immunoblotting are shown at the right of the panel. The migration positions of molecular mass markers are shown on the left.

In an effort to shed light on factor acting together with p63 in epithelial homeostasis maintenance, we carried out further investigation on selected genes. We decided to validate these targets by looking at transcript expression level.

We started by testing 60 transcript expression levels in all clone types; of these, 12 are supposed to be stem cell-specific, 48 expressed at the same level between H, EM and LM (unshown data). The expression levels of the target transcripts were assessed by quantitative

RT-PCR, with primers designed on the housekeeping gene GAPDH as a control for equal input of cDNA quantity between samples. After that, we focused our attention on 2 putative stem cell genes. In figure 31 are reported the results of the expression levels detection of the progenitor transcripts*: inhibitor of growth family, member 5* (ING5) and *prostanoid EP2 receptor* (PTEGR2). Both transcripts are wide expressed in EnSC/H and did not show any expression in differentiated cells. As outlined in the picture, qRT-PCR is able to assess a differential expression of the transcripts, as described for p63α. Interestingly, these selected genes shows decrease during clonal conversion as well. In particular, ING5 showed a marked detection only in holoclone type from protein point of view.

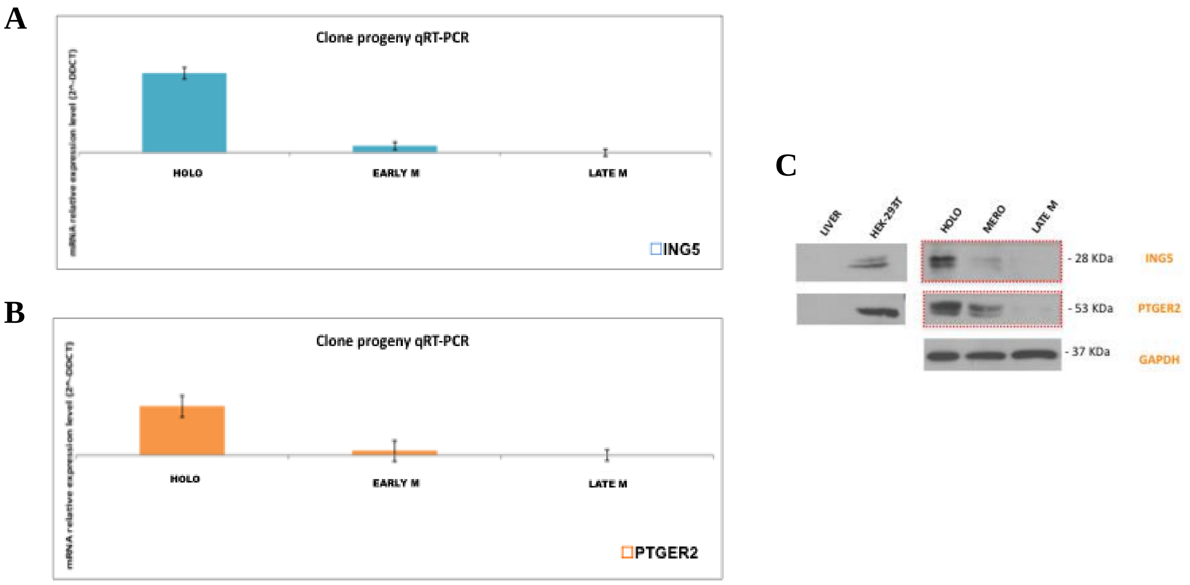


Fig. 31. Definition of KSC-specific expression transcript and protein. (A-B) Clonal conversion is coupled with a decrease in the transcript expression level of putative stemness selected genes. C) Cell lysate samples (10µg), corresponding to each clone type, were run on 4-12% SDS-PAGE gel and transferred onto nitrocellulose membrane. Liver and HEK293T cell lysates were used as negative and positive control respectively. Antibodies used for immunoblotting are shown at the right of the panel. The migration positions of molecular mass markers are shown on the right.

Altogether, these results confirm the powerful potentiality of array technique for the discovery of the gene signature of uncharacterized and not isolable stem cell populations; the preliminary data shown here might pave the way for further investigations in order to unravel the transcriptional networks that determine the progenitor and the differentiated phenotypes. Therefore, further investigation is needed to reveal a functional correlation between these selected genes and the stemness.

4. DISCUSSION

The basal layer of stratified epithelia is divided in three cellular sub-populations: keratinocyte stem cells (KSCs), transient amplifying cells (TACs) and post-mitotic differentiated cells (PMDCs). Each step, from pluripotency to terminal differentiation, requires the establishment of a specific expression program promoting specific developmental commitments that are faithfully transmitted to daughter cells. Comprehensive, genome-wide approaches, that comprehend high-throughput genomic technology, bioinformatics and biostatistics, have been developed to unveil the molecular circuitry wiring stem cell genetic programs. These advanced next-generation sequencing-based technologies have been developed to interrogate various aspects of cellular processes, such as the repertoire of transcripts of a specific cell state (the transcriptome). However, all the studies that took advantage of these techniques were performed on cell lines, while relatively little is known about human KSCs; this is essentially due to the difficulty in obtaining significant numbers of homogeneous populations of pure KSCs. The stem cell compartment represents only the 3-10% of the whole pool of keratinocytes. A number of molecular markers have been described to be preferentially expressed by putative KSCs; however, the differential expression of these molecules does not represent a distinctive feature of stem cells and selecting these surface markers does not allow to isolate KSCs, but, more simply, to enrich the fraction of cells with highest proliferative potential within a mixed population. Absence of stem cell-specific markers is a limit for the isolation and subsequent biological and biochemical characterization of epithelial stem cells. Recently, transcriptional profiling of enriched populations of putative epithelial stem cells has been used to search for keratinocyte stem cell-specific markers. Methods used to isolate the putative stem cells before the analysis include (i) rapid adherence of trypsinized epithelial cells to collagen-coated plates (i.e. epithelial cells that adhere within 20 min are thought to be putative stem cells, whereas slowly adhering cells are defined as the TA or terminally differentiated cells [135-138]; (ii) the active efflux of the DNA binding dye Hoechst 33342 by the ABCG2/BCRP1 transporter, yielding a cohort of cells known as the side population [139, 140]; (iii) the engineering of transgenic mice to express histone H2B-green fluorescent protein in a manner that specifically tags the label-retaining cells followed by FACS sorting of single cell preparations of these label-retaining cells [140]; (iv) the engineering of transgenic mice to express green fluorescent protein under the keratin 15 promoter, which preferentially tags cells in the hair follicle bulge (site of the hair follicle stem cells), followed by FACS sorting of this green fluorescent protein cell population [141]; and (v) a FACS strategy based on cell adhesion molecule expression [86, 142]. Although all of these methods yield cells for subsequent transcriptional profiling, the cells are subjected to a variety of manipulations prior to analysis, including proteolytic tissue dissociation, cell culturing, and FACS sorting. These manipulations can potentially perturb the observed genetic profiles, and will lead to stemness loss. In view of functional data demonstrating the role of integrin α6β4 in mediating adhesion of basal keratinocytes to the basement membrane via hemi-desmosomes, we reasoned that this integrin may provide a suitable marker for the isolation of proliferative keratinocytes because these cells are permanently anchored to the basement membrane. In this way, we designed a method based on magnetic microbeads integrin selection to achieve a cell population enriched in stem cell (EnSCs). Given the method to separate EnSC from TAC population and obtaining in separate experimental step a third kind of sample, the post-mitotic differentiating cell population (PMDC), we were looking to answer on various aspects of cellular processes, such as the repertoire of transcripts of a specific cell state. In this way, we primarily focused on the assay allow us to validate biological samples which were submitted to gene signature investigation. p63 gene, a homologue of the tumor suppressor p53, is crucial for the development and maintenance of squamous epithelia. Several isoforms are described and ΔNp63α is mainly expressed in the basal layers of stratified epithelial tissues. Many studies showed that this protein isoform plays a crucial role in controlling gene expression in epidermal cells, but to date the interaction with other pathways during embryonic skin development is still poorly understood. Although it is clear that p63 expression is not limited to stem cells of the epithelial tissues that express this gene, a number of studies contend that the stem cells in fact express the highest levels of p63 [121]. Here, for the first time we provide evidence about the correlation between the value of p63-bright cell percentage and the holoclone (KSC) fraction in the epidermal cell culture. We show that p63 immunoistochemical densitometry coupled to the cell size can give rise to the estimation of p63-bright percentage. This value finds a correspondence to the amount of p63-high cells only when these cells endorse a cell size cutoff in the epidermal culture. This parameter provides an amelioration method to determine the number of stem cells in culture when used to prepare a graftable epithelial sheet. In fact, the determination of the number of holoclones in a culture requires some experimentation and would be cumbersome as a standard test for the quality of cultured grafts. Fortunately, the number of aborted colonies (those that have stopped multiplication), is inversely related to the number of holoclones, and is easier to score. The identification of holoclones by immunodetection of p63, and especially the α isoform, is an important and simple means of determining the presence of an adequate number of stem cells in a cultured keratinocyte graft and, in this study, for introducing a quality control in the validation of biological state of cell populations obtained from fresh biopsy or cell culture.

The *two-step separation* method, designed for cell isolation, primarily leads to feeder-layer depletion starting from mass cell culture and then a second step ensures the recovery of 2 side cell populations: a6-integrin-bright and a6-integrin-dim cells. Following magnetic separation, the two cell populations were analyzed for the colony-forming efficiency (CFE) and the p63-bright amount counting. These parameters are in accordance each other showing stem cell enrichment and clonogenicity increase for the positive fraction (EnSC). On other hand, the negative fraction (TAC) shows discrete clonogenicity and absence of stem cell. The experimental procedure reckons on the validation of the PMDC population as well. All the three samples show differences in their biological molecular profiles analyzed by qRT-PCR and SDS-page using already described progenitor and differentiation markers. According to the notion that only the clonal analysis can reveal the effective presence of stem cell, we enlarge our results providing the evidence of the presence of holoclones only in the EnSC. This event is the most important experimental step, because both EnSC and TAC show tissue regeneration ability, but only the first cell fraction is endowed of stem cells. In this manner, to perform microarray analysis we collected 3 cell populations from five donors: EnSC with high clonogenigity and sustained by holoclone cell type; TAC population composed from committed cell expressing differentiation markers and without stem cells; PMDC population with reduced growth potential and clonogenicity and ,overall, without stem cells.

Expression profiling by microarrays has been used so far as a very successful tool for the retrieval of important information about how the transcriptome is deployed in different cell types and how it varies during differentiation. In this first part of our work, we performed the data analysis by a pair-wise comparison of two groups of arrays (1) EnSCs versus TACs and (2) EnSC versus PMDCs, estimating the number of differentially-expressed genes (DEGs). We find 804 DEGs clearly associated to progenitor/differentiated states. Using network-based Ingenuity Pathways Analysis (IPA) tool, the list of genes up-regulated in one population and the others are superimposed and defined by a prior microarray-based signal analysis (e.g. the DEGs defined by Affymetrix). The functional classification of 261 genes up-regulated in EnSCs showed statistically significant out-put score towards several gene categories. In particular, genes involved in the positive regulation of transcription, gene expression and metabolic processes, in cancer processes and molecules included in tight junction signaling were significantly more represented in the collection of genes up-regulated in EnSCs when compared to TACs and to PMDCs. Altogether these results firmly demonstrate that EnSC, TAC and PMDC populations represent distinct cell phenotypes, with distinct transcriptional identity and different functions; the whole core analysis showed that PMDCs are indeed representative of a late stage of keratinocyte differentiation, while EnSCs show precise feature similar and common to stem cells of other human tissues. Apart from the exclusive fractioned cell profile of the epidermal populations described above, important evidences about the differences in the transcriptome during clonal conversion can be extrapolated by looking at DEG by holoclone (H), early (EM) and late meroclones (LM). So far, the univocal method to define the clone type is provided by CFE analysis, counting abortive colonies. Otherwise, this is not the best manner to define the biological behavior of the clone types. In this way, we perform microarray assay on the different clone progenies. The unsupervised analysis confirmed a biological state common to the H and EM. On other hand, this approach has been a helpful tool to define the differences in the transcriptional profiling of H and LM. In particular, genes involved in the positive regulation of transcription, metabolic processes, in cancer processes and genes coding for molecules implicated in tight junction signaling were significantly more represented in the collection of genes up-regulated in H compared to LM. Interestingly, H differentially expressed genes belong to specific functional categories that were already found in the first data set analysis consisting in EnSC population. By merging the distribution of up-regulated genes in the EnSC and in H, we observed that a discrete number of differentially expressed genes were shared both in H and in EnSC, indicating a common transcriptional program which is not different between the same cell types at distinct level of stem cell enrichment. After having associated high progenitor-gene signature, we sought to identify univocal holoclone genes, as KSC markers. We considered as putative targets for further analyses all the overlapping genes identified by Affymetrix found in both EnSC and H. By looking at the evidence reported in the scientific literature concerning functional categories previous found in progenitor and holoclone populations, we selected 60 overlapping genes in EnSC/Holoclone. After that, we focused our attention on 2 putative stem cell genes: *inhibitor of growth family, member 5* (ING5) and *prostanoid EP2 receptor* (PTEGR2). These selected genes shows decrease expression level during clonal conversion. Interestingly, ING5 showed a marked detection only in holoclone type from protein point of view. A large body of evidence exists that the PG synthesis pathway, via PGE2-EP receptor signaling, plays an important role in mammary gland and colorectal carcinogenesis. The EP2 receptor deficiency was associated with decreased cell proliferation *in vivo* and resulted in decreased epidermal hyperplasia. Indeed, available data suggest that EP1, EP2, and EP4 receptors all play a role in the early stages of epithelial tumorigenesis. Other solid tumor models, which perhaps have more relevance to established colorectal cancer, have implicated EP3 and EP2 receptors in the host angiogenic response [143,144]. Moreover, we investigated ING5 as it belongs to a large family group of tissue homeostasis regulators, the MORF complex. Recently, Mulder KW. et al. revealed a strong correlation between keratinocyte stemness and this complex. Indeed, ING5 interacts directly with EZH2 and UHRF1, two main regulators of epidermal self-renewal [145]. In general, the proteins of the Inhibitor of Growth (ING) family are involved in multiple cellular functions such as cell cycle regulation, apoptosis, and chromatin remodeling. For ING5, its actual role in growth suppression and the necessary partners are not known. Studies have shown that ING proteins exert their biological function through their association with specific molecular partners, in particular ING5 was identified as one of the interacting partners of Inhibitor of cyclin A1 (INCA1). The main effect of this interaction there would be the capability of adherent cells to promote the colony formation, as shown for human tumorigenic cells [146].

The preliminary data outcome of the work consists of a list of putative genes that might be involved in the specification of a stem cell program and in epidermal homeostasis. Additional clues could be then gained by validating these KSC-specific genes and their transcripts using in vitro assays, either by testing putative transcript activity and subsequently protein functions in regenerated organotypic human epidermal tissues. In conclusion, this work defines the set of specific gene signature in human epidermal progenitor cells and paves the way for a deeper understanding of the molecular basis that underlies the biology of skin stem cells and epidermal homeostasis.

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