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The role of survivin in squamous cell carcinoma

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LIST OF ABBREVIATIONS

KSC: keratinocyte stem cell

TA: transient amplifying cell

PM: post mitotic cell

CSC: cancer stem cell

SCC: squamous cell carcinoma

BCC: basal cell carcinoma

AK: actinic keratosis

IF: interfollicular

HF: hair follicle

SG: sebaceous gland

Introduction

1.0. SKIN

The skin is the largest organ of the body and is the first lane of defense against injury, dehydration and infection and is important in thermoregulation.

Human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue (Breathnach, 1971; Goldsmith, 1991; Montagna et al, 1974, 1992; Zelickson et al, 1967).

The normal epidermis is a terminally differentiated stratified squamous epithelium. The major cell, making up 95% of the total, is the keratinocyte, which moves progressively from the attachment to the epidermal basement membrane toward the skin surface, forming several well-defined layers during its transit.

Within the epidermis, there are several other cell populations, namely melanocytes, which donate pigment to the keratinocytes, Langerhans' cells, which have immunological functions and Merkel cells.

Thus, on simple morphological grounds, the epidermis can be divided into four distinct layers (Fig 1):

- stratum basale or stratum germinativum,
- stratum spinosum,
- stratum granulosum,
- stratum corneum.

The stratum basale is a continuos layer that is generally described as only one cell thick, but may be two to three cells thick in glabrous skin and hyperproliferative epidermis. The basal cells are small and cuboidal (10–14 nm) and have large, dark-staining nuclei, dense cytoplasm containing many ribosomes and dense tonofilament

bundles.

Immediately above the basal cell layer, the epibasal keratinocytes enlarge to form the spinous/prickle-cell layer or *stratum spinosum*.

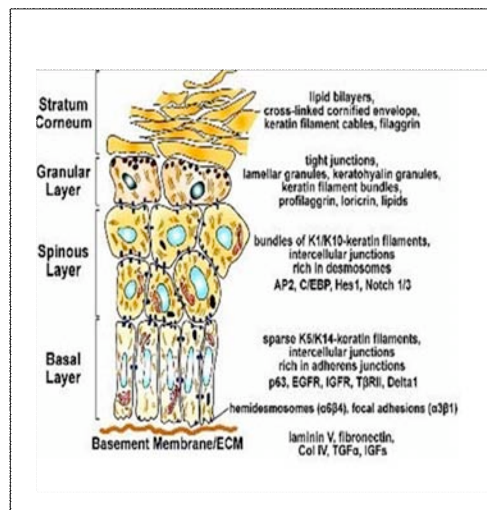


Fig 1: Structure of the epidermis

The stratum spinosum is succeeded by the *stratum granulosum* or granular layer because of the intracellular granules of keratohyalin. At high magnification, the dense mass of keratohyalin granules from human epidermis has a particulate substructure, with particles of irregular shape on average 2 nm length and occurring randomly in rows or lattices (Lavker et al, 1971). The cytoplasm of cells of the upper, spinous layer and granular cell layer also contains smaller lamellated granules averaging 100–300 nm in size, which are known as lamellar granules or bodies, membrane-coating granules or Odland bodies (Odland, 1991).

These are numerous within the uppermost cells of the

spinous layer and migrate towards the periphery of the cells as they enter the granular cell layer. They discharge their lipid components into the intercellular space, playing important roles in barrier function and intercellular cohesion within the stratum corneum.

The outermost layer of epidermis is the stratum corneum where cells (now corneocytes) have lost nuclei and cytoplasmic organelles. The cells become flattened and the keratin filaments align into disulphide cross-linked macrofibres, under the influence of *filaggrin*, the protein component of the keratohyalin granule, responsible for keratin filament aggregation (Lynley and Dale, 1983). The corneocyte has a highly insoluble cornified envelope within the plasma membrane, formed by cross-linking of the soluble protein precursor, *involucrin* (Rice and Green, 1977), following the action of a specific epidermal transglutaminase also synthesized in the high stratum spinosum (Buxman and Wuepper, 1978). The process of desquamation involves degradation of the lamellated lipid in the intercellular spaces and loss of the residual intercellular desmosomal interconnections. In palmoplantar skin there is an additional zone, also electronlucent, the *stratum lucidum* between the granulosum and corneum. These cells are still nucleated, and may be referred to as 'transitional' cells.

Epidermal differentiation comprises all the molecular, biochemical and morphological processes that promote the transformation of a basal cell into a corneocyte (Jean-Hilaire Saurat et al, 1999). The number of new keratinocytes from the basal layer is equal to those that are lost in the stratum corneum, therefore providing,

under physiological condition, a constant epidermal thickness. In normal condition, there is a perfect balance between proliferation and desquamation that entails a complete epidermal renewal every 28 days.

During this process, a wide number of marker are expressed by keratinocytes undergoing differentiation.

- Transglutaminase family protein members (TG), which comprise 8 distinct isoforms. TG type 1, 3 and 5 have a fundamental role in skin development and are responsible for the cross-linking within keratinocyte precursors and the formation of cornified envelope during differentiation. TG2 has a role in the basement membrane cross-linking. They are terminal differentiation markers (Harrison et al, 2007).
- Keratins: Type I (acid keratins) and Type II (basic keratins). About 30 different keratin chains have been identified and numbered on the base of their molecular weight. In keratinocytes we can find:
  - K15: epithelia stem cell marker of the basal layer (Qian Zhan et al, 2006). K15 expression indicates slow cycling cells and no differentiation (Watt et al, 2006);
  - K5: this molecule is expressed by basal cells together with K14 and p63;
  - K10: it is a Type I keratin; it is expressed in association with K1 (Type II) forming an hetero-tetramer in the basal layer of the epidermis (Kinouchi et al, 2002; Steinert, 1991). These molecules are synthetized superficially in the upper layers and the filaments are assembled in micro-fibrils and linked to keratoialin granules in the granulosum. The granules contain

pro-filaggrin, a phosphorylated protein and rich in hystidine (Williams, 2001).

Involucrin: it is a soluble cytoplasmic protein, riches in glutamic acid and glutamine, abundantly expressed in the epidermis starting from the stratum spinosum (Cainelli et al, 2004). It is absent in the basal layer and it appears in the upper layer during keratinocyte migration. Involucrin forms the cornified envelope of keratinocytes and its expression is correlated to the increase in calcium concentration (Alberg et al, 2007).

1.1. Epidermal homeostasis and keratinocyte stem cells (KSCs)

Epidermis serves as a barrier that protects against environmental stresses, such as microbes, water loss, physical, thermal, and mechanical injuries. To support this role, epidermis must renew constantly throughout life to maintain normal homeostasis and to repair damage after wounding. The regenerative capacity of epithelial populations have been studied for many years with the development of cultured keratinocytes derived from the epidermis, used to produce autologous graft that regenerate an epidermis over a full-thickness wound (Coolen et al, 2007).

Epidermal homeostasis depends on a balance between proliferation and differentiation/apoptosis of keratinocytes. The epidermis contains a basal layer of keratinocytes that adhere to the underlying basement membrane (BM). Periodically, these cells detach from the BM, withdraw from the cell cycle, and initiate a program of terminal

differentiation, moving upward the skin surface.
Keratinocyte stem cells (KSCs) are responsible for epidermal homeostasis and for repairing the tissue following injuries. KSCs self-reniew and generate the different lineages that form the mature tissue.
There are three pools of KSCs in different locations. The interfollicular (IF) stem cells of the basal layer, the hair follicle (HF) stem cells of the bulge, and the sebaceous gland (SG) stem cells that are located above the bulge and below the hair shaft orifice.
During physiological tissue renewal, the three skin cell populations are each believed to be maintained by their own stem cells. When tissue homeostasis is disrupted, however, any of the three stem cell populations is capable of producing all three structures (Levy et al, 2007; Fuchs and Horsley, 2008).
Human epidermis is thick with frequent cellular turnover and a tendency to produce long-term epidermal cultures in vitro. On the other hand, while the mouse can be genetically manipulated, it displays a thin, less active epidermis, whose cells fail to yield long-term cultures. In addition, some of the markers used to identify stem cells are different in mouse versus human skin. Despite these differences, common signaling pathways appear to control epithelial stem cell maintenance, activation, lineage determination, and differentiation.

1.2. Interfollicular stem cells and keratinocyte supbpopulations

Regeneration of epidermis depends on the proliferation of

a subpopulation of basal keratinocytes, known as IF stem cells.

The current dogma of the epidermal proliferative unit (EPU) defines a model where a single stem cell divides infrequently to produce a stem cell daughter and a non-stem committed progenitor cell, named transit amplifying (TA) cell. TA cells divide a small number of times before withdrawing from the cell cycle and undergoing terminal differentiation in the suprabasal layer.

The EPU model has been questioned recently by lineage tracing experiments demonstrating that normal adult epidermis is maintained by a single population of committed progenitor cells with different stochastic choices (Clayton et al, 2007). In any event, the EPU model implies that in the basal layer of epidermis there is a proliferative heterogeneity.

Clonogenicity assays allow to distinguish three types of clones: holoclones that arise from stem cells and found large colonies with the greatest regenerative capacity in long-term culture; paraclones which give raise to abortive colonies and undergo differentiation; and meroclones that originate from TA and have an intermediate proliferative capacity (Barrandon and Green, 1987).

The different clonogenic potential well correlates with the expression of β_1-integrin (Jones and Watt, 1993). Indeed, keratinocytes expressing highest levels of β_1-integrin give rise to holoclones, whereas cells with low levels of β_1-integrin produce smaller colonies. This has allowed to select a population of stem keratinocytes based on the rapid adherence to type IV collagen.

A number of markers to enrich for stem cells have been reported, including high expression of α6 integrin (α6bri) and low expression of CD71 (CD71dim). In particular, α6bri/CD71dim basal keratinocytes exhibit many features of stem cells, such as quiescence and the greatest long-term regenerative capacity (Li et al, 1998). Furthermore, melanoma chondroitin sulfate proteoglycan (MCSP) is confined to non-cycling keratinocytes expressing highest levels of β1 integrin, and can thus be considered a marker for epidermal stem cells (Legg et al, 2003). Also the EGF receptor antagonist Lrig1 has been proposed as a marker of human IF stem keratinocytes, since it maintains these cells in a quiescent state (Jensen and Watt, 2006).

While these markers have greatly helped in isolating and characterizing keratinocyte subpopulations enriched in stem cells, a definite identification of epidermal stem cells and their niche is still vague. Clusters of putative stem cells expressing β_1-integrin, MCSP, and Lrig1 are mostly detected in the upper part of the epidermal rete ridge, whereas α6bri/CD71dim keratinocytes are found at the tip of the rete ridges. On the other hand, xenograft studies using lentiviral vectors have shown that stem cells are dispersed throughout the basal layer (Ghazizadeh and Taichman, 2001, 2005).

Irrespective of the markers and the location, stem cells can be identified based on the fact that they divide infrequently, thus maintaining the incorporated radioactive thymidine for long periods of time. This provides evidence that at least mouse epidermis contains slow cycling stem keratinocytes. On the other hand, radioactive labeling

cannot be applied to humans in vivo. Yet, using a scaffold-based organotypic skin culture, it is now possible to mark keratinocytes with iododeoxyuridine and follow them for 8–10 weeks, when only <1% of basal cells have retained the label. Using whole mount preparations, Muffler and co-workers were able to demonstrate that LRCs are individually dispersed throughout the basal layer in a random distribution with no evidence of a pattern suggestive of EPU. As a similar pattern was also observed for the IFE of mouse skin (Braun et al, 2003), this is likely to be the kind of distribution of stem cells in the basal layer.

Consistent with this finding, Marconi and co-workers have shown recently that survivin identifies a population of IF stem cells expressing highest levels of β_1-integrin, and these cells are dispersed randomly in the basal layer (Marconi et al, 2007). Actually, one could comment that such a random distribution throughout the entire basal layer better fulfills the tasks of IF stem cells that would thus be ready to reconstitute epidermis wherever it is needed. While stem cells should reside in protected locations, factors, adhesion molecules, and cells participating in the niche are evenly expressed throughout the epidermis and not just at the tip or in the upper part of the rete ridges (Muffler et al, 2008).

1.3. KSC characterization and isolation

Currently, there is no known specific marker for KSCs as these cells are not located in a specific compartment and are therefore difficult to isolate and characterize. As

integrins play an important role in maintaining stem cells in the epidermis, the majority of works focused on stem cells isolation, using integrins as good surface “enrichment markers” for KSC.

Recently, a population of putative stem cells has been identified in the α6Bri/CD71dim population (Li et al., 1998). Integrin _6_4 mediates adhesion of basal keratinocytes to the basement membrane via hemi-desmosomes (Van-der-Neut et al., 1996). CD71 is the transferring receptor and is expressed in growing cells and hematopoietic and neural development. Since stem cells in epidermis are permanently anchored to the basement membrane and are mainly in a quiescent state, cells that are α6Bri/CD71dim are reasonably a good candidate for stem cells. On the contrary, cells that are α6Bri/CD71Bri seem to have properties of TA cells (Li et al., 2004). It has been also demonstrated that α6Bri/CD71Bri, α6Bri/CD71dim and α6Dim populations have equivalent tissue reconstruction ability (Li et al., 2004). This could be due to a preferential selection of a population by the assay’s conditions. For this reason, further study is required to design an experimental assay able to distinguish epidermal stem and progenitor cells (Kaur, 2004).

The isolation of cells that are positive for α6 integrin and positive for K15, a basal layer-associated keratin, allowed localizing the proliferating compartment of adult epidermis. This is mainly located in the tips or rete ridges. Moreover, α6high/K15high cells are also negative for K10 and have enriched clonogenic activity (Webb et al., 2004).

Stem cells have the intrinsic characteristic of being slow cycling or quiescent cells. Another strategy to isolate stem cells in the epidermis uses an *in vivo* labeling of human keratinocytes with a nucleoside, bromodeoxyuridine (BrdU). In the epidermis the cells that incorporate and retain the dye over a long interval, followed by a chase period in the absence of BrdU, have been believed to represent KSC and are called label-retaining cells (LRC) (Bickenbach, 1981; Morris et al, 1985; Cotsarelis et al, 1990; Bickenbach and Chism, 1998). Rapidly dividing basal cells (TA) dilute their nuclear label with every cell division. After the chase period, only non-cycling or slowly cycling keratinocytes retain the nuclear label. Recently, Vogel J.C. and colleagues compared LRC in human epidermis with the side population (SP), a group of cells that efflux Hoechst 33342 fluorescent dye (Terunuma et al., 2003). SP cells are highly enriched for long-term hematopoietic repopulation activity following transplantation, and are present in multiple mammalian species (Goodell et al, 1997). In some tissues these cells may represent primitive progenitor cells (Gussoni et al, 1999, Hulspas and Quesenberry, 2000). The ability to efflux Hoechst 33342 dye in these cells is due to the expression of some ABC transporters, a family of trans-membrane proteins involved in drug efflux from several mammalian cell types. It has been shown that SP and LRC populations in human keratinocytes are distinct and that SP cells do not over-express β1 and α6-integrins, opposite to LRC (Terunuma et al., 2003). *In vivo* assays to test the capacity of repopulating a wounded skin have been

recently developed and used to test stem cells candidates (Terunuma et al., 2004). These tools recapitulate human epidermis onto immuno-compromised mice and evaluate the repopulation ability of subsets of cells when putted into a competitive environment. Using this assay to compare SP cells and keratinocytes expressing elevated levels of α6 integrin and low levels of CD71, it has been shown that SP keratinocytes showed little competitive expansion in vivo and were not enriched for KSCs. On the contrary α6Bri/CD71dim keratinocytes expanded over 200-fold during the study, thus showing stem cell properties (Terunuma et al., 2007).

These studies suggest that stem cells in human epidermis have some known characteristics, such as the high expression levels of β1 and α6-integrins. It is also well accepted that stem cells have high proliferation potential, although residing in the tissue, under homeostatic conditions, in a quiescent or slow-cycling state.

It has been reported that cell size correlates with proliferation ability in human keratinocytes, the smallest cells retaining higher CFE (Barrandon and Green, 1985; 1987). The group of Duan E. further enriched KSC by combining human collagen IV adhesiveness and cell size isolation approach. RAD keratinocytes with the smallest size have the highest proliferation potential and show stem cells characteristics, such as high CFE, differentiation capacity, high β1-integrin expression and the ability to be incorporated in mouse embryo development (Li et al., 2004).

The characterization of KSC has also been possible by the use of *in vitro* systems. The isolation of three populations of human keratinocytes able to generate different colonies *in vitro*, has been reported by the pioneering work of Barrandon and Green in 1980's. Human KSC and TA cells when isolated in culture give rise to holoclones and paraclones, respectively. Holoclones retain the greatest proliferative potential and the capacity to generate a mature epithelium in vivo (Mathor et al., 1996) and to differentiate into distinct cellular lineages (Pellegrini et al., 1999). Using *in vitro* models, it has been proposed that p63, a transcription factor homologus to p53 essential for epithelia proliferation and development, is a good marker for KSC (Pellegrini et al., 2001).
The isolation of KSC requires the identification of a structural or functional marker, specific for this subpopulation. Stem cells are also characterized by intra-cellular pathways that decide their faith and that are necessary for stem cells maintenance. One of these pathways is the β-catenin/*wnt* pathway. β-catenin ablation in the skin results in no hair generation. In normal conditions, *wnt* pathway is silent and β-catenin participates to adherent junctions. The activation of *wnt* pathway leads to the translocation of β-catenin in the nucleus where it can regulate gene expression, in particular of the *myc* gene. A decrease in *myc* transcription depletes KSC in the epidermis (Waikel et al., 2001).
Nevertheless, a good tool to enrich human KSCs is their isolation on the bases of different levels of β1-integrins expression. β1-integrins specifically bind collagen IV, one

of the major components of the ECM. Keratinocytes that express high levels of β1-integrin, adhere rapidly to collagen IV coatings (therefore called rapidly adhering cells, RAD) within 5 minutes (Fig.2).

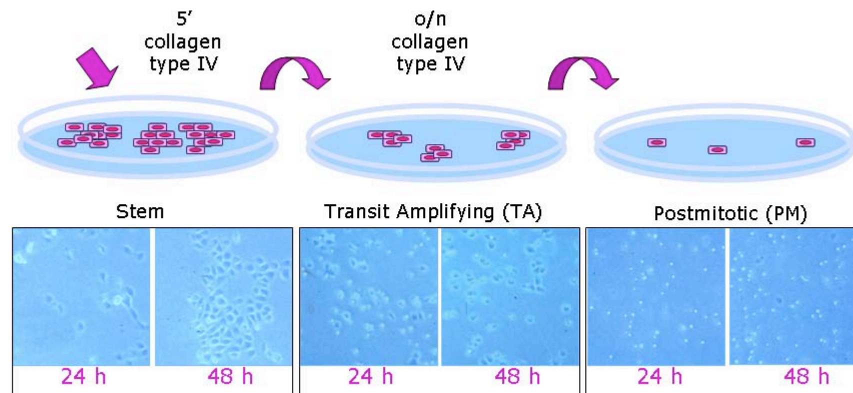


Fig. 2: KSC separation based on collagen IV adhesion

These cells lack BrdU incorporation, thus indicating their slow cycling status. Moreover, RAD do not express keratin 10 (K10), a keratin specific for the super-basal layers of the epidermis (Jensen et al., 1999) and have high colony forming efficiency (CFE) and greater _6_4-integrin and ΔNp63 expression. This population has been hypothesized to be enriched in KSC (Jones and watt, 2003). Recently, it has been shown that RAD cells expressing low levels of desmoglein-3, a major component of desmosomes (cell-cell junctions), have greater CFE and proliferative capacity (Wan et al., 2003). TA cells, express lower levels of β1 and a6β4-integrins, and differentiate after low number of cell divisions. For these reasons these cells are believed to adhere less rapidly to collagen IV coatings. In the adhesion assay, TA cells are able to adhere to collagen IV coatings during over-night incubation. The cells that do not adhere are considered PM cells.

2.0. Survivin

Survivin is one of the members of the IAP family but, unlike other IAP's, survivin not only inhibits apoptosis but also regulates cell division.
A large body of literature supports the concept that Survivin, is only expressed in cancer and in foetal tissues while it is almost absent in differentiated adult tissue (Ambrosini et al, 1997). By contrast, survivin is expressed in normal human epidermis, and in particular in KSC (Chiodino Cet al, 1999). More specifically, Survivin is almost exclusively expressed in KSC, whereas it is only slightly present in TA.
Survivin is a protein encoded by a single gene located in the chromosome 17 of human genome. Originally identified in B-cell lymphoma cells, survivin is almost absent in the majority of human adult tissues but is highly expressed in foetal and highly proliferative tissues, contributing to tissue homeostasis and differentiation (Adida et al., 1998). Survivin expression is also prominent in cancer cell lines and cancer tissues, such as lung, colon and breast (Ambrosini et al., 1997). Cancer cells seem to utilize survivin in order to progress in their growth by being less sensitive to apoptosis (Chakravarti et al., 2002). Inhibition of survivin leads to reduced tumor growth, supported by a reduction of tumor vessels (Blanc-Brude et al., 2003). Survivin over-expression correlates with poor

prognosis and resistance to chemo-therapy in several human cancers (Yamamoto et al., 2008).

2.1 Survivin structure

Survivin structure is quite unique among the other IAPs members: it contains only one Baculoviral IAP Repeat (BIR) domain and an alpha elica coiled-coil domain at the carboxy-terminal end, instead of the classical RING finger domain (Verdecia et al., 2000) (Fig.3).
Survivin was discovered to play important roles both in cell division and cell survival, as opposite to the other IAP proteins which are mainly involved in apoptosis regulation (Li et al., 1998). This dual action, which distinguishes survivin from the other IAP family members, is ensured by its unique structure. Survivin has only one copy of a modified BIR domain, which is used for the homodimerization of the protein and for its interaction with other chromosome passenger proteins.
Phosphorilation of threonine 48 in the BIR domain alters the ability of survivin to bind borealin, a chromosome passenger protein (Barrett et al, 2011).
The structure of survivin also differs from that of the other IAPs at the C-terminal domain that is substituited by a coiled-coil a-helix domain, which in turn is responsible for its regulation of cell division. (Li et al., 1998).
Survivin involvement in apoptosis seems to be mediated by caspase binding through the BIR domain.

X-ray crystallography showed that survivin forms and acts as homo-dimers.

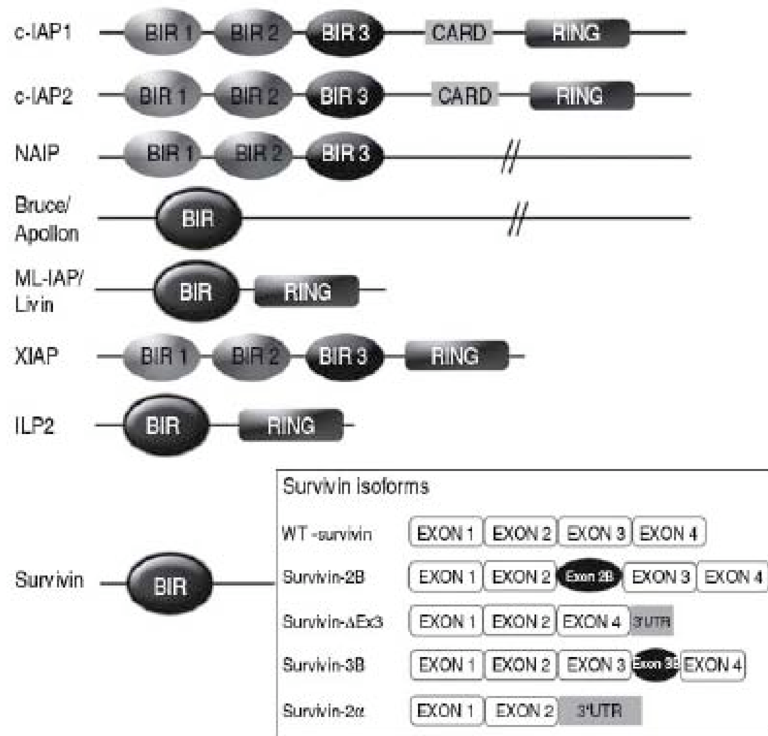


Fig 3. the inhibitor of apoptosis (IAP) members and survivin isoforms

2.2. Survivin regulation

Survivin expression is highly regulated in a cell-cycle dependent manner (Altieri, 2006). This regulation is exerted on specific sequences at the promoter level, increasing during G1 and reaching highest levels of expression in the G2-M phase of the cell cycle (Li and Altieri, 1999). Survivin regulation occurs both at the transcriptional, splicing, degradation, intra-cellular sequestration, and post-transcriptional levels (Mita et al., 2008). First of all, survivin is up-regulated by NF-kB,

Insuli-like growth factor, wnt, and some members of the ras gene family (Mita et al., 2008). On the other hand, survivin gene is negatively modulated by p53. In several human cancers p53 is mutated and lost its cyto-protective functions. The control WTp53-mediated on survivin is therefore missing, thus inducing an increase in the expression of this protein (Zhou et al., 2002).

Survivin levels are also controlled by protein degradation through the ubiquitin-proteasome pathway. In particular, survivin decreases during the G0-G1 phase of the cell cycle. Mutations in the BIR, N- or C-terminus of survivin destabilize its structure, thus sensitizing survivin to the proteasome-mediated degradation (Zhao et al., 2000).

At the transcriptional level survivin promoter is regulated by β-catenin activated transcription factor, Sp1 and Stat3, being Sp1 the dominant regulator (NGan et al., 2008)

At the post-transcriptional level, survivin is finely regulated by phosphorylation. Survivin function depends on its phosphorylation of Thr 34 by Cdc2 kinase, which renders survivin more stable and less sensitive to proteasome degradation. The lack of survivin phosphorylation on this residue leads to the dissociation of the caspase 9-complex with XIAP-survivin and the subsequent activation of caspase-9 mediated apoptotic pathway (O'Connor et al., 2000). Heat shock protein 90 (Hp90) also binds and stabilizes survivin as the disruption of the complex induces survivin degradation by proteasome (Fortugno et al., 2003). On the other hand, as the other IAP family member XIAP, survivin is able to form a complex with Smac-DIABLO. This binding leads to the activation of apoptosis

both due to sequestration of survivin and activation of caspases (Obiol-Pardo et al., 2008).
Lastly, survivin gene is alternatively spliced to give rise to several isoforms, with either anti- and pro-apoptotic properties (Sampath and Pelus, 2007). Their functions and localization will be discussed later.

2.3 Survivin compartmentalization

One distinguishing feature in survivin biology, (that could be considered a regulatory mechanism), is its intra-cellular compartmentalization. In particular several pools of survivin have been identified, including nuclear, cytoplasmic and mitochondrial pools (Fig. 4) (Altieri, 2008).
Survivin is localized at the centromeres at the beginning of mitosis and re-localizes to the overlapping microtubules of the spindle midzone in anaphase. In telophase, Survivin is localized at the midbody and is degraded after cytokinesis (Uren et al., 2000). In interphase, survivin is mainly localized in the nuclei of the cells, showing immuno-chemically and post-translationally unique characteristics. Nuclear survivin began to accumulate in S phase whether cytosolic survivin increases at mitosis (Fortugno et al., 2003). Although this fraction of survivin may lack anti-apoptotic properties (Connell et al., 2008), it is correlated with poor cancer prognosis and seems to be essential for the tumor-promoting activity of this protein (Knauer et al., 2006). In the nuclei of the cells survivin promotes G1-S transition by inducing pRb inactivation (Connell et al., 2008). This correlates with the assumption that survivin

translocation to the nucleus correlates with tumor cell dedifferentiation (Moon and Tarnawski, 2003).

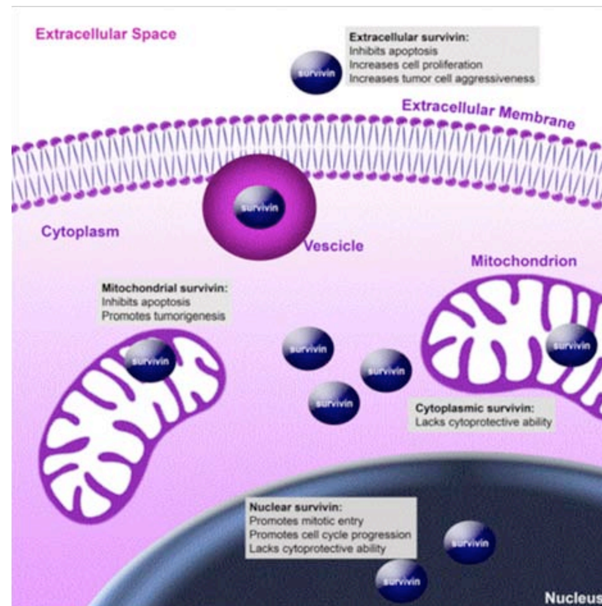


Fig. 4: Survivin pools exert different functions

Monomeric survivin can be exported form the nuclei to the cytoplasm by Crm1, an export receptor (exportin) (Engelsma et al., 2007). In the cytoplasm survivin localize on the microtubules of the mitotic apparatus participating in the assembly of a bipolar mitotic spindle (Fortugno et al., 2002). The cytoplasmic pool of survivin acts as a member of the chromosomal passenger complex (CPC) and as a regulator of microtubule dynamics. The CPC corrects attachment errors between chromosomes and the mitotic spindle, regulates the quality-control checkpoint, and ensures the correct completion of cytokinesis (Lens et al., 2006). Survivin physically and functionally interacts with several members of the CPC in a stable manner. In cells in which the CPC is dirsupted, survivin adheres to the chromosomes and no longer concentrates at the centromeres or transfers to the anaphase spindle midzone

(Wheatley et al., 2001). There are also evidences of direct association of survivin with the polymerized microtubules of cells undergoing mitotic division (Altieri, 2006). Survivin is in fact necessary for the correct localization of CPC proteins to the kinetochores (Lens et al., 2006).
Another important pool of survivin is localized in the mitochondria, especially in tumor cells (Dohi et al.,2007). There is experimental evidence that support that each compartment is semi-autonomous and independently regulated (Altieri, 2008). The mitochondrial pool of survivin is a clear example. Survivin is actively imported in the mitochondria by physical association with Hsp90, and seems to specifically inhibit apoptosis (Ghosh et al., 2008). In the mitochondria, survivin binds Smac (Sun et al., 2005) sequestering it away from XIAP (Song et al., 2003). This mechanism stabilizes XIAP which in turn inhibits caspase activation.

2.4. Survivin splicing

Another regulatory mechanism of survivin is the alternative splicing of *survivin* human gene, which produces 4 known variants: survivin-2B, survivin-ΔEx3, survivin-2α, and survivin-3B (Mahotka et al., 1999; Mahotka et al., 2002; Caldas et al., 2005; Badran et al., 2004).
Wild type (WT) survivin transcript contains 4 exons and produces a 431 bp protein. Survivin-2B has an additional exon of 23 amino acids, exon 2B, inserted in its BIR domain, which results in a 500 bp protein. Survivin-2B is

localized both in the mitochondria and in the nuclei of the cells, through the Crm1 transporting pathway (Knauer et al., 2007). Interestingly, survivin-2B is also localized in the microtubule organization center (MTOC) and, in contrast to other survivin isoforms, behaves as a pro-apoptotic molecule (Ling et al., 2007). Forced expression of survivin-2B induces mitochondria-dependent apoptosis indicated by Smac release from mitochondria, activation of caspases 9 and 3, loss of mitochondrial potential, down-regulation of Bcl-2 and up-regulation of Bax. Survivin-2B is also able to antagonize WT-survivin action by competing to the binding of polymerized tubulin suggesting that the two isoforms function by regulating each other (Islam et al., 2000). Survivin-2B pro-apoptotic activity correlates with its expression which is significantly decreased in late stage tumors, suggesting a potential inhibitory role of survivin-2B in cancer development (Mahotka et al., 2002). Being WT-survivin and survivin-2B expression regulated by p53, it has been hypothesized a regulatory mechanism driven by p53, which influence the balance of the two proteins depending on the status of the cell. This balance could be also involved in cancer progression (Islam et al., 2000).
Survivin-ΔEx3 omits exon 3 causing a frameshift that result in a COOH terminus with no homology to wild type survivin. Evidence suggests that this protein, together with Survivin-2B, is mainly involved in inhibition of apoptosis, rather that cell cycle regulation (Noton et al., 2006). Survivin-ΔEx3 preferentially localizes in the nucleus during late G1 to G2 phases of the cell cycle but is also localized in the cytosol and mitochondria of some cells (You et al.,

2006). WT-survivin and survivin-ΔEx3 form heterodimers, thereby regulating the balance between proliferation and cell death (Caldas et al, 2005). In the mitochondria survivin-ΔEx3 binds to Bcl-2 and to activated caspase-3, acting as an adaptor; the formation of this complex allows Bcl-2 to inhibit the activity of caspase-3. This variant is also able to maintain the mitochondrial transmembrane potential and to prevent the translocation of cytochrome c in the cytosol and activation of the apoptotic cascade (Malcles et al., 2007). Survivin-ΔEx3 is over-expressed in many tumors and seems to contribute to tumorigenesis by protecting malignant cells from apoptosis (Mahotka et al., 2002). Moreover Survivin-ΔEx3 may regulate angiogenesis via several mechanisms including cell invasion, migration, and Rac1 activation (Cladas et al., 2007).

Survivin-2α consists of 2 exons: exon 1 and exon 2, as well as region of intron 2. Acquisition of a new in-frame stop codon within intron 2 results in a truncated protein of about 8.5 KDa. Survivin-2α is expressed at high levels in several malignant cell lines and primary tumors. Functional assays show that survivin-2α attenuates the anti-apoptotic activity of WT-survivin by physically interaction (Caldas et al., 2005). In cells undergoing mitosis, survivin-2α is confined to the cytoplasmic compartment. Interestingly, when co-expressed with survivin, survivin-2α co-localizes with WT-survivin to the centromeres of the chromosomes in pro-metaphase, metaphase, and at the midbody during late telophase/cytokinesis. Moreover, the normal cytoplasmic localization of survivin shifted to the nucleus in interphase cells thus supporting the hypothesis that

survivin-2α exerts pro-apoptotic properties by destabilizing WT-survivin localization (Caldas et al., 2005).
Survivin-3B consists of 5 exons including novel exon 3B derived from a portion of intron 3. Acquisition of a new in-frame TGA stop codon within the novel exon 3B predicts a truncated 120 amino acid protein. As WT-survivin and survivin-2B, this variant maintains the nuclear export signal and is therefore also localized in the nuclei of the cells. Recent reports suggest that survivin-3B has cytoprotective functions and, unlike the other variants, is able to bind the CPC proteins (Knauer et al., 2007). The anti- or pro-apoptotic properties of survivin-3B have not been fully elucidated yet, but its structure suggests that it could exert anti-apoptotic properties. In fact, survivin-3B maintains the BIR domain that is sufficient to inhibit apoptosis in many cellular systems (Takahashi et al., 1998).

2.5. Survivin functions

Survivin is a multi-functional protein. Several studies suggest that survivin has definitely a role in the regulation of cell division. On the contrary, survivin role as inhibitor of apoptosis is still unclear.
Survivin role in cell division is unanimously accepted and is correlated with survivin expression pattern during the cell cycle. In particular survivin is highly expressed during G2/M phase of the cell cycle both in normal (Li et al., 1998; Kasof and Gomes, 2001) and tumor cells (Reed, J.C., and Reed, S.I. 1999). Its expression decreases during

G1 phase. At the beginning of mitosis, survivin is mainly divided in two pools. One pool of survivin is bound to polymerized tubulin on the centrosomes, microtubules and the remnants of the mitotic apparatus. A second pool of survivin localizes to the kinetochores of metaphase chromosomes and is associated to AuroraB kinase, INCENP and borealin (CPC proteins). De-stabilization of survivin interaction with microtubules leads to loss of apoptosis inhibition and increase in caspase 3 activity (Li et al., 1998). Moreover, survivin inhibition *in vitro* leads to multipolar spindle formation, thus resulting in multinucleated cells (Chen et al., 2000). In the survivin knock out (KO) mouse model, problems in chromosome segregation and cytokinesis leads to embryo lethality; all null embryos became grossly affected by day5.5 (Uren et al., 2000). Early signs of embryo deterioration include degenerating blastomeres, micronuclei formation, variable nuclear sizes, irregular nuclear morphology and multinucleation. These aberrations are characteristic of an underlying defect in mitosis. As the phenotype progressed, it revealed the absence of normal mitotic spindle structures and intercellular midbodies, with reduced microtubule networks around the cells, and bundling of microtubules. Survivin localization at this level is therefore not casual and extremely important for the fate of the cells. Recent works report that survivin mediates the proper targeting of the CPC proteins to kinetochores and stabilizes the microtubules in order to have a correct bipolar spindle formation (Altieri, 2006).

On the other hand, survivin role in <u>apoptosis inhibition</u> is still controversial. Survivin truncation at the C-terminus

leads to loss of cyto-protection (Fengzhi Li et al., 1998). Survivin inhibition results in increased caspase 3 activation in G2M phase, supporting a putative role for this protein in blocking apoptosis (Li et al., 1998). On the contrary, survivin inhibition in vertebrate cells induces an increase of multi-nucleated, but not apoptotic cells (Kallio et al., 2001). Initially survivin was postulated to selectively bind caspase 3 and 7, being directly involved in apoptosis control (Tamm et al., 1998). However, this model was challenged by the observation that survivin lacks the structural motifs that confer the ability to other IAPs to bind caspases (LaCasse et al., 1998) Later experiments reported that survivin is able to interact with caspases and blocks apoptosis *in vitro* and *in vivo* only when is in complex with XIAP, or other proteins such as HBXIP (hepatitis B X-interacting protein) (Dohi et al., 2004). Moreover, the mitochondrial pool of survivin seems to be the fraction responsible for apoptosis inhibition by sequestering Smac (Dohi et al., 2004; Song et al., 2003).

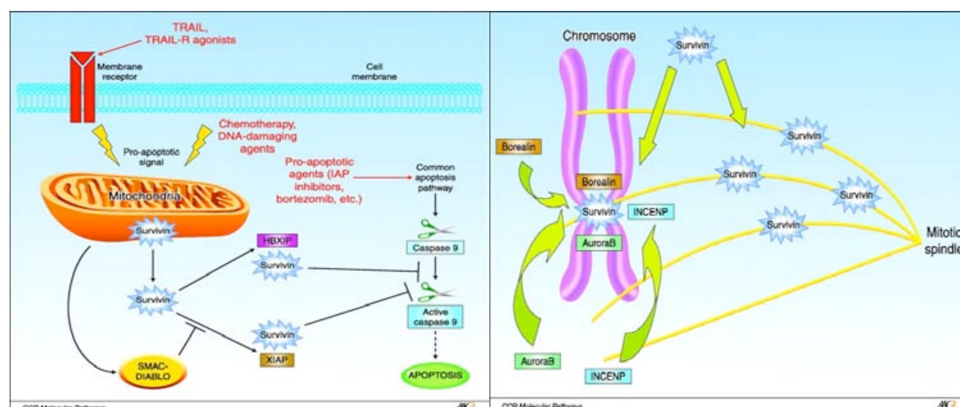


Fig. 5. Survivin as inhibitor of apoptosis and as cell cycle regulator

Survivin is also involved in apoptosis inhibition independently from caspases activation (Fig.5).

Altogether these data support the concept that survivin is necessary for proper cell division. Survivin influences apoptosis by being an important and necessary factor for the stability of the cells and guaranteeing a correct cell cycle progression, whether than directly acting as an inhibitor of apoptosis.

2.6. Survivin in the skin

As previously reported, survivin is expressed at low levels in normal adult human tissues. In early experiments, survivin expression was not detected in normal human adult skin (Grossman et al., 1999a) whether was highly expressed in the skin of fetus (Adida et al., 1998). Survivin over-expression was detected in non-melanoma skin cancers and in a keratinocyte cell line (HaCat cells) (Grossman et al., 1999b). Later experiments showed that survivin is expressed in normal human skin, in the basal layer of epidermis but not in the super-basal layers (Chiodino et al., 1999; Botchkareva et al., 2006). Transgenic mice expressing survivin in skin developed normally, without histological abnormalities or epidermal hyperplasia. Survivin over-expression did not affect keratinocyte proliferation both in homeostatic conditions and under UVB radiation. In contrast, in this mouse model, survivin expression inhibited UVB-induced apoptosis *in vitro* and *in vivo* cooperating with p53 (Grossman et al., 2001).

Being UV radiation one of the most important causes of skin cancer, and being survivin involved in UVB-induced apoptosis, several groups evaluated survivin function in both melanoma and non-melanoma skin cancers. UVB

radiation seems to rapidly up-regulate survivin expression in human keratinocytes (Aziz et al., 2004). Indeed, survivin is highly expressed in keratinocytic neoplasms and hyper-proliferative lesions such as squamous and basal cell carcinomas and psoriasis (Grossman et al., 2004; Abdou and Hanot, 2008). In skin cancer survivin seems to protect the keratinocytes from apoptosis thus leading to accumulation of mutations, malignant conversion and cancer initiation (Zhang et al., 2005).
Moreover survivin immuno-reactivity was detected in benign and malignant melanocytic lesions, with strong nuclear expression in invasive lesions of melanomas (Chiodino et al., 1999; Ding et al., 2006). Down-regulation of survivin in aggressive human melanoma cell lines resulted in increased apoptosis in these cells (Grossman et al., 1999b). Inhibition of survivin has been revealed to be a good method to sensitize aggressive cancer cells to apoptosis. In melanoma, for example, the use of survivin-specific inhibitors induces an increased sensitivity of the cells to radiotherapy (Pennati et al., 2003).

2.7. Survivin in diseased skin

Survivin is overexpressed in the majority of human cancers, including that of lung, colon, huterus, brain and ovary, as compared with the normal counterpart (Altieri et al 2001).
Indeed, survivin appears to be an important prognostic marker and/or chemoresistance predictive factor in several human tumors, in that its high expression levels in neoplastic tissues often correlate with poor prognosis

(Zaffaroni et al, 2005; Su et al, 2010; Taubert et al 2010; Xiaoyuan C et al, 2010)

In particular, the nuclear pool of survivin, rather than cytoplasmic survivin, can function as an independent prognostic factor for the clinical outcome of some human cancers and can correlate with tumor resistance to common therapeutic approaches. However, this effect seems to be tumor-type dependent, as nuclear survivin is reported to be an unfavourable prognostic factor in only some tumors (Moon et al, 2003) whereas it seems to indicate a favourable prognosis in other form of cancers, such as breast and gastric carcinomas (Okada et al, 2001; Kennedy et al, 2003)

This is in line with what happens in the regression of cutaneous T- cell lymphoma, a disease characterized by by migration of mutant T lymphocytes to the skin. In this type of cancer, nuclear survivin is predictive of systemic disease being associated with tumor progression (Goteri et al, 2007).

Because the two main pools of survivin are apparently independent in terms of mechanism of action and differentially important for cancer development and recurrence, the subcellular distribution of survivin is of clinical relevance (O'Connor et al, 2000; Xia et al, 2006; Altieri et al 2006). Consistently, the recently discovered mitochondrial pool of survivin seems to have an important role in cancer biology. Indeed, mitochondrial survivin protects cancer cells from apoptosis and promotes tumor formation (Dohi et al, 2004a; Dohi et al, 2004). Similarly, extracellular survivin has been shown to have an intriguing role in cancer maintanance. Tumor cell are able to release

survivin in the extracellular space via exosomes (Khan et al, 2011). Here, it is promptly captured by neighboring cancer cells, influencing and enhancing their proliferation, invasive capacity, and resistance to therapy (Mera et al 2008; Aspe et al, 2009).
Normal stromal cell do not absorb extracellular survivin, suggesting a tumor-specific mechanism of auto-control.
Cutaneous tumors include both nonmelanoma and melanoma skin cancers. In both types of cancer, survivin is overall upregulated compared whit normal skin (Chiodino et al, 1999; Lo Muzio et al, 2001; Bowen et al, 2004; Park et al, 2004; Bongiovanni et al 2009).

3.0. Skin Cancer

Skin cancer is the result of a malignant transformation of skin cells. Skin cancers are the fastest growing and the most commonly diagnosed type of cancer in the United States. Their incidence is alarmingly rising worldwide as a consequence of, among other factors, increased sun exposure. There is persuasive evidence that UVB radiation is involved in the initiation, promotion and progression of all the types of skin cancers (Brash et al., 1991).
Basal cell Carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma comprise the three most common types of cancer.
BCC and SCC are the so called “Non-melanoma skin cancers” and are the most common; on the contrary melanoma is the least frequent but is potentially the most serious. They respectively account for 80, 16 and 4% of all skin cancers (Bowden et al., 2004).

Between the non-melanoma skin cancers, SCCs are the least common but they carry a major risk of metastasis.

3.1. Squamous cell carcinoma

Squamous cell carcinoma is a malignant epithelial tumor which originates not only in epidermis but also squamous mucosa or areas of squamous metaplasia. Most SCCs develop from precursor lesions such as actinic keratosis (AK). Metastases, when they occur, are generally to regional lymph nodes, and are detected 1–3 years after initial diagnosis.

SCC derives from hyperproliferation of keratinocytes residing in the inter-follicular epidermis. Upon UVB irradiation, human keratinocytes can acquire several genetic alterations that lead to SCC formation. Aneuplidy is found in some SCCs (Robinson et al., 1996) and Ras mutations are present in 0-46% of human SCCs (Van der Schroeff et al., 1990). In addition, half of the diagnosed SCCs have p53 mutations and some of them (about 20%) have $p16^{INK4\alpha}$ or $p14^{ARF}$ mutations. It has been supposed the existence of a multi-step process for SCC formation, but the mechanism is still unclear.

Most of all, it is not clear which population of keratinocytes generates and maintains human SCC. Under UVB-induced skin wounding, the keratinocyte can acquire mutations and generate skin cancer if the DNA-damage is not repaired properly. It has been demonstrated that the wound is also replenished by bone marrow derived stem cells that migrate from bone marrow to the skin (Nygren et al., 2004). Nevertheless, these cells are not responsible for the

generation of skin cancer (Ando et al., 2009). Recently, the concept of stem cells at the origin and maintenance of cancer has been demonstrated to be a good model for some human tumors (Nakshatri et al. 2009, Altaner 2008 are two examples).

4.0. Survivin and SCC

Survivin is overexpressed in human and mouse SCCs as compared with normal skin (Bowen et al., 2004; Bongiovanni et al., 2009), and correlates with tumor aggressiveness and lymph node metastasis (Lo Muzio et al., 2001). This is also observed in head and neck SCCs, where survivin overexpression correlates with tumor progression and resistance to therapy. Survivin is expressed both in benign precancerous lesions, such as actinic keratosis, and in the actual SCC (Park et al., 2004), although in tumor lesions its expression is more pronounced. In contrast, human basal cell carcinomas weakly express survivin (Chiodino et al., 1999; Bowen et al., 2004; Park et al., 2004), suggesting the presence of a tumor-specific expression pattern of survivin in the skin. In sebaceous neoplasms, such as adenomas and carcinomas, only nuclear survivin is expressed, and it increases in aggressive tumors (Calder et al., 2008). In line with these findings, nuclear survivin seems to be the predominant form, being expressed in virtually 100% of human cutaneous SCC lesions, whereas cytoplasmic survivin is present in less than one-third of the cases (Bongiovanni et al., 2009).

In cutaneous SCC carcinogenesis, survivin seems to have a

crucial role. UVB, as one of the major causes of mutations in the skin, favors the survival of p53-mutated clones, whereas it induces apoptosis of normal cells. The proliferative advantage of mutated cells allows the colonization of the surrounding space leading to amplification of p53-mutated cells. When survivin is overexpressed in keratinocytes, no changes in cell differentiation and proliferation are observed.
On the other hand, survivin expression further suppresses apoptosis, increasing the number of neo-formed p53-mutated clones following UVB irradiation (Zhang et al., 2005).
Interestingly, expansion of p53-mutated clones is impaired by survivin overexpression in mouse keratinocytes. This is in line with the reduced susceptibility to form papillomas of K14-survivin transgenic mice (Allen et al., 2003; Zhang et al., 2005). These data suggest that the antiapoptotic function of survivin favors the formation of mutated clones, yet inhibiting their expansion. However, once the tumor is formed, survivin is able to convert benign lesions to invasive SCCs (Allen et al., 2003), thus having a key role in the progression of the disease.
In tumor cells, survivin is highly expressed, sustains proliferation, and protects from apoptosis. Common cancer treatments induce apoptosis by downregulating survivin expression (Roy et al., 2009). In line with this finding, resveratrol, a grape-derivative protective factor against tumor formation, reduces survivin upregulation following UVBinduced carcinogenesis (Aziz et al., 2005). It remains to be determined whether survivin also changes its intracellular localization during apoptosis in cancer cells.

We have preliminary data showing that in cutaneous SCCs, survivin shifts from the nucleus to the cytoplasm after treatment with imiquimod (Figure 3a–e). Survivin expression and function being tightly correlated is of paramount importance to elucidate the subcellular localization of survivin before and after treatment of cutaneous lesions.

5.0. Cancer stem cells (CSC)

There are two hypotheses that explain tumor progression (Fig. 6). The first one, the stochastic hypothesis, assumes that any cell within a tumor are potentially tumorigenic; it proposes that all cells within a tumor is relatively homogenous. The second, the so called hierarchical hypothesis or cancer stem cells (CSCs) hypothesis, suggests that in a heterogeneous population of cancer cells, only a small fraction of cells is able exclusively regenerate the tumor in its full complexity and heterogeneity upon transplantation. These cells share with normal stem cells some characteristics, such as the self renewal capacity and the ability to generate a differentiated progeny. For these reasons the subpopulation of cancer initiating cells has been named Cancer stem cells population (Schwartz- cruz y celis A and , Melendez Zajgla J, 2011).

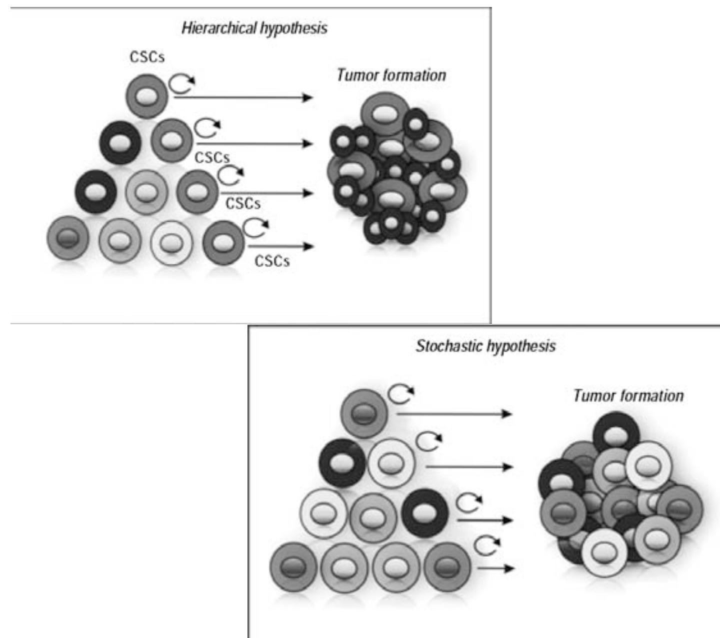


Fig 6: stochastic and hierarchical hypothesis

The idea of the cancer stem cells arose from the observation of striking similarities between the self renewal mechanism of stem cells and cancer cells (Singh SK et al, 2003).

CSC could originate by mutations in undifferentiated tissue stem cells, progenitor cells, and differentiated cells. (Singh SK et al, 2004; Jordan CT et al, 2006; Charafe-Jauffret E et al, 2009). A mutational events occurring in a progenitor cell may not be as dangerous as in the stem cells, because this cell has limites self renewal ability and it becomes clonally exhaustes as it generates differentiates cells (Singh SK et al, 2004). Additionally, it has been proposed many hypothesis concerning the origin of CSCs (Bosch J et al, 2007, Clevers H et al, 2005):

- loss of regulation of the microenvironment (including the niche)

- loss of asymmetric division. Polarity loss may lead to the lack of this type of division and the consequent accumulation of these cells. it is thought that they only divide symmetrically
- cell fusion
  - horizontal gene transfer. The stem cells have the capacity of introducing apoptotic bodies that re-programs their genetic load, turn it in tumorigenic (Fig 7) (Schwartz- cruz y celis A and , Melendez Zajgla J, 2011).

The genetic program that controls self-renewal and differentiation plays a key role in the generation of CSCs. It is known that as stem cells, CSCs have the ability to proliferate indefinitely by deregulated self-renewal capacity (Singh SK et al, 2004).

Progenitor cancer cells, which represents most of the tumor population, have a high rate of proliferation (Dontu G, 2008) and, unlike CSCs that have a stem phenotype and a small number of cells within the tumor (0,01-1%), together with a smaller rate of proliferation (Visvader JE et al, 2008). It has been suggested that eliminating this differentiated progeny and maintaining CSCs will result in tumor relapse (Dick JE et al, 2003).

Five key observation define the existence of a CSC population (table) (Dalerba P et al, 2007):

- only a minority of cells within a tumor have tumorigenic potential when transplanted into immunodeficient mice (Dalerba P et al, 2007)
- they self renew and proliferate (Sing SK et al, 2003; 2004)

- they generate clusters of clonally derived cells resembling spheres (Sing SK et al, 2003; 2004)

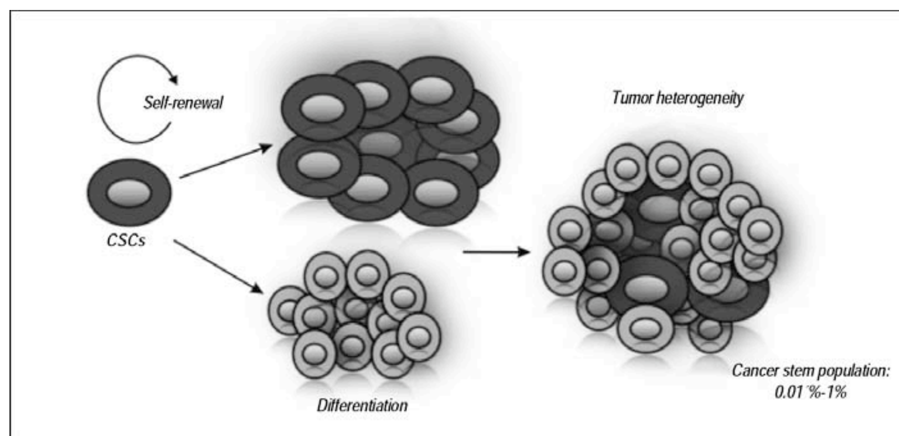


Fig 7: Characteristic of CSCs: differentiation and self-renewal

- tumorigenic cancer cells are characterized by a profile of specific surface markers and can be isolated by flow cytometry or other immunoselection methods (Dalerba P et al, 2007)
- the tumor mass derived from tumorigenic cancer cells contains mixed population of tumorigenic cancer cells and non tumorigenic, recreating a phenotipically heterogeneous population (Cheshier et al, 2009)

This process is very similar to the process where somatic stem cell can both self-renew to maintain stem population and also produce differentiates progeny to create specific mature cell types of an organ (Cheshier et al, 2009).
The symbiotic relationship with the niche is totally perturbed in CSCs, and it remains incompletely understood (Polyak K et al, 2006). The interaction within may have an important role in maintaining a population of tumor cells with characteristics of self-renewal (Sneddon JB et al, 2007).
The CSCs can be defined experimentally for their ability to continuously generate tumors. Today, the practical definition and the gold standard to define the stemness of the cancer cells has been the ability to generate a phenocopy of the original malignancy in immunocompromised mice (Ponnusamy MP et al, 2008).
Scs are rare in most tissues, therefore, they need to be identified prospectively and purified to study their properties (Reya T et al, 2001). Nowdays, SCs are recognized by their immunophenotypic profile, for example hematopoietic SCs express CD34 y CD90, neuronal stem cells CD133 and nestin (Mayani H et al, 2005; Singh SK et al, 2003; 2004).an important marker for isolation and analysis of SCs is conferred by ABC transporters that gives them the property of drug transporting cells. most cells accumulate fluorescent dyes like Hoechst 33342 and rhodamine 123, but SCs do not, since these dyes are expelled by ABCG2 and ABCB1 among others. Because they don’t accumulate these compounds, SCs can be sorted by collecting cells that contain a low level of

Hoechst 33342 fluorescence. These cells are known as “side population” or “dull cells” (Dean M et al, 2005).

Another type of isolation is the use of sphere culture system, first use by Reynold and Weiss in 1992 to identify NSCs. This method has permitted the in vitro characterization of SCs, establishing that the neurospheres are multi-potential floating clusters cells and are derived from clonal expansion of a single NSC (Singh SK et al, 2004). This culture system has also been used to identify other SCs like mammary stem and progenitor cells (Charafe-Jauffret E et al, 2009)

Despite in cutaneous squamous cell carcinoma the presence of a stem cells population has not been determined, several studies suggest that SCC can be derived from normal stem cells. It has been reported that β-catenin signal is important in SCC formation and maintenance. Moreover, a population of stem cells, maintained by this pathway in mouse SCC, has follicular bulge stem cells characteristics (Malanchi et al., 2008). Moreover, K19, a marker of epithelial stem cells, is over-expressed in cutaneous SCC compared to normal skin (Chen et al., 2008).

Altogether these studies suggest the presence of a stem cell-like population in SCC of the skin.

The successful elimination of cancer requires an anti tumor therapy that affects the differentiated cells as well as the cancer stem cell population (Chumsri S, et al 2007; Klonisch et al, 2008). It is thought that quiescent CSCs are more resistant to chemotherapy and to targeted therapies (Jordan CT et al 2006; Visvadert JE et al, 2008); this may

be due to intrinsic or extrinsic resistance that favors the selection of stem clones able to survive therapy. Today, conventional cancer therapies including surgery, chemo, radio and immunotherapy quickly eliminate differentiated tumor cells, reducing the tumor mass, but in general, leaving the potentially initiating tumor cells. (Chumsri S et al 2007; Singh SK et al, 2007; Klonisch T et al, 2008).
An ideal course of therapy should eliminate the differentiated cancer cells while at the same time, specically, selectively and rapidly eliminate also the CSCs, thus, avoiding toxic side effects to other cell types (Klonisch T et al, 2008).
This will require specific identification of therapeutic targets that occur exclusively in CSCs (Majeti R et al, 2009).

6.0. In vivo cancer model

Cancer is a multistep process requiring a series of intermediate steps, each accompanied by biochemical, morphological and cytological changes. Animal models of human cancer have evolved in attempts to capture the complexity of the human disease and to provide a new platform for cancer research. A variety of in vivo models are now available each with specific advantages and drawbacks.
Traditional in vivo models relied on the use of chemical carcinogenesis in mice (Khavari PA, 2006). In details, this is a multistage chemically induced carcinogenesis (Kemp CJ et al, 2005). In the first step, called initiation, mice are treated with a low dose of the mutagen 9,10-dimethyl-1,2

benzanthracene (DMBA). In the second step, called promotion, mice are treated continuously with a drug that stimulates epidermal proliferation, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA). During promotion, benign tumors (papillomas) arise, probably as a consequence of additional mutation, some of which will progress into invasive SCC. Using this protocol, papillomas contain activating mutations in HRas gene, suggesting that this mutation confers a selective advantage to epithelial cells (Quintanilla M et al, 1986).

Injection of cloned mouse and human cancer cell-line xenograft into immunodeficient mice has also been used extensively. These cell lines, however, have commonly undergone multiple adaptions to long-term cell culture, compromising the accuracy with which they represents cancers that arise spontaneously in a patient. In spite of advances in a diverse array of model systems, basic questions about cancer formations are unresolved and many cancer therapeutics fail during development, highlighting the need for additional, relevant model systems in which to study human cancer (Khavari PA, 2006).

More recent transformations studies of human mesenchymal and epithelial cells have used ectopic injection of large numbers of cells into the subcutaneous space of nude mice as the central tumorigenesis model (Gupta BP et al 2006; Elenbaas B et al, 2001). However, this is an artificial microenvironment that is not where the studied tumour originally developed, and represents a tissue setting in which spontaneous tumorigenesis never occurs in human.

Moreover, subcutaneous tumor formation does not model invasion well because it lacks an intact epithelial basement membrane zone and an organized underlyng extracellular matrix.

Model	Tranformed cell type	Genetically defined	Intact immunity	Intact 3D tissue
3D *in vitro* models	Mouse or human cells	Possible	No	No*
Injected xenograft	Human cell lines	Possible	No	No
Injected autograft	Mouse cell lines	Possible	Yes	No
Chemical carcinogenesis	Mouse or human tissue‡	No	Yes/no	Yes
Radiation carcinogenesis	Mouse or human tissue‡	No	Yes/no	Yes
Murine genetic models	Mouse tissue	Yes	Yes	Yes
Regenerated human tissue§	Human tissue	Yes	No	Yes

Table 1: types of experimental cancer models

Khavari's staff have recently shown that multiple cancer-relevant genes can be efficiently introduced into primary human cells, and that this can be coupled with tissue rigeneration on immune-deficient mice to produce architecturally accurate human skin malignancies (Fan et al, 1997; Chudnovsky et al, 2005). In this model, human primary keratinocytes are retrovirally transduced to overexpress two genes: on one side, oncogenic Ras, which alone is not sufficient to generate epidermal neoplasia; on the other side , IkBa, the inhibitor of NF-kB, which is able, if over-expressed with oncogenic Ras, to overcome the cell cycle arrest that NF-kB would induce after Ras hyper-activation (Fig 8).

Cells that undergo high efficiency stable retroviral gene transfer rapidly generate three-dimensionally intact human dermis and then are grafted onto immune-deficient mice.

The double transduction results in large neoplasms similar to spontaneous human SCC when transduced keratinocytes are transferred onto immune-deficient mice (Khavari PA, 2003): growth arrest triggered by oncogenic

Ras is bypassed by IkBa-mediate blockade of NF-kB, generating malignant human epidermal tissue resembling SCC.

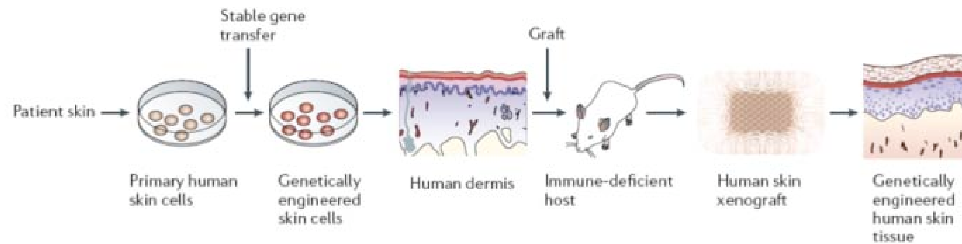


Fig. 8: Diagram of the experimental approach for generating genetically defined human tissue cancer

Human cell tumorigenesis is dependend on laminin 5 and a6b4 integrin. Thus, IkBa circumnvents restraints on growth promotion induced by oncogenic Ras and can act with Ras to induce invasive human tissue neoplasia (Khavari PA et al, 2003). Ras-IkBa epidermal tumors showed changes in protein expression that have been reported as characteristic for SCC, including increases in vascular endothelial growth factor (VEGF) and matrix metalloproteinases 3 (MMP3), and a decrease in E-cadherin. Ras-IkBa tumours also showed a more than tenfold increase in mitotic index.
Thus, Ras-IkBa epidermal neoplasia strogly resembles spontaneous human SCC.

Aim and Rationale

AIM AND RATIONALE

Survivin is mostly expressed in fetal and cancerous organs, while it is rarely detected in normal tissues. (Fukunuda S et al 2006; Jha K et al, 2009). We and others have recently confirmed cytoplasmic survivin expression in few basal keratinocytes. In particular, we have shown that survivin is mostly expressed in KSC (Marconi et al., 2007), that are considered to be the origin of SCC. When compared to normal human skin, both actinic keratosis (AK), *in situ* SCC and SCC overexpress survivin (Park et al, 2004). However, a complete evaluation of survivin expression in normal versus pre-cancerous and cancerous skin lesions is still lacking. It has been proposed that survivin serves as a prognostic marker for several cancers (Lo Muzio et al., 2001). Moreover, survivin expression seems to be correlated with skin cancer aggressiveness (Lo Muzio et al., 2001). Because survivin seems to have a key role in cancer progression (Lo Muzio et al., 2001), the possibility to characterize survivin-expressing cells in normal and pathologic human skin is challenging and has the potential to clarify the changes occuring in keratinocytes during the conversion towards the tumor phenotype. It has also been shown that survivin increases cell migration in melanoma cells (McKenzie JA et al, 2010).
The following specific aims were addressed:

to evaluate survivin cellular and subcellular expression in cancerous and precancerous lesions

to study the role of survivin in origin, development and aggressivness of skin cancer *in vitro.*

Patients and methods

PATIENTS AND METHODS

Patients and samples

For immunohystochemistry analysis, skin samples from normal individuals, AK, *in situ* SCC and WD, MD or PD cSCC were obtained from patients recruited at the Policlinico of Modena, Modena, Italy between 2009 and 2011. Inclusion criteria for lesional skin samples were the presence of the lesion in photo-exposed areas of the body, patients aged between 50 and 90 years old, no previous treatment for SCC.

For cell culture, tumor samples were obtained from MD and WD cutaneous SCC patients recruited between 2011 and 2012 at the Nuovo Ospedale Civile di Sassuolo, Modena, Italy. The Declaration of Helsinki protocols were followed and patients signed the informed consent from the Department of Dermatology and dermatological Surgery, approved by the Modena Commission of Ethics (protocol no. 184/10). Inclusion criteria were the presence of the lesion in photo-exposed areas of the body, patients between 50 and 90 years old, no previous treatment for SCC, lesion size equal or grater than 1cm diameter. Half of the sample was fixed for pathological confirmation. Only samples following the inclusion criteria were analysed.

Ex vivo studies

diagnosis	n°	well differentiated	moderately differentiated	poorly differentiated
actinic keratosis	24			
in situ SCC	18			
SCC	29	16	9	4

In vitro studies

PATIENT	DIAGNOSIS	AGE	LOCALIZATION	DIFFERENTIATION STATUS	INFILTRATION
#8	Bowen Disease	73	Leg	/	/
#9	SCC	89	Cheek	MODERATE	YES
#10	SCC	100	Noise	HIGH	YES
#13	SCC	84	Hand	MODERATE	YES
#14	SCC	84	Shoulder	POOR	YES
#16	In situ SCC	67	Face	/	/
#17	In situSCC	96	Cheek	/	/
#19	SCC	98	Hand	MODERATE	YES
#21	in situ SCC	82	Arm	/	YES
#25	SCC	84	calf	HIGH	YES

Cell culture

The spontaneously transformed keratinocyte line HaCaT was kindly provided by Dr N. Fusenig (DKFZ Heidelberg) and cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG) plus 10% foetal calf serum (FCS) as described (Boukamp et al, 1998). Total keratinocytes were obtained from normal human epidermis derived from young or adult individuals as previously described (Marconi et al, JID 2004). Briefly, total skin was treated with Dispase (Roche, Indianapolis, Indiana, USA) for 2h at 37°C. Epidermis was separated from the dermis, then minced and treated with a solution of trypsin/EDTA (0.05% trypsin, 0.02% EDTA (ethylendiaminetetraacetic acid)) at 37°C for 30'. Cells were 0.45 mm filtered and centrifuged at 1.200 rpm for 10 minutes. Total keratinocytes were then washed in PBS and lysed for Western Blotting analysis.

Primary cSCC cells were obtained from surgical excision of the affected areas. Keratinocytes were obtained from at least three different donors as previously described (Patel et al, 2011). Briefly, keratinocytes from MD or WD cSCC

were obtained mincing total skin, followed by incubation for 2 hours with Dispase (Roche) and Ultrapure Collagenase Type III (Sigma, St Louis, MO) at 37 °C. After removal of the supernatants, the remaining tissue was incubated at 37 °C for 20 minutes with trypsin 0.05% with EDTA 0.02%. Once total keratinocytes were extracted, cells were plated on Mitomycin-C treated J2-3T3 feeder layer at 10^5 to 3 X 10^5 per 100 mm dish density, in keratinocyte medium (Purdie et al, 2011). 7-10 days later, colonies start to be visible, SCC keratinocytes are detached from the plate and lysed for Western Blotting analysis.

Scratching assay

Mock and survivin overexpressing HaCaT cells or SCC primary human keratinocytes were trypsinized, counted and 50.000 cells were plated on six-well tissue culture plates and then treated with 5 mg/ml of mitomycin C for 2 hours. Twenty-four hours later, the cells were washed three times in serum-free medium and three lines for each well were drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with serum-free medium to remove all detached cells and incubated in serum-free medium. Cells were monitored at 48 hours from stimulation. The result of each experiment was expressed as the % of the mean of migrated cells from six different areas. The final results are expressed as the mean±SD of three different experiments. Student's t-test was performed for comparison of the means.

MTT assay

SCC primary human keratinocytes were plated in 96-well tissue culture plate (5000 cells / well). At different time points, proliferative cells were detected by incubating with MTT (Sigma-Aldrich) solution at 37°C for 4 h. They were solubilized with DMSO and the formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm. The results are expressed as optical density units or as viability percentage respect of control. Results are calculated as the mean ± SD of three different experiments. Student's t-test was used for comparison of the means.

Determination of Colony Forming Efficiency (CFE)

The ability of cells to form colonies *in vitro* is a frequently used tool to compare the proliferative capacity of subpopulations of cells. The CFE is determined not only on the bases of the number of colonies generated, but also of their size. The standard protocol considers cells with higher CFE as cells that have a greater proliferative potential. KSC are thought to retain high proliferative capacity and therefore show greater CFE when compared with differentiated cells (Tiberio et al., 2002).

SCC keratinocytes were detached from collagen coatings by treatment with a trypsin/EDTA solution (0.05%/0.02%). 3 dishes (9.6 cm^2) were plated for each cell population at a density of 100 cells per dish. Keratinocytes were cultured on mytomicin C-treated 3T3 cells ($2.4U10^4/cm^2$; ATCC) and cultivated in Dulbecco's modified Eagle's medium added with 10%FBS for 2 weeks.

Culture medium was changed after 6 days. Fourteen days later, dishes were fixed with 10% buffered formalin and stained with crystal violet. Colonies that contained more than 50 cells were counted and CFE was calculated. The colony number was expressed as a percentage of the number of cells plated in each dish. Results are calculated as the mean ± SD of three different experiments.

Isolation of human SCC subpopulation

For skin cancer studies, a SCC primary keratinocytes were cultured in serum-free media (KGM). To separate RAD and non-RAD human SCC cells, cultured cells were seeded onto collagen IV coated plate and separated on the basis of the expression of beta 1 integrin. Cells which are able to adhere in 5 minutes to collagen IV coated plate are considered RAD whereas not adhering cells were transferred to another coated plate and considered NRAD. The day after, both RAD and non-RAD cells were collected for the grafting system, lysed for western blotting analysis or reseeded for in vitro experiments.

Infection of HaCaT cells

A total of $16 \cdot 10^3$ /cm2 HaCaT cells were plated and 24 h later, infected twice with conditioned medium containing retrovirus for EGFP (Enhanced Green Fluorescent Protein) or survivin–EGFP (codified by pcz-CFG5.1–EGFP or pcz-CFG5.1-Survivin-EGFP retroviral vector a kind gift from Achim Temme, Technische Universitat Dresden, Germany) and polybrene to a final concentration of 0.8 lg / ml. Then, culture medium was changed, cells were trypsinized and the number of EGFP-positive cells was controlled by flow

cytometry. Only if the infection efficiency was at least 80%, HaCaT cells were used for the experiments. Survivin overexpression was controlled 24 h after infection by Western blotting, as described below. For the scratching assay, 16 · 103 /cm2 of infected cells were plated and 48h later were treated with mytomicin C, as described below.

BrdU analysis

To identify actively-cycling cell population, we have used BrdU Flow Kits (BD Pharmingen). SCC keratinocyte subpopulations were incubated with 10uM BrdU in culture medium for 24 hours. Then, BrdU-pulsed cells were trypsinized and transferred in flow cytometry tubes. Cells were fixed and permeabilized with BD Cytofix/Cytoperm Buffer for 20 minutes at room temperature, BD Cytoperm Buffer for 10 minutes on ice and BD Cytofix/Cytoperm Buffer for 5 minutes at room temperature. Subsequently, cells were treated with Dnase to expose incorporated BrdU for 1 hour at 37°C Fluorescent anti BrdU antibody was added for 20 minutes at room temperature. Finally, for cell cycle analysis, total DNA was stained with 7-AAD solution.
After every incubation, cells were washed with BD Perm/Wash Buffer 1X, centrifuged for 5 minutes at 300g and surnatanrt was discared.
Flow cytometric analysis was performed by Epic XL flow Cytometer (Coulter).

Production and maintenance of skin equivalents

Firstly, rat-tail collagen was extracted following the protocol from Rajan et al., 2006. Human fibroblasts were isolated from neonatal foreskin, cultured in DMEM

supplemented with 5% FBS, 1% Penicillin/Streptomicin/Amphotericin-B, 2% L-glutamine. Rat-tail collagen dermal equivalents were generated as follows. A cell-free solution consisting of DMEM supplemented with 10% FBS and rat tail collagen at the final concentration of 1.35 mg/ml, was seeded onto tissue culture inserts namely Corning Costar Transwell Supports (Biocompare, South San Francisco, CA, USA). A second collagen gel was prepared similarly by mixing 150,000 fifth or sixth passage fibroblasts with 1.35 mg/ml rat-tail collagen. This solution was added to the first and incubated for 4-5 days at 37°C. At this point, 250,000 transfected keratinocytes were seeded on top of dermal equivalents. After incubation for 1 h at 37°C to allow attachment of the cells, complete keratinocyte medium (DMEM/F-12 mixture 3:1, supplemented with 10% FBS, 2% L-glutamine, 1% Penicillin/Streptomicin/Amphotericin-B, hydrocortisone 0,4 μg/ml, insulin 5 μg/ml, adenine 1,8 X 10~4M, triiodothyronine 2 X 10~9M, cholera toxin 1 X 10~10M) is added to cover the surface of the gel. The skin reconstructs are maintained in submerged conditions for 4 days, followed by removal of the media from the top of the gels to allow air-liquid interface (emersion phase). At this point the medium is switched to the same as above, but that does not contain EGF, to preserve collagen degradation. The medium was changed every 2 days and skin equivalents were harvested after 14 days.

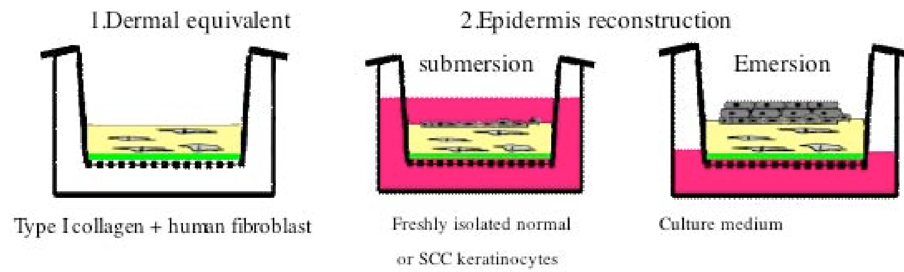


Fig. 9: Skin equivalent assesment

Squamospheres formation

SCC primary human keratinocytes were placed in culture plastic wares with nonadhesive surface. 10 cm dish are made of nonadhesive for cells by coating with 1% agarose SM/ML in DMEM. Cells were plated at the density of $15x10^4$cells/10cm dish and the culture medium was changed every other day until the sphere formation

Long term growth potential

After keratinocytes subpopulation isolation, keratinocytes were harvested by Trypsine/EDTA (0,05%/0,02%) treatment. 3600cell/cm2 were seeded on F25 flasks, collected at 80% confluence, counted and seeded onto new flask at the same density. Population doubling (PD) was calculated as follow:

$$PD=\ln (N/N_O)/\ln 2$$

Were N represents the number of the harvested cells and N0 is the number of the seeded.

The total number of the cell generated by each subpopulation was calculated as follow:

$$\text{Total cell number} = (Nt-Np)/Np$$

biological triplicate was performed and data are expressed as mean.

Survivin siRNA transfection

About 100 000 cells / well were plated on six-well plates in penicillin / streptomycin free medium. Twenty-four hours later, cells were transfected with 50 nm survivin-siRNA (Dharmacon Inc, Lafayette, CO, USA) or water as mock control, combined with Lipofectamine 2000 and Opti- MEM (both from Invitrogen Corporation, Carlsbad, CA) as datasheet suggests.

Transfection was controlled by Western blotting using anti-survivin antibody at 24, 48 up to 120 h, as described below.

Cell infection

Packaging lines for the described retroviral constructs H-Ras G12V, IkBaM and Bic Surv (figg. 10, 11, 12) were generated by transinfection in the ecotropic Phoenix and amphotropic GP.envAm12 cells, as previously described (Grande et al., 1999). HaCat cells were transduced by two cycles of infection (6 hours each) with viral supernatant in the presence of polybrene (8 mg/ml). Transduced cells were selected in the presence of 1 mg/ml puromycin (Sigma, St Louis, MO).

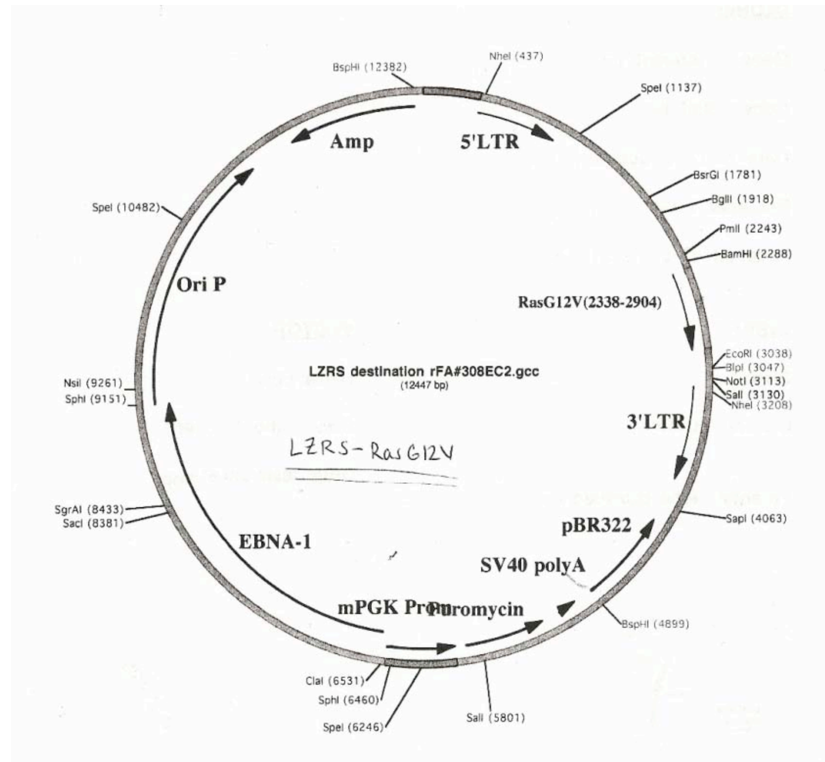


Fig.10: Ras G12V retroviral vector

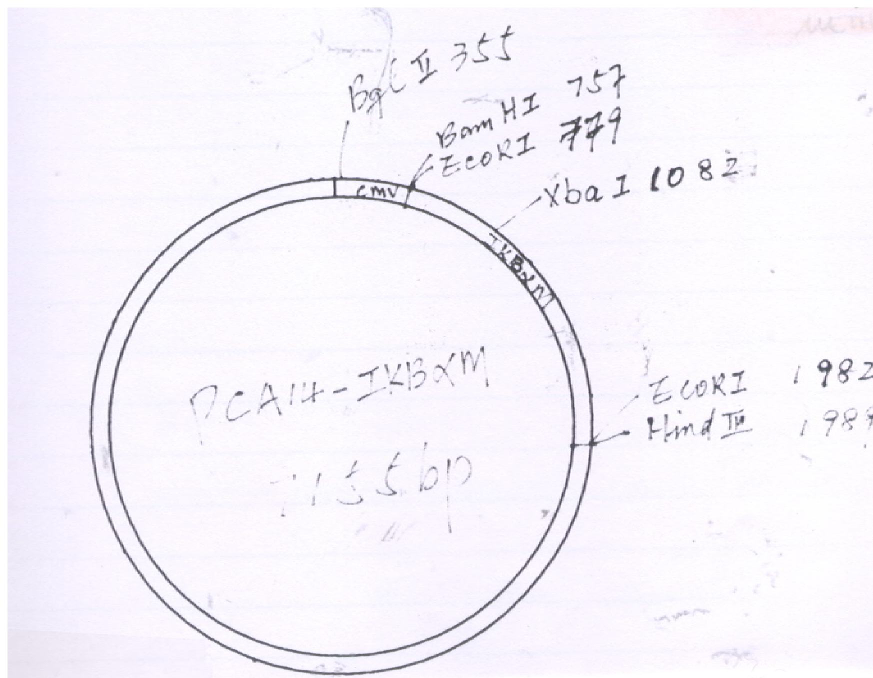


Fig.11: IkBalpha retroviral vector

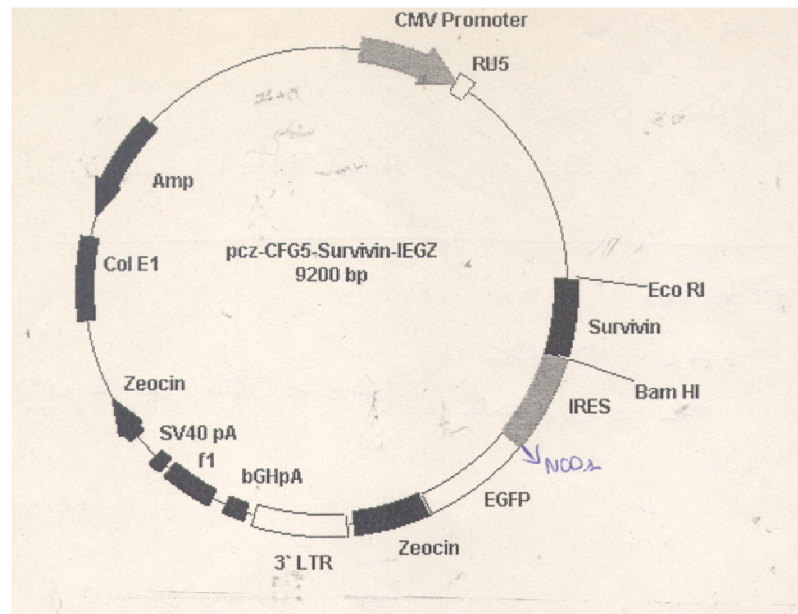


Fig 12: survivin retroviral vector

Immunohistochemistry

Four-micron sections cut from formalin-fixed, paraffin-embedded skin, were deparaffinized in xylene and rehydrated in graded alcohol. The slide were first boiled in citrate buffer, pH 6, for 20 minutes in a standard pressure cooker. Quenching of the endogenous peroxidase activity was obtained by treatment with 6% H_2O_2 in PBS. For single staining, samples were than incubated with 10g/ml anti survivin rabbit polyclonal antibody (anti- nuclear and anti-cytoplasmic survivin; Abcam) at 4°C overnight. After three 5-minute washes in PBS, slides were incubated with secondary antibody anti-rabbit biotinylated (DakoCytomation) at 1:300 dilution for 45 minutes at room temperature, followed by incubation with streptavidin–biotin peroxidase complex for 30 minutes

(BioSPA Division; Societa` Prodotti Antibiotici S.P.A., Milan, Italy, http://www.spaspa.it/biospa/home.htm) at 1:100 dilution. After three washes of 5 minutes in PBS, the reaction products were visualized with amino-methylenecarbazole. Double staining was continued by incubating the slides with 1% goat serum. Then the slides were incubated either with rabbit monoclonal anti-CK10 antibody (1 : 100; Epitomics, Inc. Burlingame, CA, USA), or with rabbit monoclonal anti-CK15 (1 : 100; Epitomics, Inc.) for 1 hour and, after three washes of 5 min in PBS, slides were incubated with secondary antibody anti-mouse (1 : 20; Dako) or anti-rabbit biotinylated (1 : 300; DakoCytomation) for 45 min at room temperature, followed by incubation with anti-mouse alkaline phosphatase complex for 30 min (1 : 20; Dako). After three washes of 5 min in PBS, the slides were stained with Fast blue (Sigma). Negative controls were obtained by omitting primary antibodies. For nuclei counting, tissue sections were counterstained with Harris hematoxylin (Sigma) for 20’’. Images were acquired at X 200 or 400 using a conventional optical microscope (Zeiss Axioskope 40). The results of the immunohystochemical staining were evaluated separately by two observers, in a blinded fashion. To quantitate nuclear survivin expression, two different calculation methods have been used:

3 to 5 pictures representative of the lesion, independently from survivin staining, have been taken. Both positive and negative areas for survivin are included in the calculation.

3 to 5 pictures of areas which display nuclear staining of survivin have been taken.

In both cases, the number of strongly or moderately stained nuclear-survivin positive cells were counted and normalized to the total number of hematoxilin-positive nuclei. 400 cells were examined in each area at X 400 magnification. The mean percentage of positive cells was determined. To evaluate the frequency of positive nuclei relative to basal cells, 300 basal cells were examined in at least 4 areas at X 400 magnification. The number of suprabasal and basal positive nuclei was counted and expressed as number of positive nuclei/basal cell. This was performed on at least 3 different sections of epidermis from adult and young donors and the results were reported as a mean. Cytoplasmic and nuclear survivin expression was scored semi-quantitatively as strong (+++), moderate (++), weak (+) or negative (-). Survivin distribution in lesional skin has been indicated as localized, when restricted to areas of the lesion, or homogeneous, when equally distributed.

Hematoxylin & Eosin Staining

Hematoxylin and Eosin staining is a popular staining method in histology. The staining method involves application of hemalum, wich is a complex formed from aluminium ions and oxidized hematoxylin. This colors nuclei of cells (and a few other objects, such as keratohyalin granules) blue. The nuclear staining is followed by counterstaining with an aqueous or alcholic solution of eosin Y, which colors eosinophilic other structeres in various shades of red, pink and orange.

Four micron sections cut from formalin-fixed, paraffin-embedded skin were deparaffinized in xylene, rehydrated

in graded alcohol and then rinse in distillated water. Slides were stained for 10 minutes with Ematoxylin solution, rinse in tap water and then stained for 5 minute in 90° Ethanol and for 15 minutes in Absolute Ethanol. Subsequently, they were rinsed for 5 minutes in Xylen, mounted with Eukit and observed at optical microscope.

Immunofluorescence staining

Four micron sections cut from formalin-fixed, paraffin-embedded skin were deparaffinized in xylene, rehydrated in graded alcohol and then rinse in distillated water. Slides were treated with cytrate Buffer for 20 minutes at 98°C and then equilibrated at room temperature for 20 minutes. Tissues were permeabilized with Tryton x100 for 5 minutes on ice and unspecific staining was blocked with 0,5% albumin-5% goat serum for 15 minutes at room temperature.

Tissues were incubated for 1 hour at 37°C with primary antibody: mouse anti survivin 1.250 (Thermoscientific); rabbit anti Ras 1:250; rabbit anti IkBalpha millipore 1:250; undiluited mouse anti keratin 34bE12 (Cell marque); anti rat CD51 1:50; mouse anti VEGF 1:50 (R&D system); rabbit anti ki67 1:200 (Epitomics). After three 5-minute washes in PBS, cells were incubated with Alexa Fluor 546 anti-rabbit (Invitrogen Corporation, Carlsbad, CA, USA) or Alexa Fluor 488 anti-mouse (Invitrogen Corporation, Carlsbad, CA, USA) for 45 minutes at room temperature. After three washes in PBS, the slides were stained for 5 minutes with 1 μg/ml Dapi (Sigma-Aldrich), washed and covered with coverslips. Micrographs were taken on a

Confocal Scanning Laser Microscopy (Leica TCS4D) (Leica, Exton,PA).

Statistical analysis

Statistical significance for nuclear survivin positivity was determined for each lesion type compared with normal or lesional skin using an unpaired 2-tailed *t* test. Significant differences were considered for $P<0.05$.

Western Blotting

HaCaT cells and total primary human keratinocytes, either obtained from adult or young specimens, were washed twice with PBS and lysed on ice separating nuclear and cytosolic components. Briefly, 1 million of cells of each cell type were lysed with Chemicon Nucelar Extraction kit (Chemicon International Inc., Temecula, CA, USA). Subcellular fractionation was performed following manufacturer's instructions (Chemicon64 #2900). Twenty micrograms from each lysate was analysed under reducing conditions for survivin, enolase and beta actin expression on 18% polyacrylamide gels, and blotted onto nitrocellulose membranes. To verify equal loading of total proteins in all lanes, the membranes were stained with Ponceau Red. The blots were blocked for 2 h in PBS blocking buffer (pH 7.4 with 0.05% Tween-20 and 5% non-fat dry milk). The membranes were incubated overnight at 4°C with anti-survivin rabbit polyclonal (1:1000; Abcam, Cambridge, UK), FABP5 goat antibody (1:1,000; R&D Systems, Inc. Minneapolis, MN, USA), or with an anti-human Cytokeratin 10 rabbit antibody (1:50000; Epitomics, Burlingame, CA, USA) or with an

anti-human involucrin mouse mAB (1:6,000; Sigma-Aldrich, Solms, Germany or anti-enolase goat polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a cytosolic positive control or with anti-b-actin, mouse monoclonal antibody (1:5000; Sigma, St Louis, MO, USA) as a loading control. Then, membranes were washed in PBS / Tween, incubated with peroxidase-conjugated goat anti-rabbit antibody or anti-mouse antibody (1:3000; Biorad, Hercules, CA, USA) for 45 min at room temperature, washed, and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, UK). The band intensities were quantitatively determined using ImageJ software, and protein levels intensity were normalized to b-actin expression. In each experiment, cell lysates from at least three different samples were analysed and band intensity is the result of the mean between the different experiments.

Tumorigenesis assay

This assay has been previously shown to be a good tool to evaluate the tumorigenic capacity of sub-populations of cells to generate tumors (Worst et al., 1982). This method is based on the use of a silicone chamber inserted into the skin of immunocompromised mice

The grafting system

This small chamber has a hole on the top. RAD and non-RAD cells from both SCC-13-derived tumors and cultured. SCC-13 have been counted and mixed with 1 million of human fibroblast. After harvesting, each sub-population

mix has been injected in the hole of the chamber. The chamber itself is a closed environment. After one week, the upper part of the chamber is removed to create the microenvironment that resembles normal skin to air exposure. One week after the chamber is cut, the chamber is totally removed and the graft is continuously monitored. This system is more efficient than the simple sub-cutaneous injection because the presence of cells that are in the skin in homeostatic conditions constitute a good microenvironment to sustain the tumor growth. Depending on the aggressiveness of the cells and their growth ability, the tumor became visible after a period of 1-3 months after grafting.

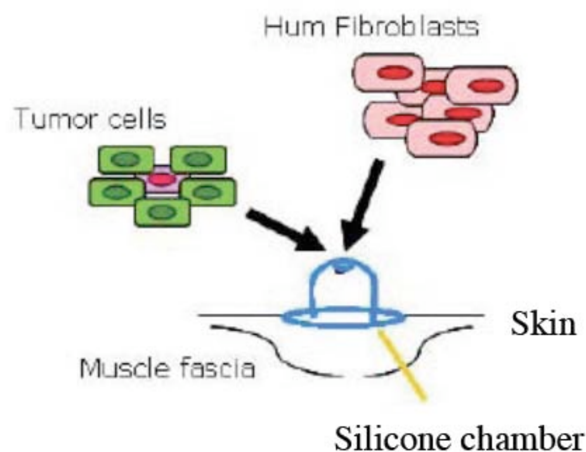


Fig. 13: graft of silicone chamber

Subcutaneous tumorigenicity

For in vivo tumorigenicity assays, normal human keratinocytes were infected with the LZRS-H-Ras G12V or/and IkBalphaM or survivin retrovirus. Each infection

was be performed for 24hours. After retroviral infection, cells were transfected with 50uM specific survivin siRNA (Dharmacon Inc, Lafayette, CO, USA). 24 h after transfection, were brought into suspension, admixed with Matrigel (Millipore, Chemicon), and injected (10^6 cells per injection) subcutaneously in CB17 SCID/SCID mice. Tumors were dissected 6 wk later.

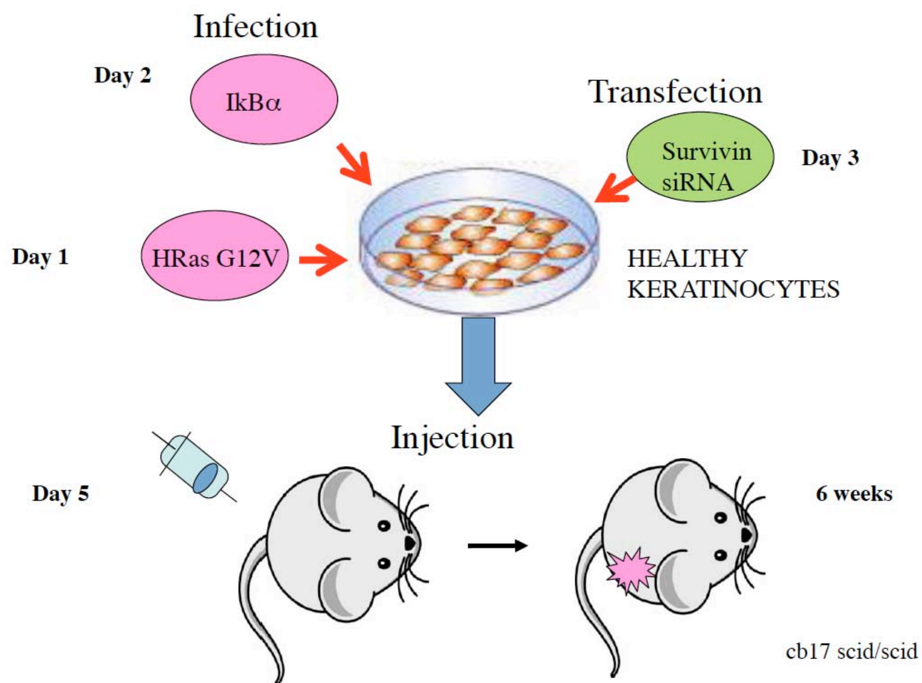


Fig 14: In vivo tumorigenesis model

Results and discussion

PART I

Survivin expression in cancerous and precancerous skin lesions

Survivin is expressed both at the nuclear and cytoplasmic level in normal human epidermis

Survivin is a key molecule for the maintenance of epidermal homeostasis. We have previously shown that survivin sustains keratinocyte proliferation, and highest levels of survivin protect keratinocyte stem cells (KSC) from apoptosis (Marconi et al, 2007; Dallaglio et al, 2009). However, survivin expression in normal human epidermis of adult individuals has been controversial for years. In this respect, some reports show that survivin is absent in normal epidermis (Fukunuda S et al, 2006, Jha K et al, 2012) while others, including our group, have recently confirmed the presence of survivin in the cytoplasm of basal keratinocytes. Nuclear survivin, which seems to be associated to cell proliferation, has been detected in hyperproliferative skin conditions such as psoriasis and cancer (Altieri et al, 2001). However, to date there are no reports showing nuclear survivin expression in the epidermis of healthy individuals.

Survivin expression in human lesional and healthy skin has been deeply analyzed in different works (Chiodino et al, 1999; LoMuzio et al, 2001). However, a unique report including a deep analysis of survivin expression in healthy skin, premalignant and malignant keratinocytic neoplasms,

particularly looking at their differentiation grade, has not been previously published. In addition, the use of a highly sensitive immunohystochemical technique can improve the detection of survivin in analysed sections, thus providing new information.

We therefore performed an optimized immunohystochemistry assay using a highly sensitive immunohystochemical method, based on standardized and precise temperature and time conditions. The assay has the advantage to employ an anti-survivin antibody able to detect both the nuclear and cytoplasmic pool of the protein, ensuring a complete evaluation of the marker.

We evaluated survivin expression in normal human skin and in skin from AK, *in situ* SCC and SCC patients. As shown in figure 1, both nuclear and cytoplasmic survivin are expressed in normal human epidermis, as compared to the negative control where primary antibody was omitted. To our knowledge, this is the first report showing nuclear survivin expression in healthy epidermis. While cytoplasmic survivin is present in basal cells only, as previously reported (Marconi et al, 2007), nuclear survivin (N-surv) positive cells are located in basal cell layers or at the boundary between basal and suprabasal layers. To exclude N-surv expression due to unspecific staining, we performed western blotting on nuclear and cytosolic extracts from human primary total keratinocytes derived from healthy epidermis. We confirmed survivin localization in both keratinocyte nuclei and cytosol from total normal keratinocytes and, in particular, we show that cytosolic survivin is more abundant as compared to nuclear one. Enolase, an enzime with cytoplasmic only activity, is

expressed in the cytosolic fraction only thus confirming the correct separation of nuclear and cytosolic compartments (Fig 15).

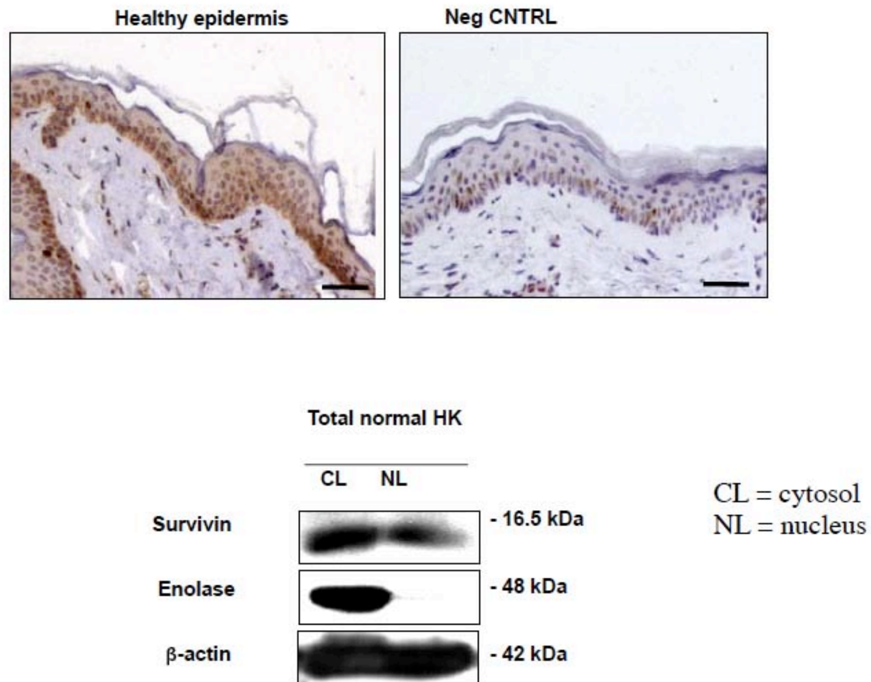


Fig. 15: Differential expression of nuclear and cytoplasmic survivin in normal epidermis

Nuclear survivin is overexpressed in AK, in situ SCC anc SCC

We performed the previously optimized immunohystochemistry assay for survivin expression also in actinic keratosis (AK), *in situ* SCC and SCC. Because these lesions occur mainly in adult and old patients, we used normal adult skin for the comparisons. SCC have been divided into three main categories based on the differentiation grade of the tumor. Being UVB one of the most common risk factor associated with SCC

development, only skin biopsies from sun-exposed areas have been included in the study. As shown in figure 16, while cytoplasmic survivin is expressed in healthy epidermis from adult donors, it is absent in lesional epidermis. On the other hand, while positive nuclei are rare in normal skin and, when present, are mainly located in the basal or lower suprabasal cell layers, N-surv is overexpressed in AK and *in situ* SCC and it is mainly localized suprabasally, involving also higher spinous and granular layers. In SCC, only N-surv is expressed and its levels are highest in poorly differentiated tumors. To confirm survivin overxpression in the nucleus of SCC cells, we obtained nuclear and cytosolic extracts from SCC13 (a cell line previously obtained from a cSCC of the face (Wu and Rheinwald et al,1981) and cultured keratinocytes isolated from a primary cSCC (see M&M section). In figure 2 we confirmed that nuclear survivin is mainly expressed in SCC cells while the cytosolic fraction is scarsely expressed in both cell line and primary SCC cells. To compare survivin expression in the different skin lesions, we calculated the % of positive nuclei/total counted cells. Recently, it has been shown how intra-tumor heterogeneity is a key factor in the global evaluation of a tumor section, as it may influence, among other factors, the choice of the theraputic treatments for the patients (Minner et al, 2012; Potts et al, 2012). First, we found that survivin expression was localized only in some areas of AK, moderately (MD) and well differentiated (WD) cSCC lesions. As opposite, survivin expression was diffuse and homogenously distibuted in *in situ* and poorly differentiated (PD) cSCC (Figure 2). In figure 2, the

percentage of positive nuclei has been calculated by evaluating survivin expression in the whole lesion, taking into consideration the heterogeneity of tumor samples. We found that N-surv is significantly more expressed in AK and cutaneous SCCs as compared to healthy skin. In addition, N-surv expression is higher in *in situ* SCCs than total SCC, WD and MD tumors. On the other hand, survivin expression is similar between *in situ* SCC and PD tumors, while PD SCC display the highest levels of N-surv expression. We can conclude that, the percentage of survivin positive cells is markedly higher in SCC than in in situ tumor only when a global evaluation of the lesion is performed.

In accordance with previous results, we have observed a shift of survivin from the cytoplasm to the nucleus of keratinocytes in AK, *in situ* SCC and SCC sections. However, N-surv is not only located in basal cell layers but also in suprabasal keratinocytes from AK and SCC lesions, suggesting that the appearance of positive nuclei in high suprabasal cell layers is a hallmark of disrupted epidermal homeostasis. Interestingly, the percentage of survivin positive cells is significantly higher in *in situ* SCC as compared to WD and MD SCC. On the other hand, the percentage of positive cells are comparable between PD SCC and *in situ* lesions. This is probably due to the diffuse distribution of survivin-positive cells in both tumor types, while the high amount of differentiated cells in WD and MD tumors that are negative for survivin expression, contribute to the decrease in the percentage of survivin positive cells. While survivin expression does not distinguish between *in situ* and PD SCC, it is significantly

different among SCCs depending on the differentiation grade, in that PD SCC display higher levels of N-surv than MD and WD tumors. This indicates that survivin expression correlates with SCC aggressiveness, in accordance with previous reports (Lo Muzio et al, 2001).

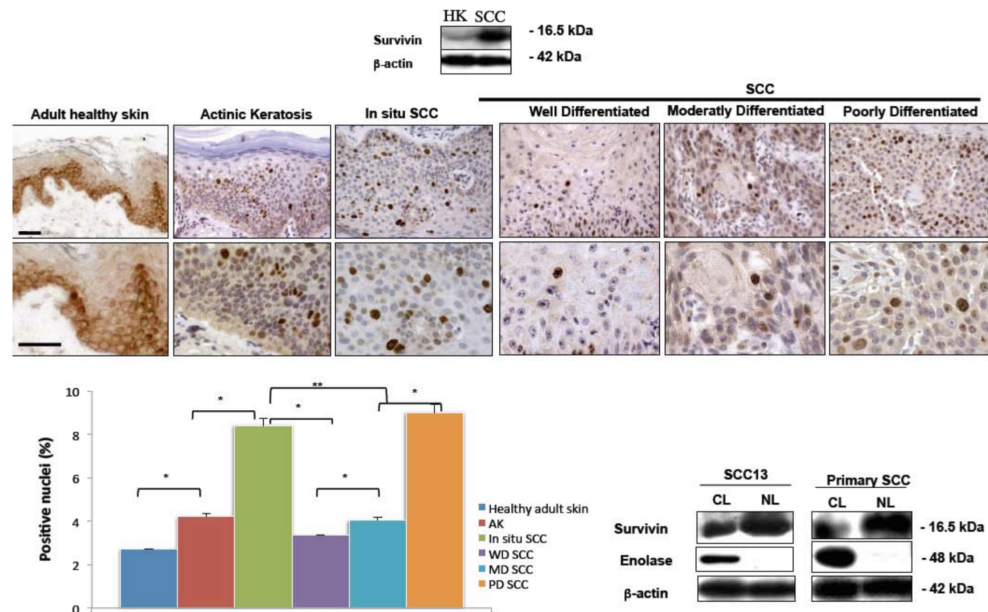


Fig. 16: expression of survivin in AK, in situ SCC and SCC

Survivin expression correlates with invasiveness in poorly differentiated SCC

Although survivin distribution was mainly homogeneous in PD SCC lesions, we observed a clear change of survivin subcellular localization in different areas of the lesions. As shown in fig. 17, survivin is mainly cytoplasmic in non-lesional epidermis adjacent to tumor, and it is expressed only

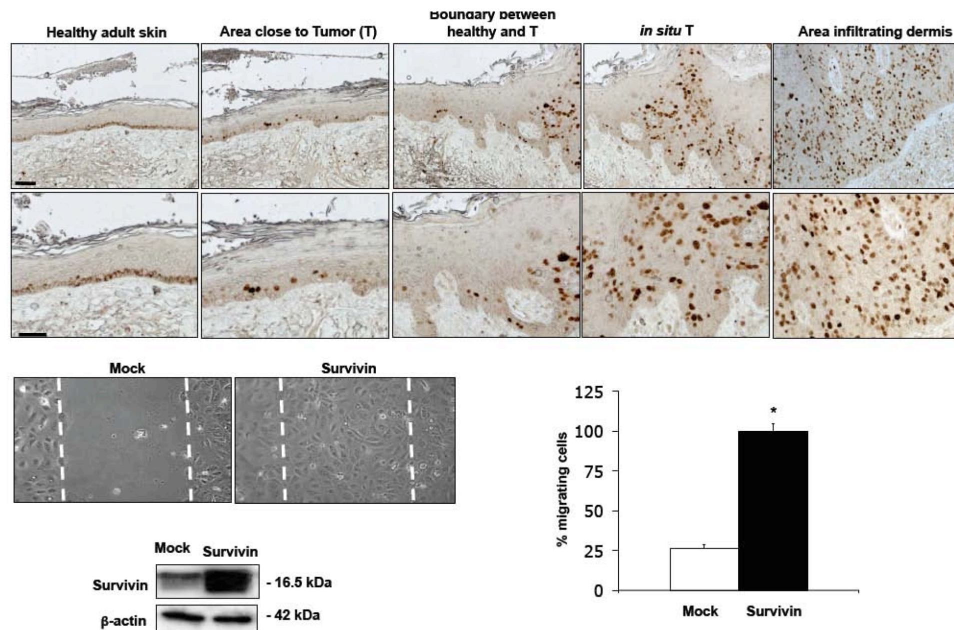


Fig. 17 Nuclear survivin is mainly located in invasive SCC nests and sustains keratinocyte migration

in basal keratinocytes. When shifting towards cancerous epidermis, we observed a clear change in subcellular localization of survivin, which becomes nuclear and marks cells also in suprabasal cell layers. This is more evident in cancerous epidermis where only few cells located in upper cell layers are negative for survivin. Moreover, we observed a marked increase of survivin positive cells in deeper cell nests of the tumor. Because survivin overexpression in tumors infiltrative compartment often correlates with tumor metastasis (LoMuzio et al, 2001), and some lines of evidence suggests a clear involvement of survivin in cell migration (McKenzie et al, 2010), we wanted to address if survivin overexpression in deeper cell nests may contribute to their potential to migrate and generate metastasis. To this purpose, we overexpressed survivin in HaCaT cells and confirmed survivin

overexpression by western blotting at 24h. The same number of mock and survivin-overexpressing cells were then plated, treated with mytomicin C and scratched with a tip. At 24h, we fixed, colored the cell monolayer and counted migrated cells. We observed increased keratinocyte migration in survivin-HaCaT keratinocytes as compared to mock cells. Altogether, these data suggests that survivin sustains an active role in keratinocyte migration and its overexpression in infiltrative keratinocyte nests of cSCC tumors may reflect the ability of survivin-positive cells to invade the dermis and migrate.

The increased ability of survivin-overexpressing keratinocytes to migrate in wounded areas *in vitro* further confirms the relationship between survivin expression and SCC cell aggressiveness. This is in line with previous reports showing survivin ability to sustain cell migration in cutaneous neoplasms (McKenzie at al, 2010; McKenzie and Grossman, 2012). However, which survivin pool is involved in the migration process is still unclear and needs further investigation.

Survivin is predominantly expressed in cSCC cells negative for K10 but positive for K15.

To evaluate if N-surv positive cells display the same expression pattern in normal and lesional skin, we performed a double staining for survivin and either K10 or K15 in AK, *in situ* SCC and SCC lesions. In figure 18, we show that K10 expression is confined to suprabasal keratinocytes in both AK and *in situ* SCCs. However, in AK,

only basal cells are negative for K10 while also few suprabasal keratinocytes are K10-negative in *in situ* lesions. This may reflect changes in epidermal structure often seen in neoplastic epidermis. Interestingly, in both AK and *in situ* SCC survivin-positive cells are also positive for K10, as observed in normal epidermis. By contrast, in SCC lesions, K10 is distibuted in both basal and suprabasal cells and the majority of survivin positive cells do not express K10. This is more evident in PD SCC as compared to MD and WD SCC.

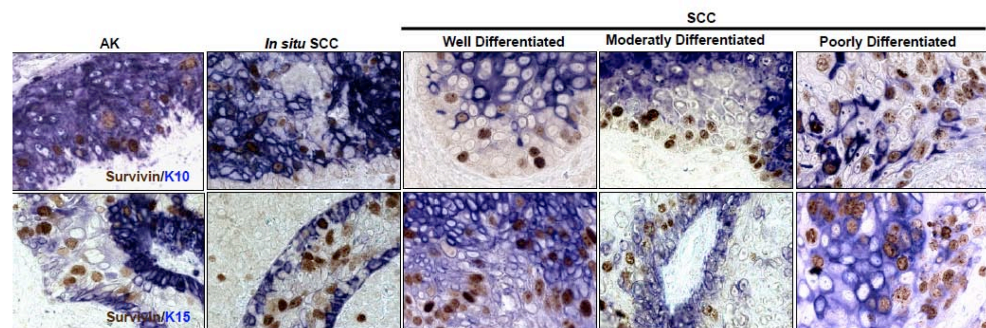


Fig 18: Double staining of representative AK, *in situ* SCC and cutaneous SCC sections for survivin and either K10 or K15

At variance with previous results (Abbas and Bhawan, 2011), we detected K15 expression in all SCC lesions, though at different levels. In AK and *in situ* SCC K15 is mainly basal and it is barely or not expressed in survivin-positive cells. On the other hand, in SCC lesions, K15 is distributed in all cell layers that also express survivin, with no appearent differences between tumors differentiation grade.

In normal epidermis, N-surv positive cells express K10, but not K15, thus suggesting that N-surv is not a marker of

stem keratinocytes *in vivo.* Alternatively, *N-surv* could identify a population of cells starting to express K10, located at the boundary between the basal and suprabasal cell layers. This is consistent with the definition of transit amplifying cells (TA), which are committed to terminal differentiation, while retaining some proliferative activity.

PART II

In vitro characterization of primary SCC keratinocytes: role of survivin

Characterization of SCC keratinocytes in vitro

For in vitro studies, we isolated primary keratinocytes from ten SCC biopsies of sun-exposed areas and from healthy skin. In order to evaluate in vitro differences between primary healthy human keratinocytes and SCC primary keratinocytes, we performed MTT assay at 24, 72, 120 and 144 hours. SCC keratinocytes displayed higher proliferation rate ($p<0,05$) as compared to healthy keratinocytes (fig. 19).

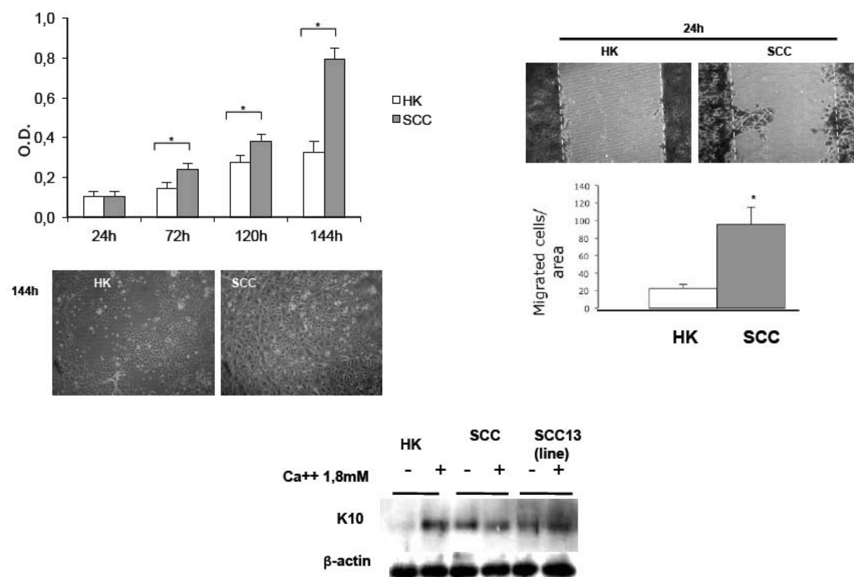


Fig 19: in vitro characterization of primary SCC and healthy keratinocytes.

Scratching assay demonstrates that treatment with Mytomicin C prevents migration of normal keratinocytes. On the other hand, SCC keratinocytes retain the ability to migrate ($p<0,05$), suggesting that these cells are more invasive and aggressive as compared to healthy keratinocytes (Fig 19).
To evaluate the behaviour of SCC keratinocyte after differentiative stimulus, cells were seeded at the same density and stimulated with 1,8mM $CaCl_2$. As shown by western blotting (Fig 19), while healthy keratinocytes express high levels of K10, SCC keratinocyte are insensitive to Ca++, showing K10 expression levels similar to normal controls.

3D SCC models

Substantial evidences from recent studies show that solid tumors contain a subpopulation of CSCs. (Jordan et al, 2006). It is well known that CSCs play an important role in tumor initiation, progression, metastasis, and resistance to therapy (Costea DE et al, 2006; Locke M et al, 2005; Chiou SH et al, 2008). The isolation of CSCs from cancer cells has been achieved successfully via the use of different techniques. Concurrent studies confirmed that the sphere culture system is an efficient method for separating CSCs from many solid tumors or cancer cells lines. These studies have suggested that CSCs can be enriched in spheres when these are cultured in serum-free medium supplemented with adequate mitogens, such as the basic fibroblast growht factor (bFGF) and epidermal growht

factor (EGF)(Chiou SH et al, 2008; Lee J, et al, 2006). When SCC keratinocytes are cultured under non adhering conditions, they are able to form squamospheres in vitro (Figure 20). However, when squamospheres are disgregated and analyzed by western blotting, they have high levels of K10 and fail to express survivin. We hypotesized that the serum used in culture medium would trigger the differentiation process and preventing proliferation. These findings suggest that squamosphere method is not suitable for our experiments.

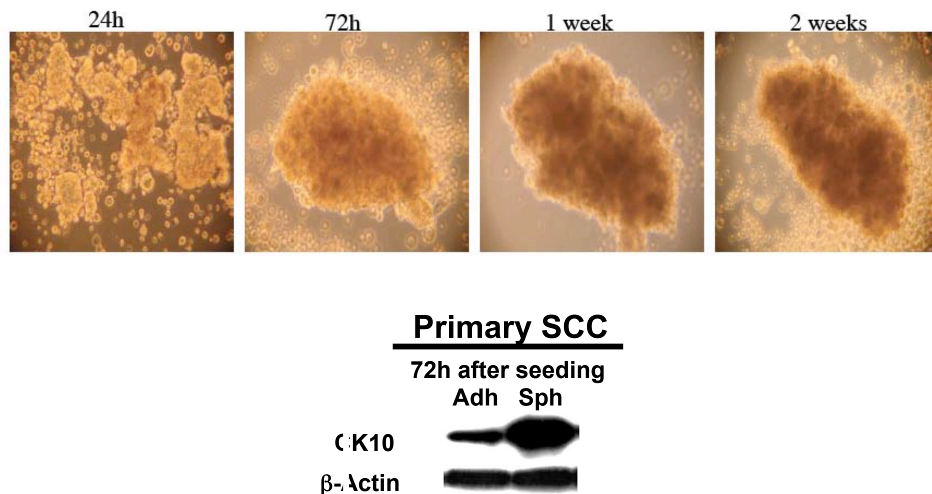


Fig 20: primary SCC keratinocytes-derived spheres

Similarly to squamospheres, also skin equivalent can be useful to better understand many features of cancer such as metastasis and invasion.
To evaluate if skin equivalent can be used as a model for SCC study, we generated skin equivalents with SCC cells and normal keratinocytes. Hematoxylin and Eosin staining highlight some cancer hystologic features including invasion through intact basement membrane into

supporting stroma (figure 21). SCC keratinocytes formed squamous cell nests in the dermal equivalent of some SCC models, which was not observed in healthy control skin reconstructs.
Based on these observation, we decided to use skin equivalent as a suitable SCC model in vitro.

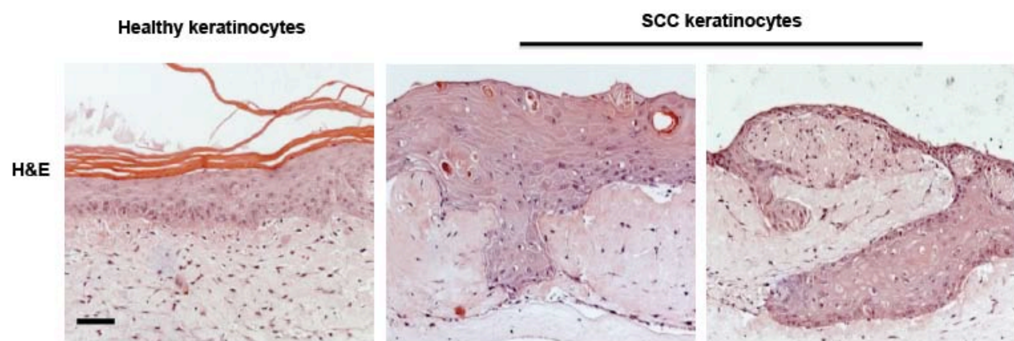


Fig 21: SCC skin equivalent

Survivin silencing reduces proliferation and colony forming efficiency of primary human SCC keratinocytes

To better clarify the role of survivin in skin cancer, we evaluated the effect of silencing survivin mRNA.
We have previously demonstrated that survivin siRNA decreases proloferation and induces apoptosis in normal human keratinocytes, in a mode consistent with the mitotic catastrophe (Dallaglio K et al, 2009). Thus, we silenced survivin with a specific siRNA. The efficiency of downregulation was confirmed by western blotting. Survivin silencing alters keratinocyte morphology and causes the formation of vacuoles in the cytoplasm (Fig 22).

CFE, shows that after 72 hours from transfection, survivin siRNA-treated keratinocytes loose their capacity of forming large colonies and presente many vacuoles. These pictures clearly suggest that inhibiting survivin markedly affects keratinocyte colony formation.

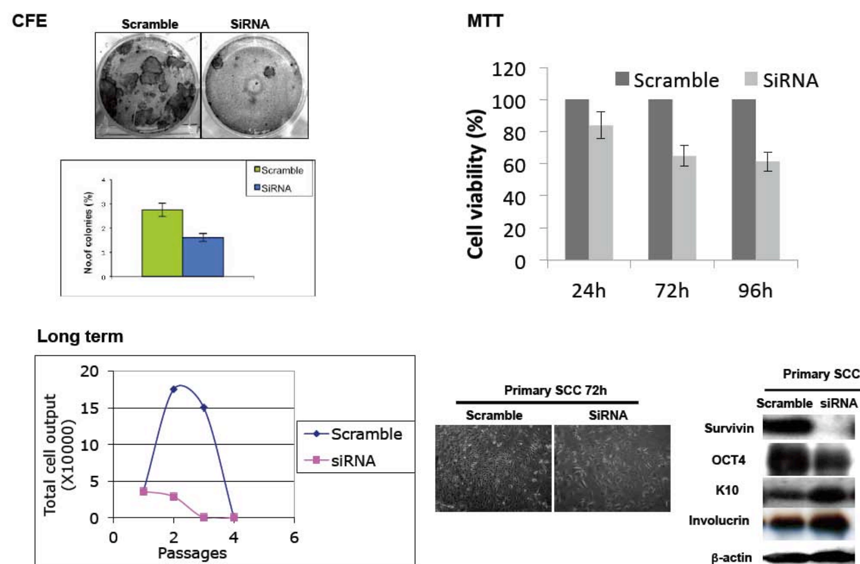


Fig 22: in vitro characterization of primary SCC keratinocytes

We next investigated the functions of survivin with regard to keratinocyte proliferation. Silencing survivin caused a significant and time-dependent decrease in keratinocyte proliferation from 24 to 96 h (Fig.8). Inhibition of survivin also induced a significant reduction in ability to grow long term, exausting the proliferative potential before scramble SCC transfected cells. These observations confirm that survivin plays an important role in cell division and could play an important role in skin cancer.

siRNA-treated SCC keratinocytes were lysed and analyzed by western blotting: expression of OCT4, a marker of

stemness, decreased, whereas differentiation markers such as K10 and involucrin increased. This suggests that survivin identifies SCC stem cells.

Survivin silencing reverts SCC to a normal phenotype in a skin equivalent model

To confirm the role of survivin in SCC, we generated a skin equivalent model with SCC keratinocytes in which survivin was downregulated. When survivin was silenced, the thickness of skin equivalent, as measured by ImageJ software, was reduced as compared to controls. Moreover, when survivin is downregulated, the number of Ki67 positive cells decreases (fig. 23) These findings not only confirm the role of survivin in keratinocyte proliferation, but also suggest that survivin silencing reverts SCC to a normal phenotype in a skin equivalent model.

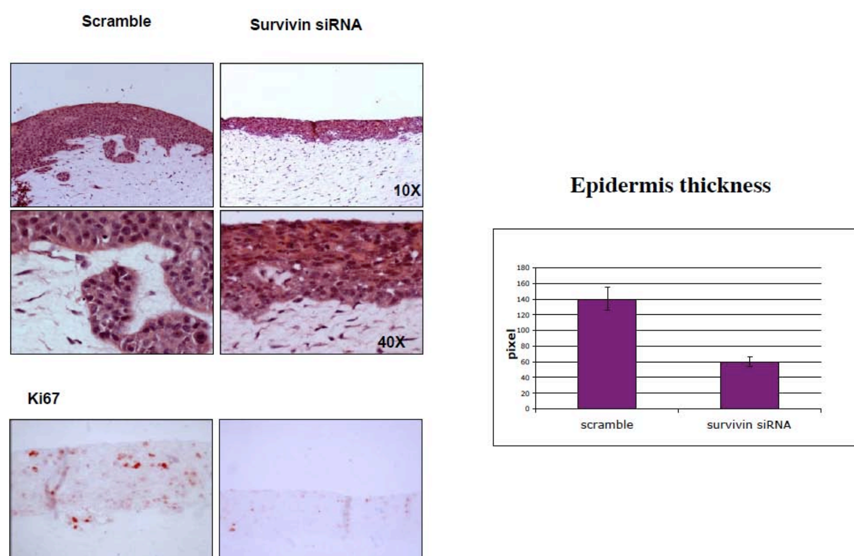


Fig 23: SCC skin equivalent after survivin silencing

PART III

SCC SUBPOPULATION: IN VITRO ANALYSIS

Isolation of primary SCC subpopulation

Basal keratinocytes express high levels of beta1 integrin, which binds to collagen fibers thus allowing anchorage of keratinocytes to extra-cellular matrix (ECM). When beta1 integring levels decrease, keratinocyte fail to adhere to ECM and undergo terminal differentiation (Jensen P and Watt FM, 1993). According to the cancer stem cells (CSC) hypothesis, tumor maintenance and recurrence following conventional treatment is sustained by a subpopulation of cells endowed with stem cell features such as in vivo self-renewal (Locke M et al, 2005; Prince ME et al 2007).

It has been shown that SCC cell lines including skin SCC-derived SCC13 and SCC12B2 contain subpopulations of stem cells. SCC keratinocytes able to adhere to type IV collagen within 20 minutes (rapidly adhering cells), show stem cell properties *in vitro,* such as higher colony forming efficiency and expression of stem-cell markers as compared to non rapidly adhering SCC keratinocytes (Jensen, Jones and Watt, 2008). We have previously demonstrated that cell adhesion to type IV collagen-coated plates within 5 minutes allows the isolation of normal human keratinocyte with the highest expression of beta1 integrin. These cells represent a population further enriched in keratinocyte stem cells (KSC), as compared to the population originally described by Watt’s group.

Our method has been used to further characterize SCC keratinocyte subpopulations. In addition, at variance with

Watt’s group, we have performed our study on cultured primary keratinocytes from SCC specimens instead of SCC cell lines mostly because primary cell cultures retain some features seen in patient tissues, such as the ability to differentiate, while cell lines have intrinsic limits due to long-passage in culture.
We have thus isolated two populations: SCC cells adhering to type IV collagen within 5 minutes (RAD) and cells that do not adhere within this time frame (NRAD). As shown in fig. 24, RAD cells express higher levels of survivin than NRAD.
Moreover, RAD cells express lower levels of K15, EFABP and involucrin than NRAD, suggesting that these cells are less differentiated. While in normal human epidermis, K15 positive cells are scattered in the basal cell layer, in skin tumor, K15 expression is down-regulated and seems to correlate with differentiation (Khanom R et al, 2012). K15-positive cancers tend to show a well-differentiated phenotype, and K15 is downregulated mainly in high grade squamous intraepithelial neoplasms (Khanom R et al 2012).

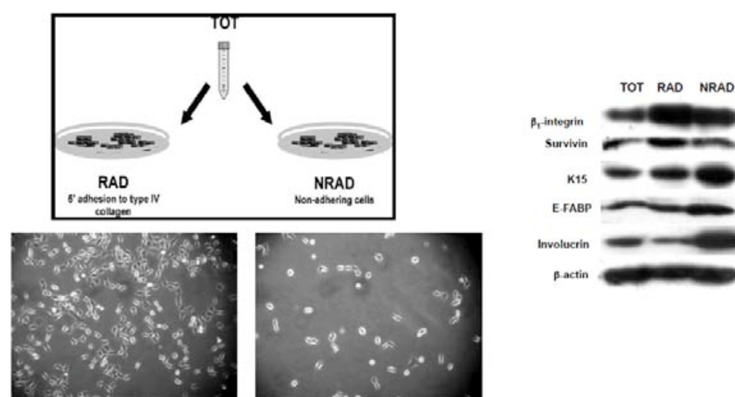


Fig 24: isolation of primary SCC subpopulation on the

basis on their ability to adhere in 5 minutes to a collagen IV coated plate

RAD cells proliferate and migrate more than NRAD cells and have high colony forming efficiency

To confirm stem cell-associated features in RAD cells, we performed a proliferation/viability assay. RAD cells proliferate with significantly greater extent and have higher colony forming efficiency in vitro, as compared to NRAD cells. Cell cycle analysis of RAD/NRAD cells shows increased S-phase in RAD cells, consistent with viability and CFE results.

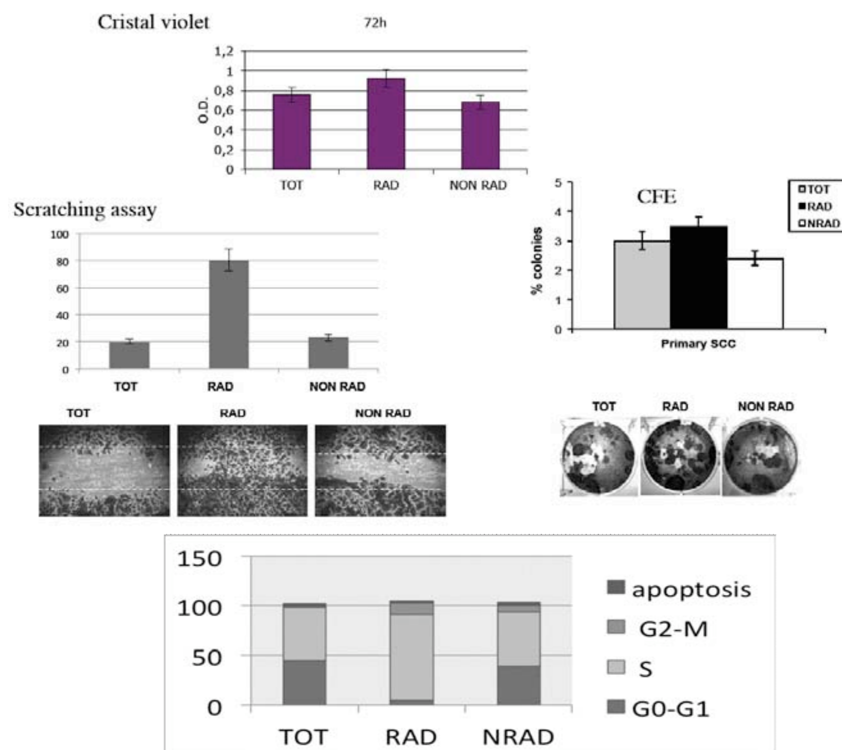


Fig 25: SCC subpopulation: in vitro analysis

The ability of cancer cells to migrate is the expression of

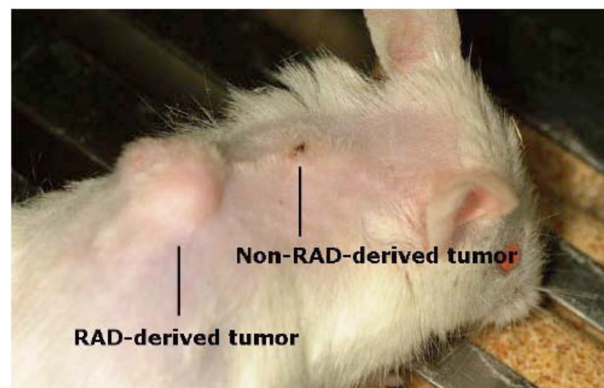
invasion potential and tumor malignancy. To evaluate RAD versus NRAD cell aggressiveness, we performed a scratching assay that measures the ability of the different subpopulations to migrate and invade *in vitro*. RAD cells migrate to a greater extent than NRAD keratinocytes, suggesting that RAD cells represent a more aggressive subpopulation (fig. 25).

PART IV

SCC SUBPOPULATION: IN VIVO ANALYSIS

In vivo tumorigenesis assay

We can summarize by saying that RAD SCC cells from primary tumors display stem cell features in vitro. However, to date, the tumorigenic ability of RAD versus NRAD cells has not been shown. We therefore performed an in vivo tumorigenic assay by xenografting RAD and NRAD cells onto immune-compromised mice. As device, we used a silicon chamber in which we seeded SCC keratinocytes and fibroblasts. When grafted onto the back of nod scid mice, RAD cells were able to form tumors 2-4 times bigger than those derived from the NRAD cells.



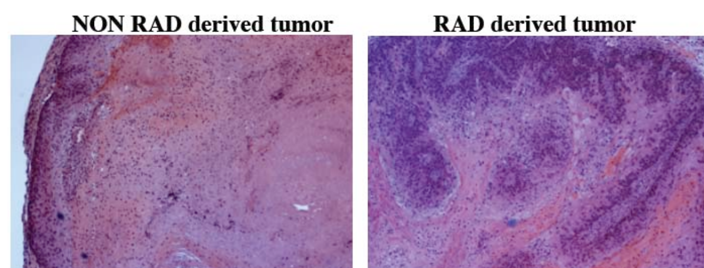


Fig 26: Rad and NRAD derived tumor in mouse and H&Estaining

Ematoxilin and eosin staining of tumors sections show that RAD-derived tumors are more aggressive and invasive than neoplasms derived from NRAD cells. External margins of NRAD tumors are interested by a lower number of proliferating cells as compared to RAD tumors, whereas the tumor core is characterized by keratin accumulation (fig. 26). By contrast, RAD derived tumors have more proliferating cells. Moreover, by counting the number of mitosis/total cells, RAD derived tumors display a mitotic index 2,68 fold higher than NRAD derived neoplasms. Consistently, survivin and Ki67 expression levels are higher in RAD tumors, whereas keratin 10, EFABP and involucrin are mainly detected in NRAD tumors, suggesting a pattern of decreased differentiation in RAD tumors.

OCT4 is a marker for CSC. High levels of OCT4 and survivin are correlated with poor survival in patients with esophageal squamous cell carcinoma (Chunguang L et al, 2012). High levels of OCT4 expression in RAD tumor confirm that RAD keratinocytes are a subpopulation enriched in CSC. These data suggest that SCC RAD keratinocytes have a key role for tumor growth and invasiveness. RAD population in primary skin SCCs not only express stem-cell associated markers and have higher CFE (Jensen, Jones and Watt, 2008), but it also displays higher tumorigenic ability in vivo (fig. 27).

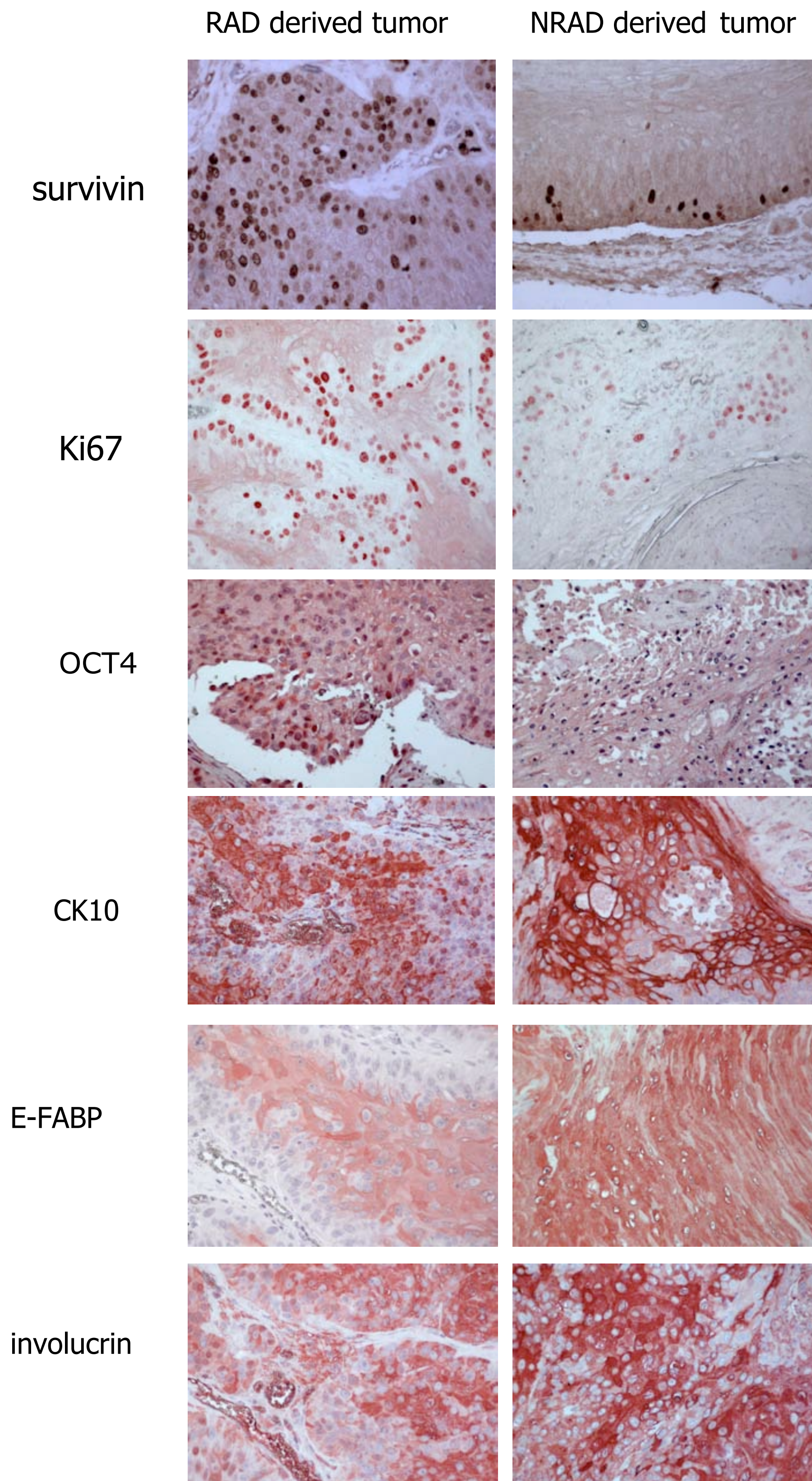


Fig. 27: characterization of RAD and NRAD derived tumor

PART V

In vivo tumorigenesis model

Mutations in Ras gene have been found in multiple types of human cancer, where it plays a critical role. In normal human epidermal cells, oncogenic Ras induced several changes that promote G1 arrest. Notably, coexpression of Ikba, the inhibitor of NFkB, overcomes the cell cycle arrest that NF-kb would induce after Ras hyper activation.

Human primary keratinocytes were retrovirally tranduced to overexpress two genes: the oncogenic H-Ras and/or IkBalpha. NoLac Z empty vector was used as negative control.

To assess whether and how this gene overexpression affects cell proliferation, we performed an MTT assay. As shown in fig 28, when H-Ras or IkBalpha are overexpressed proliferation decreases. On the other hand, when they are co-expressed, proliferation increases. In this model, in order to confirm the role of survivin in skin tumorigenesis, we downregulated survivin with a specific siRNA.

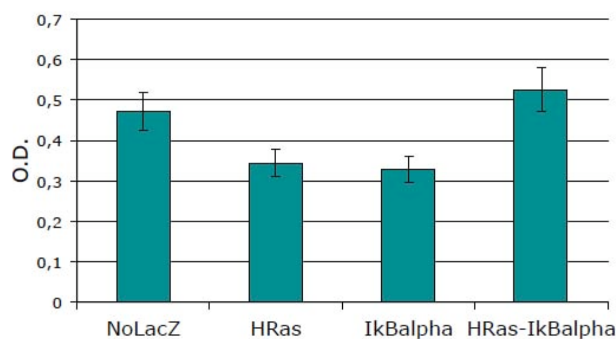


Fig 28: proliferation status of Hras and/or IkBalpha infected keratinocytes

After survivin silencing, we further confirmed a decreased proliferation both in No Lac Z and in H-Ras-Ikbalpha infected keratinocytes (fig 29).

Moreover, through scratching assay, we found that overexpression of both HRas and IkBalpha increases migratory ability, suggesting that double transduced keratinocytes are more invasive as compared to the negative control (fig 29).

Mutational activation of Ras proteins promotes oncogenesis by disturbing a multitude of cellular processes, such as gene expression, cell cycle progression and cell proliferation, as well as cell survival, and cell migration. Ras signalling pathways are well known for their involvement in tumour initiation, but less is known about their contribution to invasion and metastasis.

Recent reports have described an increased of Ras mediated cell growth by some members of the chromosomal passenger protein complex: Aurora kinase A and B and inner centromeric protein (Kanda A et al, 2005; Vernos I et al 2004). Because Aurora B has a functional interaction with survivin to modulate cell proliferation, we wanted to address if H-Ras overexpressing keratinocytes show high levels of survivin as compared to the negative control. Indeed, by western blot analysis, we found that H-Ras-IkBalpha overexpression enhances survivin expression (Fig 28).

Transformed cells were subcutaneously injected in nod scid mice. After 6 weeks, only H-Ras-IkBalpha infected keratinocytes were able to induce tumors. After 8 weeks,

we observed tumor formation in all 4 cases.

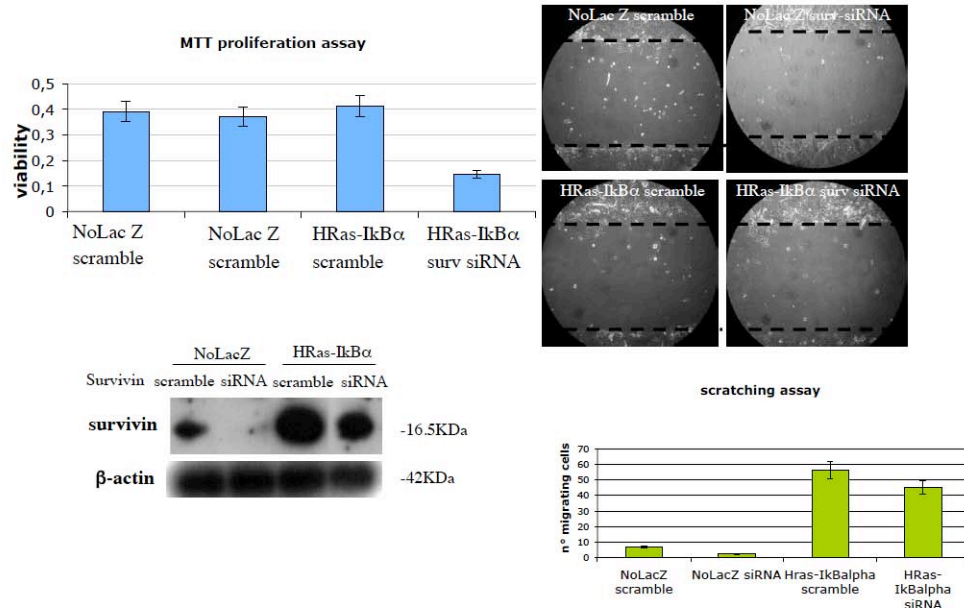
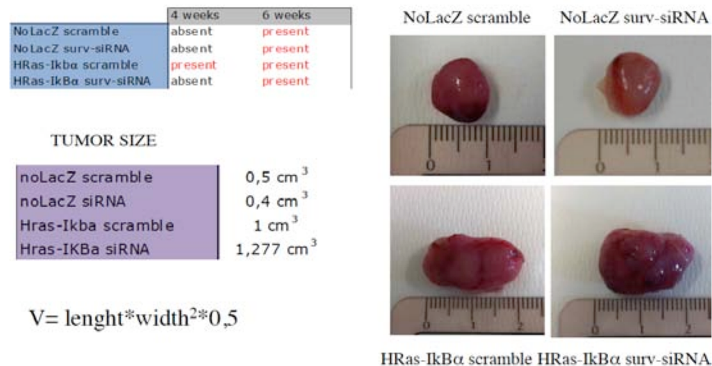


Fig. 29: in vitro characterization of H-Ras-IkBalpha infected keratinocytes after survivin silencing

In particular, coexpression of HRas and IkBalpha induced a bigger nodule than NoLac Z. Yet, when survivin was silenced, no significative differences in terms of tumor size were detected. The size of the tumor were carried out with the formula:

Tumor volume= lenght *width²*0,5

Tumor sizes are reported in table in fig 30.



	4 weeks	6 weeks
NoLacZ scramble	absent	present
NoLacZ surv-siRNA	absent	present
HRas-Ikbα scramble	present	present
HRas-IkBα surv-siRNA	absent	present

TUMOR SIZE

noLacZ scramble	0,5 cm ^{3}
noLacZ siRNA	0,4 cm ^{3}
Hras-Ikba scramble	1 cm ^{3}
Hras-IKBa siRNA	1,277 cm ^{3}

V= lenght*width²*0,5

Fig. 30: macroscopical analysis of mouse isolated tumor

Hystological analysis was performed by hematoxilin and eosin staining. As shown in fig. 31, differences between No Lac Z and H-Ras-IkB alpha are considerable. Whereas in No LacZ derived mass only cellular hyperproliferation is detected, H-Ras-IkB-alpha overexpression induces malignant neoplasia. In particular, we can observed a big necrosis area in tumor core, a more accentuated vascularization, plump nuclei, prominent nucleoli and dispersed chromatin. Tumors derived from H-Ras and IkB-alpha overexpressing keratinocytes were significantly more aggressive than tumors where survivin was silenced (mitotic index: 10,2 +/- 2,28 vs. 5,4 +/- 1,81). These findings suggests that survivin downregulation induces less severe hystologic features.

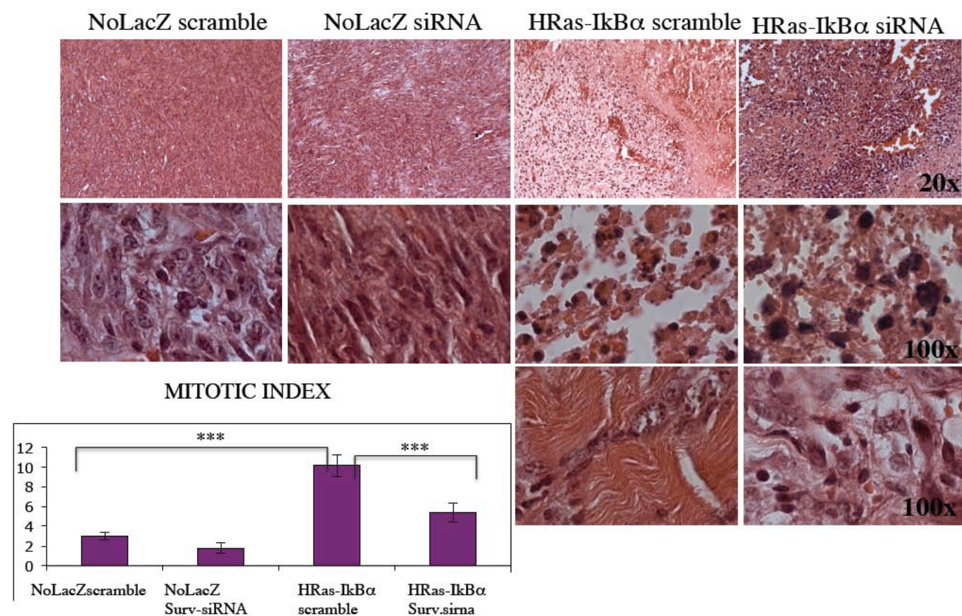


Fig. 31: Hematoxilin and eosin staining and evaluation of mitotic index of H-Ras and IkBalpha derived tumor

Staining of tumor with an anti pankeratin antibody shows

that the H-Ras-Ikb alpha derived neoplasia are of epidermal origin (fig. 32). Tumors derived from H-Ras-IkB alpha overexpressing keratinocytes express high levels of survivin (fig. 33). This result is in line with in vitro results (fig 29).

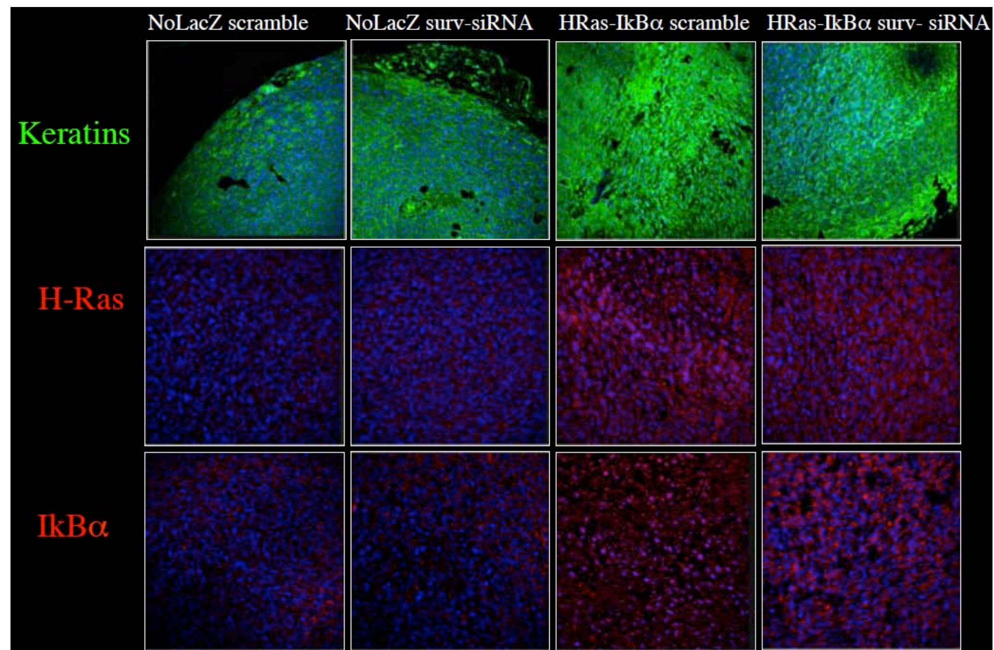


Fig 32: keratins, H-Ras and IkB-alpha expression in H-Ras-IkB alpha derived tumor after survivin silencing

We observed also that all nodules are vascularized suggesting the activation of angiogenetic process: blood vessels penetrate into cancerous growths, supplying nutrients and oxygen and removing waste products.

Hypoxia is a common feature of solid tumor, which not only is a major problem for radiation therapy but it also leads to resistance to most anticancer drugs, and appears to accelerate malignant progression and to increase metastasis. Thus, we evaluated neoangiogenesis and hypoxia in H-Ras-IkBalpha derived tumor by using anti-

VEGF, anti-CD51 (integrin alpha V) and anti-HIFalpha antibodies.

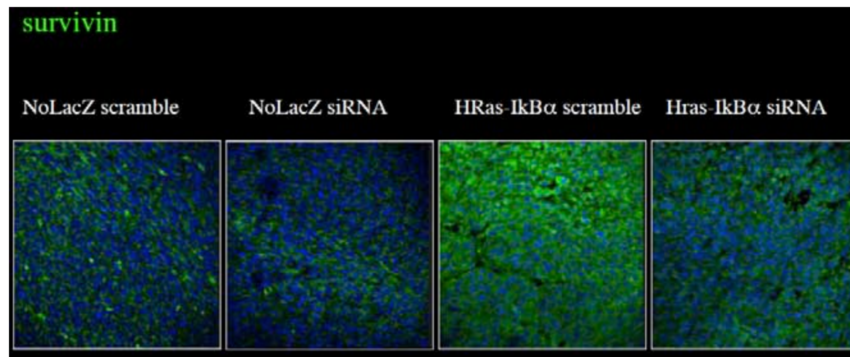


Fig 33: survivin expression in H-Ras-IkB-alpha derived tumor after survivin silencing

The hypoxya-inducible factor 1 (HIF-1), which is a heterodimeric protein consisting of an alpha and beta subunit, has an important role in tumor hypoxia.
VEGF is a selective mitogen for vascular endothelial cells, and it directly stimulates neovascularization and increases microvascular permeability and correlates with metastatic and poorer outcome in cancer patients. CD51, VEGF and HIF1alpha are more expressed in H-Ras-IkBalpha overexpressing tumor as compared to NoLac Z ones. Moreover, their expression levels decreased when survivin was silenced, suggesting that survivin silencing induces a less severe phenotype (fig. 34).
It has been demonstrated that expression of HIF1 alpha significantly correlates with the expression of survivin and VEGF, which indicates that these proteins may have a synergistic effect in carcinogenesis and development of esophageal carcinoma (Zhang H et al, 2007).

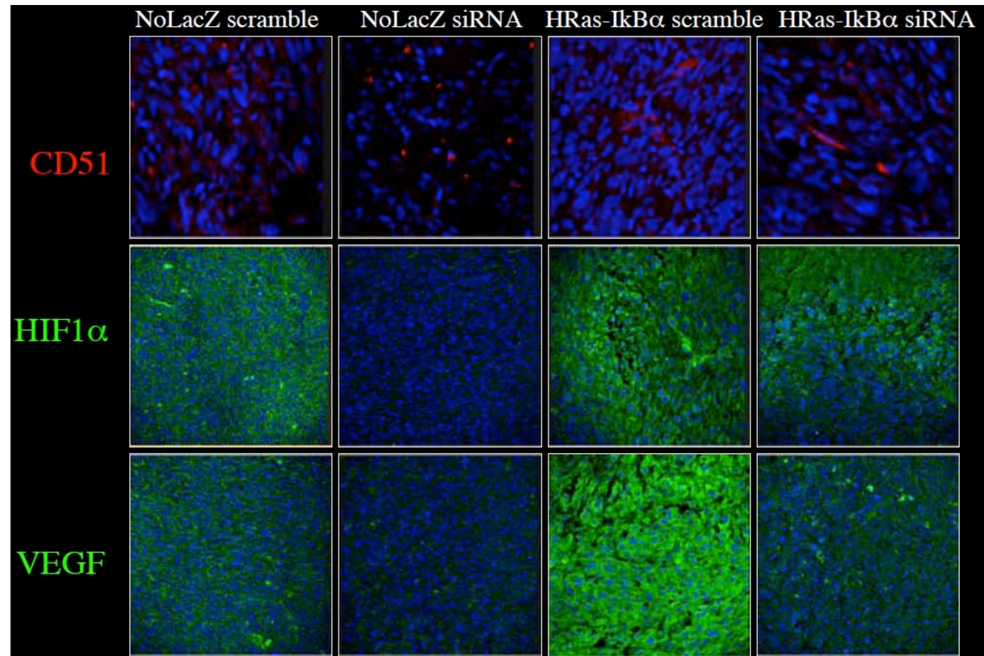
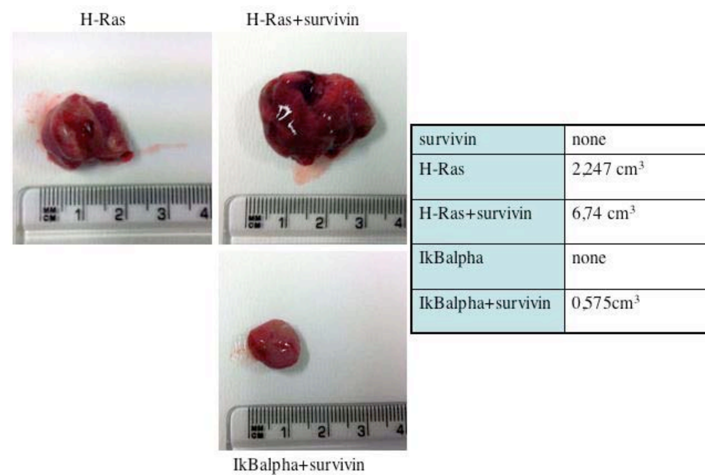


Fig 34: CD51, HIF1alpha and VEGF expression in H-Ras-IkB alpha derived tumor in association with survivin silencing

In order to confirm that survivin has a key role in skin cancer, we overexpressed survivin, H-Ras or IkB alpha alone and survivin in association with H-Ras or IkB alpha.
Overexpression of survivin or IkB alpha alone are not sufficient to induce tumor formation. According to the role of Ras in origin of cancer, big nodules are observed when H-Ras overexpressing keratinocytes are injected in mice. Interestingly, coexpression of Ras and survivin induced formation of three fold bigger tumor (6,74 cm^3) as compared to one in which Ras alone is overexpressed (2,247 cm^3) (fig 35).
Coexpression of IkBalpha and survivin induce the formation of a small lamp (0,575 cm^3).



survivin	none
H-Ras	2,247 cm^3
H-Ras+survivin	6,74 cm^3
IkBalpha	none
IkBalpha+survivin	0,575cm^3

Fig 35: macroscopical analysis of mouse isolated tumor

By hematoxilin and eosin staining, we observed that Ras and Ras-survivin overexpressing tumors have a spindle phenotype and necrotic features. However, Ras-survivin overexpressing tumor appeared more aggressive because of less differentiated cells and a bigger necrotic area.

IkBalpha-survivin tumor appeared only as a neoformation of spindle cells without necrotic area or malignancy features (fig 36). Given that IkBalpha is responsibile fot the tumor formation only when is coexpressed with survivin and taking into account that H-Ras-survivin overexpressing tumor are more aggressive that H-Ras overexpressing one, we conclude that survivin plays an important role in tumor aggressivness.

Keratin staining suggests that all tumor are epithelial in origin (fig 37). Macroscopical analysis shows an important vascularization in tumors.

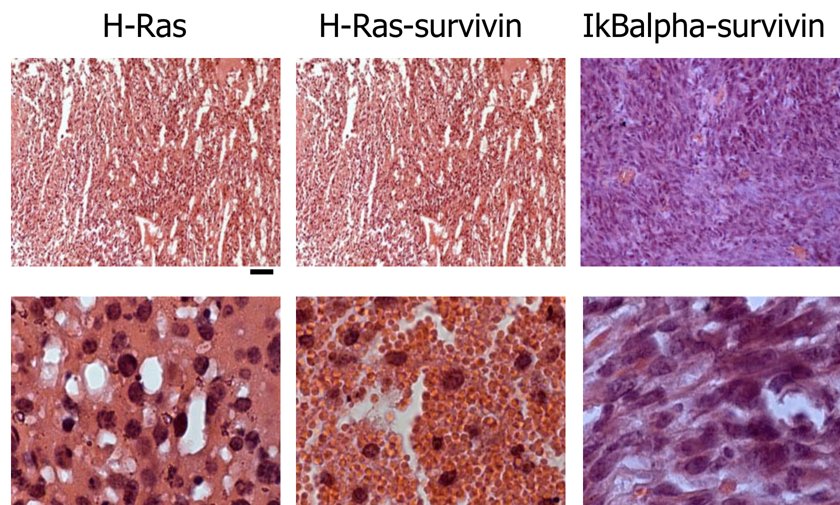


Fig.36: Hematoxylin and eosin staining

New blood vessel development is an important process in tumor progression. It favors the transition from hyperplasia to neoplasia i.e. the passage from a state of cellular multiplication to a state of uncontrolled proliferation characteristic of tumor cells.

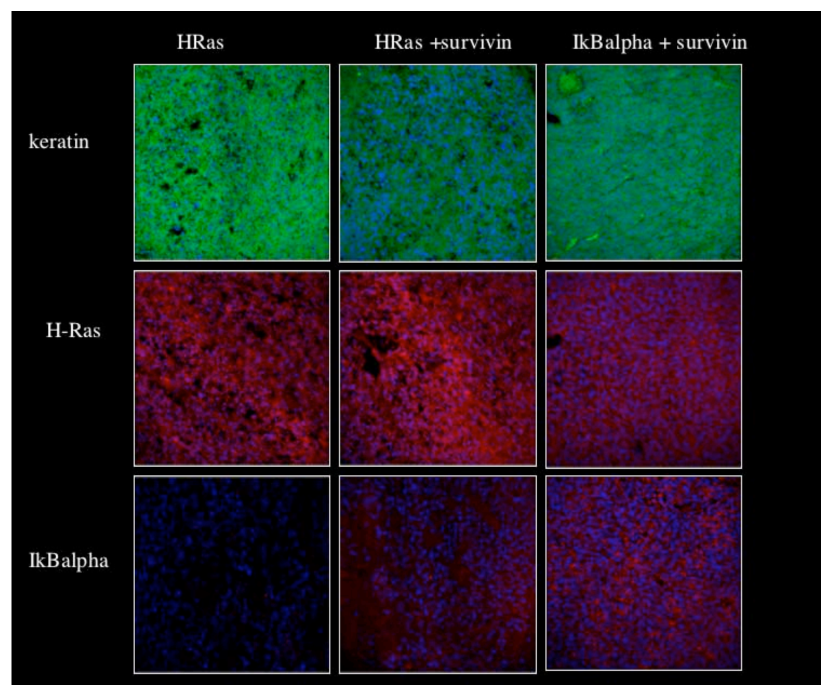


Fig. 37: immunofluorescence staining for keratin, H-Ras and IkBalpha

Neovascularization also influences the dissemination of cancer cells throughout the entire body eventually leading to metastasis formation. The vascularization level of a solid tumor is thought to be an excellent indicator of its metastatic potential.
All tumors were positive for VEGF, HIF1 alpha and CD51 staining but no significative differences have been detected in their expression levels (fig 38).

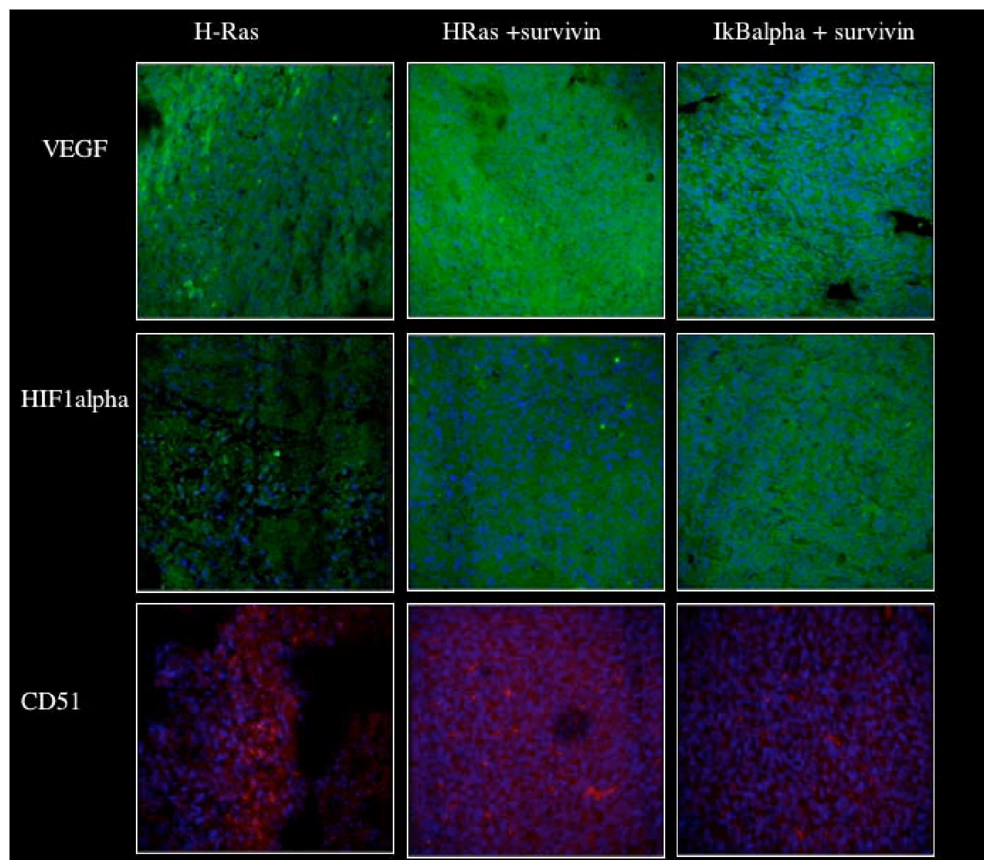


Fig. 38: immunofluorescence staining for VEGF, HIF1alpha and CD51.

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