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p63 and epithelial homeostasis

Studies of p63 under normal, hyper-proliferative and malignant conditions

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学而不思则罔，思而不学则殆
Learning without thinking leads to confusion
thinking without learning ends in danger

孔子
Confucius

In memory of my parents

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Abstract

Background: The *p63* gene is a member of the p53 transcription factor family and can produce six different proteins using two promoters and differential splicing. Expression of p63 is required for proper formation of epithelial tissues. Studies on the transcriptional control of specific genes involved in cell survival, proliferation, differentiation and adhesion have revealed the contributions of p63 to the continuously renewing stratified epithelium. In this thesis, the aim was to improve our understanding of the roles of p63 in epithelial homeostasis by investigating expression of p63 in normal and benign hyper-proliferative epithelia and exploring the influence of p63 deregulation on cancer progression.

Materials and methods: Using quantitative real time RT-PCR and immunohistochemistry, we first examined the expression of different p63 isoforms in patients diagnosed with psoriasis - a benign hyper-proliferative and inflammatory skin disease. Afterwards, we investigated responses of p63 in psoriatic epidermis upon Narrowband-UVB (NB-UVB) phototherapy. At the same time, we studied the potential impact of p63 in carcinogenesis by searching for p63 transcriptional targets in a cell line derived from squamous cell carcinoma of the head and neck (SCCHN) - the sixth most common cancer worldwide with over-expression of the ΔNp63α protein as a common feature. p63 gene silencing and microarray were used to identify p63 regulated genes. Real time RT-PCR, western blot, immunohistochemistry, chromatin immunoprecipitation, transient transfection and reporter assays were performed to confirm specific genes as direct p63 targets.

Results: Significant down-regulation of p63 mRNA levels was found in psoriatic lesions compared to patients' own clinically normal skin. Moreover, a trend of decreased TAp63 mRNA levels was seen in patients' normal skin compared to age- and sex-matched healthy controls. Following NB-UVB phototherapy, an effective first line therapy for psoriasis, expression of p63 was not significantly affected. However, significant changes in p53, FABP5, miR-21 and miR-125b were found. Surprisingly, location and expression levels of p63 proteins detected by immunohistochemistry were similar under all skin conditions. A direct transcriptional regulation of TRAF4 by p63 was seen in the SCCHN cell line and we further found that the localization of the TRAF4 protein was associated with histological differentiation of SCCHN cells. However, unlike its over-expression in SCCHN, similar TRAF4 mRNA expression levels were seen in psoriatic lesions as compared to healthy controls. Besides TRAF4, a total of 127 genes were identified as potentially p63 regulated in the SCCHN cell line and strikingly, about 20% of these genes are involved in cell adhesion or migration.

Conclusions: Dysregulation of p63 isoforms in psoriatic epidermis, especially decreased TAp63 expression, and their resistance to NB-UVB phototherapy implicated a contribution of p63 to the psoriasis phenotype. Transcriptional regulation of genes involved in multiple biological pathways indicated that over-expression of p63 in SCCHN might account for altered cell differentiation, adhesion and migration, thus contributing to SCCHN. In conclusion, our studies have found additional mechanisms through which p63 guarded homeostasis of the established epithelium. Deregulation of p63 might play a role in distinct pathological conditions by participating in diverse cellular pathways under different microenvironments.

Keywords: p63, psoriasis, SCCHN, epithelium, homeostasis

Original Articles

I. Gu X, Lundqvist EN, Coates PJ, Thurfjell N, Wettersand E, Nylander K (2006) Dysregulation of TAp63 mRNA and protein levels in psoriasis. *J Invest Dermatol* **126**:137-141.

II. Gu X, Nylander E, Coates PJ, Nylander K. Little effect on p63 but significant effect on miR-21 and miR-125b by NB-UVB phototherapy on psoriatic lesions. *Manuscript.*

III. Gu X, Coates PJ, MacCallum SF, Boldrup L, Sjostrom B, Nylander K (2007) TRAF4 is potently induced by TAp63 isoforms and localised according to differentiation in SCCHN. *Cancer Biol Ther* **6**:1986-1990.

IV. Gu X, Coates PJ, Boldrup L, Nylander K (2008) p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck. *Cancer Lett* **263**:26-34.

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Abbreviations

ALOX12	arachidonate 12-lipoxygenase
AP-1	activator protein 1
AQP3	aquaporin 3
ChIP	chromatin immunoprecipitation
CT	carboxyl-terminal basic domain
DBD	DNA binding domain
DLX-3	distal-less homeobox 3
EGFR	epidermal growth factor receptor
ERK1	mitogen-activated protein kinase 3
ERK2	mitogen-activated protein kinase 1
FASN	fatty acid synthase
FRAS1	Fraser syndrome 1
HAT	histone acetyltransferase
HNRPK	heterogeneous nuclear ribonucleoprotein K
HPV	human papillomavirus
IGFBP3	insulin-like growth factor binding protein 3
IHC	Immunohistochemistry
IKKα	inhibitor of κB kinase α
IKKα	I-kappaB kinase alpha
IL	interleukin
ITGA3	integrin, alpha 3
K1	keratin 1
K10	keratin 10
K14	keratin 14
K5	keratin 5
KC	keratinocyte
miRNA	micro RNA
MDM2	mouse double minute 2
MMP	matrix metalloproteinase
NB	narrowband
NFκB	nuclear factor-κB
NIR	novel INHAT repressor

OD	oligomerization domain
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p300	E1A binding protein p300
p38	mitogen-activated protein kinase 14
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
PERP	p53 apoptosis effector related to PMP-22
PRR	proline rich region
PTEN	phosphatase and tensin homolog
PUVA	psoralen and ultraviolet A irradiation
Rb	retinoblastoma
RT-PCR	reverse transcriptase PCR
S100A2	S100 calcium binding protein A2
SAM	sterile alpha motif
SCCHN	Squamous Cell Carcinoma of the Head and Neck
SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
TAD	transactivation domain
TGF	transforming growth factor
TID	transactivation inhibitory domain
TPM1	tropomyosin 1 (alpha)
TRAF4	tumor necrosis factor receptor-associated factor 4
UV	ultraviolet
UVR	ultraviolet radiation
WB	Western blotting

Introduction

p63 belongs to the p53 family

An older member of the p53 family

The *p63* gene was discovered in 1997 and 1998 by different laboratories and was shown to be a member of the p53 family of transcription factors (Augustin *et al*., 1998; Osada *et al*., 1998; Schmale and Bamberger, 1997; Yang *et al*., 1998). *p63* is located on chromosome 3q27-29 and consists of 15 exons. The gene expresses two fundamentally different classes of proteins, designated TAp63 and ΔNp63, by the use of alternative promoters and transcription start sites. p63 TA and ΔN isoforms are further subjected to alternative splicing at their carboxy termini, resulting in α, β and γ variants. From these combinations, six different isoforms are expressed (Figure 1).

The p53 transcription factor family includes p53, p63 and p73. p63 represents the most ancient member of the p53 family and the *p63* gene is extraordinarily conserved. Human and murine p63 proteins show 99% amino acid identity (Yang *et al.*, 1998), and there is 93% amino acid identity between human and frog (Lu *et al.*, 2001). Similar to p63, p53 and p73 are also expressed as many different isoforms. They possess a central DNA binding domain (DBD) and a C-terminal oligomerization domain (OD), but differ greatly in their N and C-terminal regions. The highest degree of homology is seen within the DBD. p63 shares about 65% amino-acid identity with the DBD of p53 and there is about 35% sequence identity in the OD between p63 and p53. As observed for p53, the OD of p63 and p73 can independently fold into stable homotetramers. p53 OD does not associate with that of either p73 or p63, however, multiple isoforms of p63 as well as those of p73 are capable of interacting via their common OD (Davison *et al.*, 1999).

The N-terminus is the least conserved domain among the family members with about 25% sequence identity. TA isoforms contain an amino terminal transactivation domain (TAD). p53 TAD is regulated by ligases and co-activator proteins and the functional conformation of this region appears to be an alpha helix which is necessary for its appropriate interactions with several proteins including MDM2. However, for p63 TAD, the helical propensity is very low, but still suitable for regulatory bindings to occur (Mavinahalli *et al.*, 2010). A putative TAD might exist within the N-terminus of the ΔNp63 variants

(Dohn *et al.*, 2001; Helton *et al.*, 2006) and a second TAD has been suggested (Ghioni *et al.*, 2002). The p53 tail is a basic domain (CT) that has been shown to possess sequence-nonspecific nucleic acid binding ability, whereas both p63 and p73 have a sterile alpha motif (SAM) domain which could be involved in oligomerizaion, protein-protein and protein-RNA interactions. p63α isoforms also contain a transactivation inhibitory domain (TID) in their C termini (Serber *et al.*, 2002).

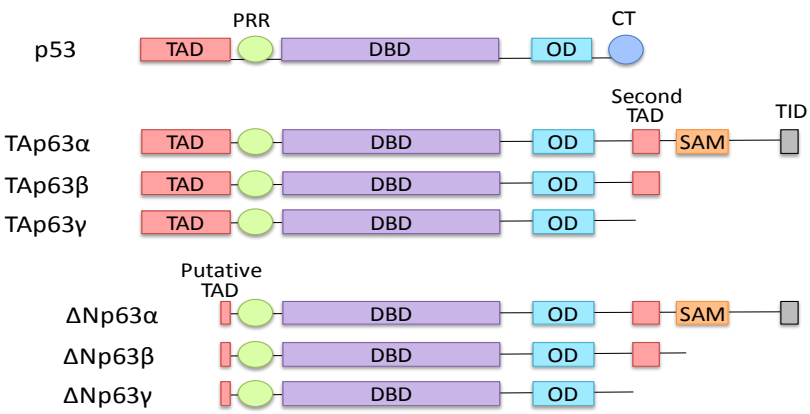

Figure 1. Domain structure of full-length p53 and p63 isoforms. PRR is short for proline rich region.

Transcriptional regulation similar to p53

Consistent with the sequence and structural homology of the p53 and p63 DNA binding domains, p63 proteins can bind to p53 consensus DNA binding sites for transcriptional control. An overlap in downstream regulated genes between p53 and p63 has been identified (Yang *et al.*, 1998). In addition, distinct subsets of p63 targeting genes were found, probably due to the existence of unique p63 consensus recognition sites. It has been shown that tetrameric p63 preferentially binds to two consecutive 10-mer sequence motifs with the consensus (rrrCGTGyyy), (t/a,a/t,a,C,A/T,T,G,t,t/a,t), or (rrrC,A/G,T/A,Gyyy), whereas tetrameric p53 preferentially recognizes (rrrC,A/T,A/T,Gyyy) (r = purines, y = pyrimidines) (Heyne *et al.*, 2010; Ortt and Sinha, 2006; Osada *et al.*, 2005).

p63 isoforms differ in their transcriptional capacities. TAp63γ is a p53-like protein and is the most potent isoform for transactivation. The transactivation activity of TAp63α is dramatically lower than that of β and γ isoforms due to the presence of a SAM domain (a dominant transcriptional repression module) and TID (Ghioni *et al.*, 2002; Serber *et al.*, 2002). It seems that ΔNp63 isoforms mainly act as transcriptional repressors, or dominant negatives, competing for DNA target sites or forming transcriptionally inactive hetero-complexes (Westfall *et al.*, 2003; Yang *et al.*, 1998). Transactivational ability of ΔNp63 isoforms were also shown, probably due to the putative TAD within the truncated N-terminus and a second TAD located between exons 11 and 12 (Dohn *et al*., 2001; Ghioni *et al*., 2002; Helton *et al*., 2006; Lin *et al*., 2009).

p63 transcriptional activity is associated with protein stability and might be regulated by post-translational modifications such as phosphorylation, ubiquitination and sumoylation (Ghioni *et al*., 2005; Li *et al*., 2008; Rossi *et al*., 2006; Westfall *et al*., 2005; Vivo *et al*., 2009). In general, ΔNp63 isoforms are expressed at higher intracellular levels and have greater stability than their TA counterparts (Petitjean *et al.*, 2008). The low stability of the TA proteins might be due to the TAD, which can regulate protein stability in a proteasome-dependent manner (Osada *et al.*, 2001). Interestingly, it has been suggested that, similar to p53, TA isoforms might induce expression of genes involved in their own degradation (Ying *et al.*, 2005).

Transcriptional co-activators can interact with numerous transcription factors and the basal transcription machinery and act to increase the expression of their target genes. Gene transcription by the p53 family of proteins is known to be regulated by p300, a transcriptional co-activator and histone acetyltransferase (HAT). p300 could bind to N-terminal domain and stimulate TAp63γ-dependent transcription, whereas ΔNp63γ inhibited transcription induction (MacPartlin *et al.*, 2005). The novel INHAT Repressor (NIR) is an inhibitor of HAT and could bind to the TAD and the OD of TAp63 thus acting as a repressor of TAp63-mediated transactivation (Heyne *et al.*, 2010).

Overlapping and distinct functions to p53

p53 plays an instrumental role in the induction of cell cycle arrest, DNA repair, senescence and apoptosis in response to DNA damage by transcriptionally regulating a multitude of target genes. In this way, p53 acts as a prototypic tumor suppressor ensuring genomic integrity and eliminating damaged cells.

The highly structural and biochemical similarities between p53 and p63 led to the early hypothesis that p63 would likewise have tumor suppressive functions. However, in human tumors, contrary to the common mutation and loss of p53, p63 is rarely mutated but over-expressed in various malignancies. Germline mutations in p63 are actually underlying a number of human ectodermal dysplasias. Three anomaly groups are associated with p63 mutations, including ectodermal dysplasia, cleft lip or palate and limb malformations (Rinne *et al*., 2007; van Bokhoven and Brunner, 2002), indicating the relevance of p63 to normal ectodermic development in humans. In addition, unlike the universal expression of *p53* transcript in a wide range of somatic cells and the accumulation of p53 protein in response to stresses, p63 protein is found in several stratified epithelial tissues such as stratified squamous epithelium (epidermis, oral mucosa, and cervical epithelium), transitional epithelium (found in the mucosa of the urinary bladder) and complex glands (prostate, mammary, salivary, and lacrimal glands) (Barbieri and Pietenpol, 2006). Tissue specific location of p63 also indicates its functional difference compared to p53.

Indeed, targeted gene disruption studies in mice revealed critical roles for p63 in embryonic development. The phenotypes of severe abnormalities observed in two independent lines of p63 (-/-) mice shared remarkable similarities (Mills *et al.*, 1999; Yang *et al.*, 1999). The most profound finding was the absence of stratified epithelia and their ectodermal derivatives, including epidermal appendages, mammary, lacrimal and salivary glands. They suffered severe dehydration and died shortly after birth due to the absence of an epidermal barrier. An ancillary finding was the marked reduction in normal limb, tail, facial, and external genital development. All of these structural defects could be traced to the fact that the epithelium failed to develop and stratify, a prerequisite for the necessary epithelial-stromal interactions that typically promote limb and appendage elongation and remodeling (Crum and McKeon, 2010). Hence, numerous studies focused on the contribution of p63 to morphogenesis of stratified epithelia.

p63 is a critical regulator during epithelial morphogenesis

Based on the epidermal phenotype of the p63 null mice reported by two independent laboratories, a role for p63 in either of two processes critical to normal epidermal morphogenesis was proposed: maintenance of the stem cell population in an already committed stratified epithelium (Yang *et al.*, 1999), or

commitment from immature ectoderm to stratified epithelial lineages (Mills *et al.*, 1999). After a decade's research, it's getting clear that during epithelial morphogenesis, the *p63* gene acts as a master regulator for maintaining basement membrane integrity, initiating keratinocyte (KC) terminal differentiation and also maintaining proliferative potential of epithelial stem cells (Crum and McKeon, 2010; Koster *et al.*, 2007; Koster and Roop, 2007; Senoo *et al.*, 2007).

Detecting temporal expression patterns of different p63 isoforms during development can provide valuable clues for understanding the developmental roles of various p63 isoforms. However, the temporal expression patterns of ΔNp63 and TAp63 transcripts during mice development were reported differently from different labs (Koster *et al.*, 2004; Laurikkala *et al.*, 2006). Nevertheless, it is agreed that p63 is expressed in the surface ectoderm prior to stratification and continues to be expressed during embryonic development. In mouse embryonic day 13 skin samples, ΔNp63 isoforms represented 99% of the p63 transcripts while the remaining 1% of the transcripts was TAp63 (Laurikkala *et al.*, 2006).

Selective genetic complementation in p63 null mice was performed to study functions of TAp63 or ΔNp63 isoforms in epidermal development (Candi *et al*., 2006). Results showed that both TAp63 and ΔNp63 isoforms were important in epidermal morphogenesis. ΔNp63α might control expansion of progenitor cells in the basal layer and TAp63α might regulate differentiation of upper epidermal layers synergistically and/or subsequently with ΔNp63α. However, using TAp63 knockout mice, it was suggested that TAp63 isoforms were dispensable for epidermal development (Guo *et al.*, 2009; Su *et al.*, 2009). They showed that the TAp63-null mouse appeared normal and suffered none of the cutaneous or physical anomalies that characterize loss of ΔNp63 expression.

Whatsoever, numerous studies have demonstrated that ΔNp63α is the main regulator in epithelial development. ΔNp63α induces target genes at different developmental stages, including genes involved in KC adhesion, proliferation, terminal differentiation and basement membrane formation (Koster and Roop, 2008). For example, the earliest known gene induced by ΔNp63α during epidermal morphogenesis (shortly after commitment to epidermal cell fate) is PERP, a tetraspan membrane protein localizes specifically to desmosomes and important for tissue integrity (Ihrie *et al.*, 2005). Subsequently, ΔNp63α induces FRAS1 for maintaining the integrity of the epidermal-dermal interface at the basement membrane (Koster *et al.*, 2007). After commitment to terminal

differentiation, ΔNp63α induces IKKα for the formation of spinous layers (Koster *et al.*, 2007). Finally, ΔNp63α contributes to epidermal barrier formation by inducing expression of ALOX12 (Kim *et al.*, 2009).

p63 is essential for maintenance of epithelial homeostasis

Location in stratified squamous epithelium

Stratified epithelial tissue is the primary barrier that protects the organism from mechanical trauma, chemical damage and microbial insults. Stratified squamous is the epithelium most frequently found and can be subdivided into non-keratinized (eg. buccal mucosa, esophagus and vagina) or keratinized types (eg. epidermis, palate and gingival). Normal epithelium is separated by a well-delineated basement membrane (basal lamina) from the dermal or stromal compartment. KCs represent more than 90% of the epithelial cells and are organized into several cell layers with layer-specific expression of structural and enzymatic markers, such as markers for basal KCs K5/K14 and markers for early differentiation K1/K10. Keratins are the most abundant cellular proteins which are attached to the cell surface via desmosomes, a type of cell-to-cell adhesion complexes that link epithelial cells to each other. Other types of intercellular junctions include adherens-, tight- and gap junctions. Langerhans cells, melanocytes and Merkel cells are also found in stratified epithelium and responsible for immunological defence, pigmentation and sensory, respectively.

Stratified squamous epithelium is a continuously renewing tissue through an intricate balance between KC proliferation and differentiation. The regenerative capacity of epithelium is sustained by proliferating cells in the basal layer. For example, in epidermis, proliferating KCs in the basal layer detach from basal lamina and move outward to the surface of the skin by first differentiating to spinous cells, then to granular cells, then terminally differentiating as cornified, anuclear cells, and ultimately shed from the body surface (Figure 2). A finite time for each KC to undergo terminal differentiation is required to maintain the dynamic equilibrium state of epidermis. Establishing, maintaining and restoring epithelial homeostasis also highly rely on a proper communication between epithelial cells and the underlying stroma which consists of fibroblasts, endothelial cells and immune cells. Many molecules have been highlighted in control of epithelial homeostasis, including myc, Notch and NFκB (Truong and

Khavari, 2007). The process of KC proliferation and differentiation in mature epithelium is somehow similar to that during epithelial morphogenesis. Therefore, it is not surprising that p63 is required not only for the formation of stratified epithelium during development, but also for developmentally mature KC to regenerate a stratified epithelium.

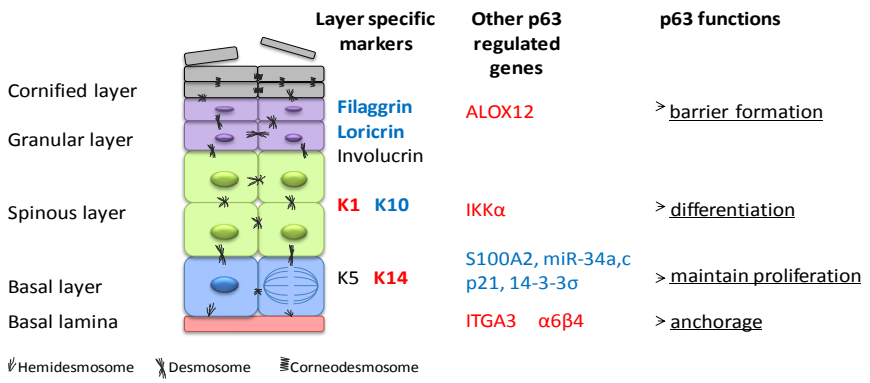

Figure 2. Differentiation of KCs in mature stratified epithelium. Most layer specific markers are up-regulated (symbol in red) or down-regulated (symbol in blue) by ΔNp63. Other selected genes regulated by ΔNp63 are also shown, demonstrating roles of p63 in maintenance of epithelial homeostasis.

It has been shown that expression of ΔNp63 in stratified epithelium is a dynamic process. The highest expression levels are observed in the basal proliferative compartment of epithelium, where progenitors reside (Yang *et al.*, 1998). In the overlying differentiated layers, ΔNp63α expression is down-regulated. Low expression of TAp63 proteins could be found through the epithelial thickness (Nylander *et al.*, 2002). *In vitro* experiments showed that during differentiation of primary murine epidermal KCs, levels of TAp63 protein were elevated coincident with the decline of ΔNp63α (King *et al.*, 2006).

Maintains basal cell proliferation and prevents premature differentiation

Similar to the role of p63 in epithelial development, it was suggested that ΔNp63α is required for maintenance of the proliferative potential of basal KCs

and for the initial commitment to the differentiated phenotype (Parsa *et al.*, 1999). Experiments showed that human primary KC with decreased ΔNp63 expression resulted in hypo-proliferation (Truong *et al.*, 2006). Increased ΔNp63α expression in basal cells could block KC stratification and differentiation, probably by inhibiting the induction of differentiation related markers such as K10, loricrin, and filaggrin (King *et al.*, 2006) and preventing Ca^{2+} induced differentiation (King *et al.*, 2003). ΔNp63α might inhibit cell cycle withdrawal and terminal differentiation of KCs by repressing expression of cell cycle regulatory proteins such as p21 and 14-3-3σ (Westfall *et al.*, 2003). In mouse KC, it was shown that expression of ΔNp63α maintained the immature state of basal KC by blocking Notch1 dependent cell cycle withdrawal and commitment to differentiation (Nguyen *et al.*, 2006; Okuyama *et al.*, 2007). Similarly, S100A2, which is also required for proper KC differentiation, was transcriptionally repressed by ΔNp63α predominantly in proliferating cells (Lapi *et al.*, 2006). Thus ΔNp63α plays an important role in the proliferative capacity of basal KCs and could also prevent basal KCs from premature differentiation.

As mentioned above, TAp63 is dispensable for the genesis of skin. However, mice lacking TAp63 aged prematurely and developed blisters, skin ulcerations, senescence of hair follicle-associated dermal and epidermal cells, and decreased hair morphogenesis. In normal mice, TAp63 is expressed not only in epidermal cells, but also in the dermal sheath and dermal papilla, niches for dermal precursor cells known as skin-derived precursors. The phenotypes seen in TAp63-null mice were likely due to loss of TAp63 in dermal and epidermal precursors since both cell types show defective proliferation, early senescence, and genomic instability. These data indicated that TAp63 served to maintain adult skin stem cells by regulating cellular senescence and genomic stability, thereby contributing to tissue homeostasis and preventing premature tissue aging (Su *et al.*, 2009). A role for TAp63 in maintaining progenitor cell proliferation and inhibiting terminal differentiation was also demonstrated by showing that over-expressing TAp63α in primary KCs resulted in failure of differentiation after Ca^{2+} stimulation. Over-expression of TAp63α in the basal layer of mouse epidermis induced severe hyperplasia and a delayed onset of differentiation (Koster *et al.*, 2004).

Induces terminal differentiation

Epidermal-specific ΔNp63 down-regulation in mice resulted in failure of KCs to undergo terminal differentiation and the development of severe skin fragility characterized by multiple skin erosions (Koster *et al.*, 2007). Induction of IKKα and K1 by ΔNp63α at early phases of KC differentiation may be required for correct exit from the cell cycle upon differentiation stimulus (Koster *et al.*, 2007; Marinari *et al.*, 2009). A complex cross-talk between p63 and Notch1, a key molecule for promoting KC commitment to terminal differentiation, has been shown to be involved in the balance between KC self-renewal and differentiation (Nguyen *et al.*, 2006).

It seems that ΔNp63 is required for the initial commitment to the differentiated phenotype, but in order for differentiation to proceed, it must be down-regulated. Decreased ΔNp63α expression in the differentiated layers might be due to the induction of miR-203 expression. MicroRNAs (miRNAs) form a class of small non-coding RNAs (19–24 nucleotides) with key roles in the regulation of gene expression in several cellular events ranging from organogenesis to immunity and carcinogenesis. miRNAs can bind to partially complementary sites in the 3' untranslated regions of their mRNA targets and inhibit gene expression either by interfering with translation or by destabilizing the target mRNA (Bartel, 2009). It has been shown that ΔNp63α was one of the targets of miRNA-203, which was induced in the skin concomitantly with stratification and differentiation, and promoted epidermal differentiation by restricting proliferative potential and inducing cell cycle exit in both human and mouse KCs (Lena *et al.*, 2008; Yi *et al.*, 2008). A feedback regulatory loop between p63 and DLX-3 was also implicated in this process. DLX-3 which is transactivated by TAp63 at the onset of epidermal terminal differentiation could induce proteasome-mediated degradation of ΔNp63α, thus cooperating to accomplish the program of terminal differentiation (Di Costanzo *et al.*, 2009; Moretti and Costanzo, 2009; Radoja *et al.*, 2007).

Regulates adhesion and maintains integrity of basal membrane

By transactivating ITGA3, it was proposed that p63 allows epidermal stem cells to express laminin receptor $\alpha_3\beta_1$ for anchorage to the basement membrane (Kurata *et al.*, 2004). ΔNp63α has the ability to induce expression of integrin $\alpha_6\beta_4$, which promotes attachment of basal cells to basal membrane thereby keeping cells in immature state (Okuyama *et al.*, 2007). ΔNp63 expression in

the basal and suprabasal layers of the epithelium may be required to maintain a pattern of adherens junctions compatible with cell proliferation; its down-regulation in parabasal layers may facilitate the expression of tight junction components which is incompatible with cell division (Thepot *et al.*, 2010).

Knockdown of p63 expression caused down-regulation of cell adhesion-associated genes, cell detachment and anoikis in mammary epithelial cells and KCs, implicating p63 as a key regulator of cellular adhesion and survival in basal cells of the mammary gland and other stratified epithelial tissue (Carroll *et al.*, 2006). Results from another study showed that loss of endogenous p63 expression resulted in up-regulation of genes associated with invasion and metastasis, and predisposed to loss of epithelial and acquisition of mesenchymal characteristics. p63 may define the difference between epithelial cells and stromal cells at the interface between these populations, while still allowing the flexibility for physiological and pathological epithelial to mesenchymal transitions (Barbieri and Pietenpol, 2006; Barbieri *et al.*, 2006). Interestingly, it was recently found that Snail and Slug, transcription factors known to promote epithelial-to-mesenchymal transitions during development and cancer, could repress ΔNp63 expression and lead to an up-regulation of TAp63, thus reducing cell-cell adhesion and increasing the migration of squamous malignant cells (Herfs *et al.*, 2010).

Responds to DNA damage

Induction of cell cycle arrest and apoptosis in response to cellular stress response is the key function to ensure genomic integrity and prevent propagation of genetic errors that leads to tumor formation. It has been shown that p63 was required for p53-dependent apoptosis in response to DNA damage (Flores *et al.*, 2002). TAp63 splice variants increased due to UVC irradiation (Katoh *et al.*, 2000; Okada *et al.*, 2002). Ectopic expression of TAp63 induced apoptosis and cell growth arrest (Gressner *et al.*, 2005). TAp63 has been shown to be the guardian of the female germ cell genome, akin to p53 in somatic cells. DNA damage induced both the phosphorylation of p63 and its binding to p53 DNA binding sites, events that were linked to oocyte death (Suh *et al.*, 2006). ΔNp63α transcript levels declined in epidermal tissue after treatment with DNA damaging agents such as UV radiation, cisplatin, or adriamycin (Harmes *et al.*, 2003; Liefer *et al.*, 2000). The increased phosphorylation of ΔNp63α following cellular stress resulted in its ubiqitination and proteosomal degradation

(Fomenkov *et al.*, 2004; Westfall *et al.*, 2005). After apoptotic doses of UVB radiation, ΔNp63 was rapidly phosphorylated by p38 kinase, thus leading to the detachment of ΔNp63 proteins from p53-dependent promoters and to the induction of apoptosis (Papoutsaki *et al*., 2005). All these findings suggest that TAp63 is the major regulator in response to DNA damage similar to p53, and that down-regulation of the dominant negative ΔNp63 can promote functions of TAp63 and p53. Interestingly, a recent study showed that both TAp63 and ΔNp63 isoforms were able to transactivate genes involved in homologous DNA repair in response to DNA damage, with ΔNp63 being the stronger transactivator, thus making the role of ΔNp63 even more complex (Lin *et al.*, 2009).

Contributes to cellular senescence and aging

The potential for p63 to act as an oncogene or as a tumor suppressor and its interaction with other p53 family members continues to be the focus of research. Mice deficient for p63 were developed by different groups to study the role of p63 in tumorigenesis, however, with differing conclusions. In a study by Flores *et al*. (Flores *et al.*, 2005), it was found that p63 (+/-) mice had a predisposition towards tumor development and that the p53 family of genes might work interdependently of each other in the suppression of tumorigenesis, with dual heterozygous p63 (+/-) p53 (+/-) mice exhibiting enhanced tumor formation and a highly aggressive and metastatic phenotype as compared to p53 (+/-) mice. In contrast, in another study (Keyes *et al.*, 2006), no evidence of enhanced tumor formation was found in p63 (+/-) mice, furthermore, dual heterozygous p63 (+/-) p53 (+/-) mice carried a reduced tumor burden compared to mice heterozygous for p53 alone.

Differences between these findings might be due to the use of different mouse strains and different targeting constructs. Nevertheless, transgenic mice from these studies manifested decreased longevity associated with characteristics of accelerated aging, including skin lesions and alopecia. Organism aging is linked to cellular senescence, which is a tumor-suppressive mechanism to prevent progression of pre-malignant lesions. Both germline and somatically induced p63 deficiency activate widespread cellular senescence. Using an inducible tissue-specific p63 conditional model, it was also shown that p63 deficiency induced cellular senescence and caused accelerated aging phenotypes in the adult (Keyes *et al.*, 2005). Transgenic mice that over-expressed ΔNp63α in the

skin also exhibited an accelerated aging phenotype in the skin characterized by striking wound healing defects, decreased skin thickness, decreased subcutaneous fat tissue, hair loss, and decreased cell proliferation (Sommer *et al.*, 2006). Using a new TAp63-specific conditional mouse model, it was demonstrated that TAp63 isoforms were essential for Ras-induced senescence through p53-independent pathways (Guo *et al.*, 2009). As mentioned above, Su *et al.* demonstrated that TAp63 was essential for maintenance of epidermal and dermal precursors and that, in its absence, these precursors senesced and skin aged prematurely (Su *et al.*, 2009).

Contributes to carcinogenesis

An aberrant over-expression of ΔNp63 was found in many epithelial carcinomas, such as SCC from head and neck, skin, lung and cervix (Di Como *et al.*, 2002; Nylander *et al.*, 2002). Over-expression of p63 may be caused by amplification of the genomic region which harbors p63 (Hibi *et al.*, 2000; Yamaguchi *et al.*, 2000). Further experiments showed that over-expressed ΔNp63α seen in human cancers maintains KC proliferation under conditions that normally induce growth arrest (King *et al.*, 2003). p63 knockdown in squamous cell carcinoma or immortalized prostate epithelial cells caused a decrease in cell viability by inducing apoptosis without affecting the cell cycle. Pro-survival ability of p63 is mediated by the regulation of fatty acid synthase (FASN), a key enzyme that synthesizes long-chain fatty acids and is involved in both embryogenesis and cancer (Sabbisetti *et al.*, 2009). ΔNp63α over-expression in squamous carcinoma cells suppressed a TAp63-dependent proapoptotic program and promoted cellular survival (Rocco *et al.*, 2006) and p63 knockdown led to TAp73-mediated apoptosis (DeYoung *et al.*, 2006). An oncogenic property of p63 was shown in squamous cell carcinoma of head and neck (SCCHN) cells by maintaining cell survival. Inhibition of endogenous p63 expression sensitises cells to the effects of ionizing radiation and cisplatin (Thurfjell *et al.*, 2005). Over-expression of p63 in SCCHN cells induced expression of the cancer stem cell marker CD44, indicating its role in the regulation of adhesion, metastasis and the cancer stem cell phenotype (Boldrup *et al.*, 2007). Elevated p63 in cancers could cause aberrant activation of cell growth progression genes, indicating its contributions to cancer initiation or progression (Lefkimmiatis *et al.*, 2009). p63 over-expression was associated with poor prognosis in SCCHN (Lo Muzio *et al.*, 2007; Lo Muzio *et al.*, 2005).

However, lower p63 expression was associated with poor prognosis in esophageal squamous cell carcinoma (Takahashi *et al.*, 2006). Loss of ΔNp63 expression was found in bladder cancer and was associated with increased invasion and metastases and poorer prognosis (Koga *et al.*, 2003; Urist *et al.*, 2002). High ΔNp63 protein levels in primary tumors accurately predicted response to platinum based chemotherapy and a favorable outcome in head and neck cancer patients (Zangen *et al.*, 2005). Similarly, p63 expression was associated with favorable prognosis in patients with lung cancer (Massion *et al.*, 2003). Whereas, by examining 106 patients with oral squamous cell carcinoma, no significant association between p63 expression and survival, recurrence or metastasis was reported (Oliveira *et al.*, 2007). The controversial role of p63 in different cancer types might be due to the multi-faceted functions of p63. It is possible that ΔNp63α acts to promote early steps in tumorigenesis by protecting cells from growth arrest and apoptosis, while at the same time acting as a metastasis suppressor by maintaining the epithelial character of cancer cells (Barbieri and Pietenpol, 2006).

Higher TAp63 expression has also been seen in human squamous cell carcinoma (Koster *et al*., 2006; Thurfjell *et al*., 2004) and high-grade follicular lymphomas (Pruneri *et al.*, 2005). The recent report that TAp63 could trigger senescence and halt tumorigenesis irrespective of p53 status highly supported TAp63 as a *tumor* suppressor (Guo *et al*., 2009). Over-expression of TAp63 in human lung, gastric and pancreatic cancer cells revealed that TAp63 could cooperatively enhance the anti-tumor effects of p53 (Kunisaki *et al.*, 2006). Of clinical relevance is that TAp63α was induced by many chemotherapeutic drugs and that inhibiting TAp63 function led to chemoresistance (Gressner *et al.*, 2005). A trend for decreased TAp63 levels has been correlated with poor clinical outcome in buccal (Chen *et al*., 2004) and laryngeal squamous cell carcinomas (Pruneri *et al.*, 2002). However, induced TAp63α expression during chemically-induced skin carcinogenesis dramatically accelerated tumor development and progression frequently resulting in epithelial-mesenchymal transitions to spindle cell carcinomas and lung metastases (Koster *et al.*, 2006).

Psoriasis

Epidemiology, symptoms and histological features

Psoriasis is one of the most common human skin diseases and is considered to have key genetic underpinnings (Lowes *et al.*, 2007). Estimates of the

prevalence of psoriasis vary from 0,5% to 4,6%, with rates varying between countries and races. Psoriasis tends to be more frequent at higher latitudes than lower latitudes and in more caucasians than in other races (Lebwohl, 2003). Psoriasis can begin at any age and develops about equally in males and females.

There are five types of psoriasis: plaque, guttate, pustular, inverse, and erythrodermic. About 80% of patients with psoriasis have plaque psoriasis, also called "psoriasis vulgaris" (Biondi Oriente *et al.*, 1989). Plaque psoriasis is characterized by the presence of red, raised scaly plaques that can cover any body surface. The most common areas of involvement include elbows, knees, lower back, and scalp. Approximately one-third of patients with plaque psoriasis are classified as having moderate-to-severe disease, on the basis of either the body surface area involved or significant impact on psychological and/or physical health (Gottlieb, 2005). Nail changes occur in most patients and between 5% and 42% of patients with psoriasis have psoriatic arthritis, a destructive and occasionally disabling joint disease (Lebwohl, 2003).

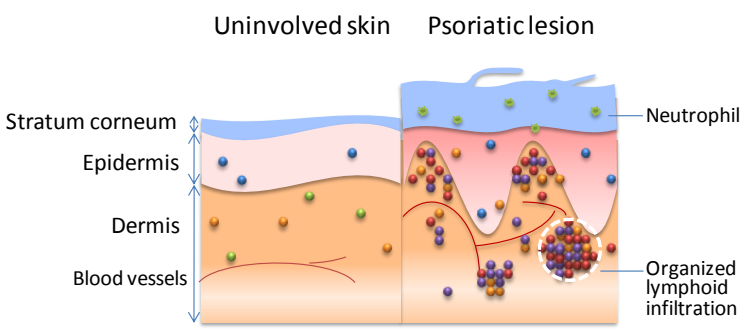

Figure 3. Histological features of psoriasis. Adapted from Lowes *et al.* (Lowes *et al.*, 2007) and Pittelkow (Pittelkow, 2005).

Histologically, psoriasis is characterized by altered homeostasis of KCs and infiltration of inflammatory cells. There is marked thickening of the epidermis, due to increased proliferation of KCs in the interfollicular epidermis. Epidermal rete ridges become very elongated and project downward into the dermis. Altered differentiation of KCs results in a stratum corneum with incompletely differentiated KCs that aberrantly retain cell nuclei, known as parakeratosis. Neutrophils are found in the stratum corneum and mononuclear infiltrates are seen in the epidermis. The dermis is also heavily infiltrated with T cells and

dendritic cells, and there are enlarged blood vessels in the papillary dermal region (Figure 3) (Lowes *et al.*, 2007).

Genetic and environmental triggers

Psoriasis has both genetic and environmental etiologies. The genetic basis of psoriasis has been investigated well in studies of families and twins. High concordance for psoriasis was seen in twins, with a higher degree of concordance in monozygotic than dizygotic twins. Furthermore, disease in monozygotic twins tended to be similar in age of onset, distribution, severity and course, whereas this was not seen for dizygotic twins. Many psoriasis susceptibility loci have been mapped to several regions on different chromosomes (Valdimarsson, 2007).

Most investigators regard psoriasis as a multifactorial disease in which several genes interact with one another and with environmental stimuli. A wide range of environmental agents can cause psoriasis flares, such as physical injury to the skin (the Koebner phenomenon), inflammation induced by cytokines or chemicals, rapid withdrawal of immunosuppressive drugs, such as corticosteroids, and bacterial or viral infections (Bowcock and Krueger, 2005; Gottlieb, 2005; Lebwohl, 2003).

Pathogenesis and treatment

The current paradigm indicates that psoriasis is driven and maintained by T cell–mediated immune responses targeting KCs though its classification as an autoimmune disease is only provisional (Bowcock and Krueger, 2005). The dominant role of immune cells in psoriasis pathogenesis was supported most directly from clinical studies of disease following treatment with a range of immune antagonists. Many therapeutic agents targeting T cells but not KCs (e.g. DAB389IL-2) show good clinical efficacy and are able to fully remit psoriasis in patients (Tonel and Conrad, 2009). At present, treatment of psoriasis ranges from topical therapies for mild disease to systemic therapy for more widespread disease. The immune system is the main target of almost all systemic treatments available for this disease (Papoutsaki *et al*., 2009). A "three-phase immune reaction" model for the pathogenesis of psoriasis has been suggested (Sabat *et al.*, 2007).

However, psoriasis cannot be explained solely on the basis of T-cell activation (Albanesi *et al*., 2007). Interactions between resident skin cells and elements of the immune system conspire to produce a disease that can last for decades in focal regions of the skin (Lowes *et al.*, 2007). Cytokines and growth factors secreted by KCs can stimulate and recruit inflammatory cells, and in turn lead to KC hyper-proliferation. Inherent alterations in epidermal KCs may play a very relevant role in the disease (Albanesi *et al.*, 2007; Lowes *et al.*, 2007). The transgenic mouse model with constitutive over-expression of activated STAT3 in the basal layer of epidermis supported the essential role of both KCs and immunocytes (mainly T cells) in psoriasis by demonstrating that both activated STAT3 in KCs and activated T cells in dermis and epidermis of the transgenic mice were required for development of psoriasis (Sano *et al.*, 2005).

Psoriatic and uninvolved skin show significantly different expression of hundreds of genes, involved in both immune response and in regulation of cellular differentiation and proliferation. Many intracellular signaling pathways have been found altered in psoriatic KCs, including STAT1-, STAT3-, NF-κB-, AP-1-, p38-, and ERK1/2 kinase-activated pathways (Albanesi *et al.*, 2007). Various cytokines and growth factors are over-expressed in psoriatic epidermis. Key cytokines produced by KCs and involved in psoriasis pathogenesis include TGF-α, TGF-β, IL-1, IL-6 and IL-8 (Krueger and Ellis, 2005).

Recently, it was shown that three miRNAs (miR-146a, miR-125b and to some extent miR-203) deregulated in psoriasis, might act as negative regulators of cytokine response, which could be responsible for the aberrant cytokine signaling and local inflammation in psoriasis (Sonkoly *et al*., 2008). Interestingly, miR-125b is an important negative regulator of p53 and p53-induced apoptosis during development and during stress response (Le *et al*., 2009). miR-203 can regulate ΔNp63 levels upon genotoxic damage, thus controlling cell survival (Lena *et al*., 2008). Mir-21, which is commonly over-expressed in many types of malignancies, was also found to be up-regulated in psoriasis. miR-21 acted as an oncogene through regulation of multiple tumor suppressor genes such as PTEN (Meng *et al.*, 2007), TPM1 (Zhu *et al.*, 2007), PDCD4 (Asangani *et al.*, 2008) and HNRPK (Papagiannakopoulos *et al.*, 2008), and stimulated not only tumor growth, but also invasion and metastasis. Notably, down-regulation of miR-21 in glioblastoma cells led to repression of growth, increased apoptosis and cell cycle arrest, by regulation of TAp63 expression (Papagiannakopoulos *et al*., 2008).

Phototherapy

Phototherapy is an old and established treatment modality in the management of many skin diseases. In general, ultraviolet radiation (UVR) exerts its effect in skin by phototype I (a direct change of molecular structure due to photon absorption) and phototype II reactions (generation of reactive oxygen species) (Schneider *et al*., 2008). Various skin biology are affected by UVR, including induction of erythema, pigmentation, vitamin D synthesis and inducing the innate but suppressing the adaptive immune system (Schwarz, 2010). The immunomodulatory effect of UVR on skin is critical for the therapeutic efficacy of UV phototherapy. UVR can induce the production of anti-inflammatory or immune suppressive soluble mediators such as some cytokines, neuropeptides and prostanoids. Expression and function of cell-surface associated molecules such as adhesion molecules, cytokine and growth factor receptors, can also be modulated by UVR. Most importantly, UVR can induce apoptosis of pathogenetically relevant cells such as skin infiltrating T cells (Krutmann and Morita, 1999).

There are different types of phototherapy, such as climatotherapy, broadband UVB, narrowband UVB (NB-UVB) and PUVA. The relatively newly invented NB-UVB phototherapy, also known as TL-01 phototherapy, is a convenient first-line treatment of psoriasis. NB-UVB phototherapy is superior to conventional broadband UVB in treatment of psoriasis and as effective as PUVA therapy (Green *et al.*, 1988; Schneider *et al.*, 2008). The effect of UVR on psoriasis can be divided into 2 groups, immediate and delayed effects. Immediate effects are largely cytopathic and induce growth arrest or even apoptosis, whereas delayed effects are modulations of the psoriasis microarchitecture (Schneider *et al.*, 2008). NB-UVB irradiation could cause apoptosis in cultured epidermal KCs (Aufiero *et al.*, 2006), however, induction of T cell apoptosis has been shown as the main mechanism by which NB-UVB resolves lesions (Ozawa *et al.*, 1999).

Squamous cell carcinoma of the head and neck

Epidemiology and risk factors

Head and neck cancer is a broad category of diverse tumor types arising from various anatomic structures including the craniofacial bones, soft tissues, salivary glands, skin, and mucosal membranes. The vast majority (more than

90%) are squamous cell carcinomas (SCCHN) arising from the epithelium lining the sinonasal tract, oral cavity, pharynx, and larynx and showing microscopic evidence of squamous differentiation (Pai and Westra, 2009). SCCHN is the sixth most prevalent neoplasm in the world. The median age at diagnosis is early 60s. A slight decrease in the overall incidence of SCCHN has been seen over the past two decades; however, an increase in cancer in the base of tongue and tonsillar cancer has been noted, and also more commonly in young adults in the USA and European countries (Argiris *et al*., 2008).

Carcinogen exposure, diet, oral hygiene, infectious agents, family history, and preexisting medical conditions all play a role, individually or in combination, in the development of SCCHN. Of these, tobacco smoking is well established as a dominant risk factor, and also correlated with the intensity and duration of smoking (Pai and Westra, 2009). Oncogenic human papillomavirus (HPV), particularly type 16, has been established as a causative agent in up to 70% of oropharyngeal cancers (D'Souza *et al*., 2007).

Diagnosis and treatment

Diagnosis is often made at a late stage of SCCHN development. Staging of SCCHN is performed using the Tumor-Node-Metastasis (TNM) classification system which describes the anatomical extent of the disease based on: T - extent of the primary tumor, N - absence or presence and extent of regional lymph node metastasis, and M - absence or presence of distant metastasis. Based on the TNM system tumors can be classified into 4 different stages. For patients with early stage disease (stage I and II), surgery and/or radiation therapy are persuaded with curative intent. However, about two-thirds of patients with SCCHN present with advanced stage disease, commonly involving regional lymph nodes. Distant metastasis at initial presentation is uncommon, arising in about 10% of patients. In these instances multimodality therapy in combination with chemotherapy and radiotherapy has been used (Argiris *et al.*, 2008; Nagaraj, 2009).

Prognosis has improved little in the past 30 years. In those who have survived, pain, disfigurement and physical disability from treatment have had an enormous psychosocial impact on their lives (Chin *et al.*, 2006). At least 50% of patients with locally advanced SCCHN develop locoregional or distant relapses, which are usually detected within the first 2 years after treatment. These features, along with the frequent occurrence of late-stage diagnosis, contribute

to a relatively poor five-year survival of about 50% including all SCCHN subgroups.

Histologically tumors are classified as well (grade 1), moderately (grade 2), or poorly differentiated (grade 3) (Woolgar and Scott, 1995). The prognostic value of histologic grading, however, remains controversial (Odell *et al*., 1994; Silveira *et al*., 2007). Nevertheless, in patients with oral squamous cell carcinoma, grade 3 regional metastasis was more frequent (Okada *et al*., 2003) and was associated with decreased survival compared with other tumor grades (Kademani *et al*., 2005).

Molecular mechanisms for carcinogenesis

Understanding the molecular basis for development of SCCHN can facilitate the integration of diagnosis and improve treatment. Several attempts have been made at identifying genetic and epigenetic biomarkers which could improve early diagnosis, predict prognosis and establish targeted treatments. A hypothetical progression model for SCCHN carcinogenesis was raised showing progression from simple squamous hyperplasia through the advancing stages of squamous dysplasia to invasive squamous cell carcinoma. It is believed that head and neck carcinogenesis is a multistep process involving the accumulation of multiple genetic and epigenetic alterations, leading to the inactivation of tumor-suppressor genes and/or activation of proto-oncogenes. Clones of phenotypically intact but genetically damaged cells can populate extended tracts of the mucosa giving rise to second tumors (Pai and Westra, 2009).

Dysregulation of several cell cycle regulated genes are found in SCCHN, such as loss of p53, p16, Rb, PTEN and over-expression of cyclin D1. Over 90% of SCCHN over-express EGFR, a central transducer of multiple signaling pathways involved in a variety of cellular responses including cell growth, angiogenesis, invasion and metastasis (Kalyankrishna and Grandis, 2006). And as mentioned above, amplification and over-expression of ΔNp63α is also the most common oncogenic event in primary SCCHN.

Aims

It is indisputable that the *p63* gene plays a central role in epithelial morphogenesis during development and homeostasis in established tissue. p63 isoforms exert their function mainly by transcriptional regulation of multiple targets, thus influencing cell biology in both physiological and pathological contexts. The unique and common function of p63 isoforms and their balance could give rise to diverse biological outcomes in specific cellular backgrounds. Therefore in this thesis, we chose psoriasis and SCCHN, two fundamentally different diseases but with cell hyper-proliferation as a basic feature, as two different pathological models for studying p63 status and target-genes, in order to increase our understanding of the roles of different p63 isoforms in maintenance of epithelial homeostasis.

Specific aims:

Paper 1: To map expression of variant p63 transcripts in healthy skin, normal and psoriatic skin from patients with psoriasis, in order to study the involvement of p63 in this chronic inflammatory skin disease with KC hyper-proliferation.

Paper 2: To investigate whether dysregulated p63 found in psoriasis responds to NB-UVB phototherapy, in order to further elucidate the role of p63 in psoriasis and also to increase our understanding of the mechanisms of phototherapy.

Paper 3: To investigate whether TRAF4 (a gene transcriptionally regulated by p53) is regulated by p63 in SCCHN and to evaluate TRAF4 expression patterns in SCCHN, in order to study potential contributions of the putative p63-TRAF4 pathway in SCCHN.

Paper 4: To identify potential p63 regulated genes in an SCCHN cell line in order to achieve further insight into how p63 over-expression could participate in the pathogenesis of SCCHN.

Materials and Methods

Paper I and Paper II

Skin samples

For the first study, punch biopsies were taken from plaque type psoriatic lesions, clinical normal skin of the patients, and healthy skin from age- and sex-matched controls. Biopsies were snap-frozen in liquid nitrogen or fixed in neutral buffered formalin for downstream applications. Patients diagnosed with plaque type psoriasis who would receive NB-UVB phototherapy were included in the second study. Punch biopsies were taken from psoriatic lesions prior to UV treatment, during and before the last session of treatment. Biopsies from healthy age- and sex- matched controls were also collected. Samples were embedded in Tissue Tek OCT compound (Miles Inc., Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. Clinical data on patients are summarized in Table 1.

Quantitative RT-PCR

In the first study, total RNA from whole skin biopsies was extracted using an RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) and stored at -80°C until use. cDNA was synthesized using a "1st strand synthesis kit for RT-PCR (AMV)" (Roche Diagnostics, Mannheim, Germany). Quantitative RT-PCR for amplifying different p63 splice variants was performed with a human p63/β-actin multi-parametric kit (Search LC, Heidelberg, Germany) and analyzed on a Lightcycler from Roche. In the second study, laser microdissected epidermis and basal layer epidermis were collected for total RNA extraction containing small RNAs. Total RNA was extracted using Trizol and Qiagen RNeasy micro kit (Qiagen, GmbH, Hilden, Germany). cDNA was synthesized using First-Strand cDNA Synthesis Kit with 10× Primer Mix (USB, Cleveland, USA). For miRNA analysis, First-strand cDNA synthesis kit and miR-specific RT primers from the miRCURY LNA™ microRNA PCR system (Exiqon, Vedbaek, Denmark) was used. Real time RT-PCR was performed using an IQ5 multicolor real-time PCR detection system with IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Mercury LNA™ microRNA assays (Exiqon) were used to quantify miR-203, miR-125b and miR-21 respectively. Sequences of self-designed primers are shown in Table 2.

Table 1. Patient data in study I and II

	Patient ID	Age	Gender	Years of disease	Comments
Study I	1	24	F	> 20 y	
	2	53	M	> 10 y	
	3	43	M	> 30 y	
	4	55	F	> 40 y	Psoriasis arthritis
	5	61	F	> 40 y	
	6	23	F	> 10 y	
	7	48	F	> 20 y	
	8	46	M	> 30 y	
	9	68	M	> 40 y	
	10	25	M	> 20 y	
	11	69	F	> 30 y	
	12	29	M	> 2 y	
	13	73	F	> 30 y	
	14	50	F	> 30 y	
	15	25	M	> 10 y	
Study II	1	40	M	> 20 y	
	2	73	F	Unknown	
	3	59	M	> 20 y	
	4	67	M	< 1 y	
	5	26	M	1 y	Stopped treatment half way
	6	17	M	> 10 y	Treatment suspended for 3 weeks
	7	47	F	> 10 y	
	8	43	M	> 10 y	
	9	46	F	> 10 y	
	10	74	F	2 y	
	11	33	F	> 10 y	
	12	28	F	> 10y	

Table 2. Primer sequences used in the different studies

Name	Forward	Reverse	Use	Study
p53	CAGTCAGATCCTAGCGTCGAG	GGGACAGCATCAAATCATCC	qRT-PCR	II
TAp63	GTCCCAGAGCACACAGACAA	TGCGGATACAGTCCATGCTA	qRT-PCR	II
ΔNp63	CTGGAAAACAATGCCCAGAC	AGAGAGCATCGAAGGTGGAG	qRT-PCR	II
FABP5	AAGAAACCACAGCTGATGGC	CATGACACACTCCACCACTA	qRT-PCR	II
TUBA6	CCGGGCAGTGTTTGTAGACT	TTGCCTGTGATGAGTTGCTC	qRT-PCR	II
RPL13A	GTACGCTGTGAAGGCATCAA	GTTGGTGTTCATCCGCTTG	qRT-PCR	II
DNp63a	CCAGACTCAATTTAGTGAGC	ACTTGCCAGATCATCCATGG	RT-PCR	III, IV
TRAF4	GCCTGGCTTCGACTACAAGT	GGATAGGCAGGCCCAATACT	RT-PCR	III, IV
β-actin	ACCATGGATGATGATATCGC	TTGCTGATCCACATCTGCTG	RT-PCR	III, IV
TRAF4a	AGCCTGGATGACAGAGCAAG	AAGCTAGGCAGGCCTAATGG	PCR	III
IGFBP3	GTGAGTGGGACTTTGGCATT	TCCAGCTCAGATGGGAAAAC	PCR	III
TRAF4b	GGTACCTGGAGGCTAAGGCAAGAGAA	CTCGAGGGATGAAAGTGTAGGGGAGGT	PCR	III
IGFBP3b	GGTACCGTGAGTGGGACTTTGGCATT	CTCGAGTCCAGCTCAGATGGGAAAAC	PCR	III
ANXA1	GGTCTACAGAGAGGAACTGAAGA	GTCACCCTTAGCAAGAGAAAGC	RT-PCR	IV
AQP3	CATCTACACCCTGGCACAGA	TCCAGAGGGGTAGGTAGCAA	RT-PCR	IV
FABP5	AAGAAACCACAGCTGATGGC	CATGACACACTCCACCACTA	RT-PCR	IV
IGFBP3	ACAGCCAGCGCTACAAAGTT	CTGGGACTCAGCACATTGAG	RT-PCR	IV
LYN	TCCCTGTATCAGCGACATGA	AGTTCCTGGCTTCAGGGTTT	RT-PCR	IV
MMP1	TGGATCCAGGTTATCCCAAA	TCCTGCAGTTGAACCAGCTA	RT-PCR	IV
MMP10	CATGCCTACCCACCTGGAC	GAGCAGCAACGAGGAATAAATTG	RT-PCR	IV
MMP14	GAAGCCTGGCTACAGCAATATG	CCGTAAAACTTCTGCATGGCA	RT-PCR	IV
PVRL1	GGCTTGACCGCATTCTTCCT	TGCAGTGCAGAACCACGTC	RT-PCR	IV
RAB38	TGCACCAGAACTTCTCTTCG	GCACCCATAGCTTCTCGGTA	RT-PCR	IV
SERPINE1	TGGAGAGAGCCAGATTCATCA	AGTAGAGGGCATTCACCAGCA	RT-PCR	IV
SERPINI1	CTGCTGCTGTCTCAGGAATG	TCAGGATGCATGACTCGTCC	RT-PCR	IV
UBE2E3	AAGGTTACTTTCCGCACCAGA	AATAGTCAAAGCGGGACTCCA	RT-PCR	IV
ANXA1	GGTATTAGGATTGGGGCAGA	AAAGGAAGCCACACCTAGCA	PCR	IV
AQP3	GGGTAAGTCAGATGGGAGAGG	GTGTCTACACATGGCGGATG	PCR	IV
IGFBP3	GTGAGTGGGACTTTGGCATT	TCCAGCTCAGATGGGAAAAC	PCR	IV
LGALS1	AAAGGACAGGGTGCACAGAG	CTCCTCGGGAAGGCTAAAGA	PCR	IV
MMP14	TCTCCCTCTGCAGGTCTCAT	GGATGTGGGAGACTTTGTCC	PCR	IV
PAI-1	CAGAGGGCAGAAAGGTCAAG	CTCTGGGAGTCCGTCTGAAC	PCR	IV
PVRL1	CATGGACGCCTGCAAGTT	CACGAGTCATGCCCCTTC	PCR	IV
SERPINI1	TACCAGCAACTGAGGCACTG	ACGAGTCCCCATAAGCCTCT	PCR	IV

Immunohistochemical staining

In the first study, formalin-fixed biopsies were paraffin embedded and analyzed using immunohistochemistry. Antibodies against TAp63, ΔNp63, p63α, p53 and phosphorylated STAT3 were used. In the second study, immunohistochemistry was performed on fresh frozen samples. An antibody recognizing all p63 isoforms and an antibody against p53 were used in the staining procedure. Staining was performed using a Ventana staining machine (Ventana Medical Systems Inc., Tucon, AZ). Antibodies used in all studies are summarized in Table 3.

Table 3. Antibodies used in the different studies

Antibody	Company	Dilution	Study
TAp63 (Long-2)	Own production	1/750	I
ΔNp63 (Δ2)	Own production	1/2000	I
p63α (Alfa)	Own production	1/450	I
p53 (DO-7)	Novocastra	1/25	I
Phospho-STAT3	Cell Signaling Technology Inc	1/20	I
p63 (4A4)	Abcam	1/50 (IHC); 1/2000 (WB)	II, III, IV
p53 (DO-7)	Dako	1/50	II
HA tag	Abcam	1/1000	III
TRAF4	Hypromatrix	1/1000 (WB); 1:50 (IHC)	III
p53 (DO-1)	Kindly provided by Dr. B Vojtesek	1/1000	III
β-actin	Chemicon Int.	1/10000;1/20000	III,IV
ANXA1	Abcam	1/5000	IV
PAI-1	Abcam	1/200	IV
FABP5	BioVendor	1/20,000	IV
Lyn	BD Transduction Laboratories	1/250	IV
PVRL1	Invitrogen	1/500	IV
anti-mouse	Pierce	1/50000	III,IV
anti-rabbit	Pierce	1/50000	III,IV

Statistical analysis

All analyses were carried out using SPSS statistics. For pair-wise analysis of data from the same patient, paired-sample T-test was conducted. Independent-sample T-test was used to compare gene expression in psoriasis vs. healthy controls. Pearson coefficient correlation was used for correlation analysis. In all

statistical tests, two-tailed *P* values < 0,05 were considered statistically significant.

Paper III and Paper IV

Cell culture and siRNA transfection

The human cell line FaDu originating from an SCCHN of the hypopharynx (ATCC) and the human osteosarcoma cell line Saos-2 (ATCC) were cultured in DMEM containing 10% FCS (Invitrogen, Grand Island, NY, USA). The human osteosarcoma cell line U2OS (ATCC) was cultured in McCoy's 5A medium containing 10% FCS (Invitrogen). p63 siRNA duplex targeting all p63 isoforms (Dharmacon, Lafayette, CO, USA) was used to knockdown p63 expression. A control siRNA targeting luciferase served as negative control. FaDu cells were transfected with the oligonucleotide duplexes (100 nM) premixed with Oligofectamine (Invitrogen).

Microarray sample preparation and data analysis

FaDu cells where endogenous p63 had been quenched by siRNA were used for microarray analysis. Total RNA extracted using Trizol was used for preparation of double-stranded cDNAs and biotinylated cRNA was hybridized to HG-U133A chips (Affymetrix, Santa Clara, CA, USA). Arrays were quality assessed by array images, RNA degradation profiles, histograms and box plots pre- and post-normalization in BioConductor, and calculation of percentage outliers in dCHIP. Expression values presented were calculated with dCHIP software using the default setting (PM-MM model). By comparing the two controls with three independent p63 siRNA-treated samples, genes were identified that had a fold change of at least 1.3-fold at 90% confidence, a mean expression level of at least 30 in either treated or control arrays and a difference of at least 10.

PCR analysis and western blotting

The same cDNA used in microarray analysis was used for microarry data confirmation. Semi-quantitative RT-PCR analysis was performed for p63 using AmpliTag Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA) in a GeneAmp 9600 thermal cycler (Perkin-Elmer). Quantitative RT-PCR was performed for 14 selected genes using the LightCycler (Roche Diagnostics Corp) and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Cort). Total protein extracts from siRNA transfected FaDu cells or from

luciferase assay lysate of U2OS cells were separated on 10% SDS–polyacrylamide gels. Antibodies used are listed in Table 3.

Formaldehyde crosslinking and chromatin immunoprecipitation (ChIP)

A 1 kb region upstream of exon 1 of the validated potential p63 target genes was examined for presence of putative p53-type response elements. Known p53 consensus sequences located in the TRAF4, AQP3, IGFBP3 and PAI-1 genes were used with IGFBP3 as the positive control for the assays. ChIP experiments were performed using the EZ ChIP™ chromatin immunoprecipitation kit (Upstate, Lake Placid, NY, USA). Polyclonal antibodies specifically recognizing ΔNp63, TAp63 or p53 were used for immunoprecipitation. A pre-immune serum was used as negative control. Eluted DNA was PCR amplified with primers specific for the regulatory regions of the candidate genes. Primer sequences for ChIP are listed in Table 2.

Plasmids and luciferase assay

The promoter region containing putative p63-REs was cloned into pGL3-Basic reporter plasmid (Promega). For luciferase assays, U2OS or Saos2 cells were seeded into 12-well plates 24 h prior to transfection. Cells were cotransfected with reporter plasmid containing promoter response elements, pRL-TK encoding Renilla luciferase cDNA and pcDNA3 vector either empty as control or containing p53, p63 or p73 isoforms using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). At 48 h after transfection, luciferase activity was measured and the value obtained by control transfection was arbitrarily set at 1.

SCCHN tissue and immunohistochemical staining

Tissue microarrays comprising representative areas from 20 tumors as well as whole sections from another 4 tumors were used for studying TRAF4 expression in SCCHN. Normal buccal mucosa from healthy volunteers was used as controls. Immunostaining was performed using a Ventana staining machine and reagents according to the supplier's recommendation.

Results and Discussion

Paper I

We first examined expression of different p63 mRNA transcripts in skin under three conditions: healthy skin from controls, uninvolved skin of psoriasis patients separated from the psoriatic lesion by several centimeters, and psoriatic lesions. Due to the lack of isoform specific primer sets, we used five primer sets specifically amplifying the two N-terminal variants (ΔNp63 and TAp63) and the three C-terminal variants (p63α, p63β, and p63γ) in quantitative RT-PCR analysis. In accordance with previous results, the main isoforms expressed in adult epidermis were p63α and ΔNp63. TAp63, p63β and p63γ were also detectable, but at lower levels. No significant differences in p63α and p63β expression were found. Similar ΔNp63 mRNA levels were seen in healthy and uninvolved skin from psoriasis patients, however, a slight reduction was found in psoriatic lesions compared to patients' own clinically normal skin. Intriguingly, TAp63 and p63γ mRNA levels were significantly lower in lesions compared to patients' own normal skin and matched healthy controls. Notably, when comparing patients' own uninvolved skin to age and sex matched healthy controls, TAp63 was the only isoform showing a trend of decreased expression. Together with the finding that TAp63 was most down-regulated in psoriatic lesions with highest statistical significance ($p < 0{,}001$), a role for TAp63 in psoriasis was indicated.

Expression of different p63 isoforms in adult epidermis was studied by immunohistochemistry using polyclonal antibodies specifically recognizing TAp63, ΔNp63, and p63α. Again, in accordance with previous results, nuclear ΔNp63 proteins were seen in basal and suprabasal cell layers of healthy epidermis. TAp63 and p63α proteins were seen throughout the whole epithelium with weaker immunoreactivity than that of ΔNp63. Immunostaining of psoriatic lesions and clinically normal epidermis from the same patient showed similar p63 signal intensity and the same protein expression pattern. It is not surprising that mRNA levels and protein expression were not concordant. A discrepancy between p63 protein expression and mRNA levels has previously been reported, showing accumulation of TAp63 after treatment with DNA-damaging agents without accompanying increasing levels of mRNA (Katoh *et al*., 2000; Okada *et al*., 2002). Post-translational modifications of p63, such as

phosphorylation, ubiquitination or sumoylation have been reported and account for protein stabilization, activation or degradation. Even if the exact mechanism is far from understood, deregulation of p63 is likely to disturb epidermis homeostasis and participate in progression of psoriasis.

Paper II

NB-UVB phototherapy for psoriasis is an effective treatment for most patients. Therefore, in this study, we wanted to know whether phototherapy affects p63 expression. We examined p63 levels in healthy epidermis and also psoriatic epidermis following phototherapy (prior to phototherapy, during phototherapy and before the last session of phototherapy). Similar to our previous finding, epidermal TAp63 and ΔNp63 mRNA levels were significantly down-regulated in psoriatic epidermis prior to phototherapy compared to healthy controls. Overall, there was no significant difference in TAp63 expression following NB-UVB phototherapy though an obvious increase in TAp63 was seen in some patients. For ΔNp63 transcripts, no significant increase following phototherapy was seen either. Immunohistochemical staining using an antibody recognizing all p63 isoforms showed p63 expression to be similar under all conditions.

Epithelial KCs exert the front-line defense of the body from the outside environment. Intrinsic defects in epithelial KCs may be important in initiating, sustaining, and amplifying the inflammatory responses by expressing molecules involved in T-cell recruitment, retention and activation (Albanesi *et al.*, 2007; Lundqvist and Egelrud, 1997). As p63 was dysregulated in psoriasis but not fully rescued by NB-UVB phototherapy, it is likely that abnormal p63 contributes to psoriasis progression and recurrence.

Following NB-UVB phototherapy, significantly increased miR-125b and significantly decreased miR-21 expression were found in psoriatic epidermis. When looking at the most basal layers only, no effect on miR-125b was seen, whereas expression of miR-21 was significantly down-regulated following phototherapy and treated psoriatic epidermis showed similar miR-21 level as healthy controls. These results indicate that NB-UVB phototherapy effectively targets miR-21 and miR-125b in psoriasis. Down-regulation of miR-21, especially in the basal layers, might help to reduce cell proliferation. Levels of epidermal miR-21 expression were plotted against p63 from all psoriasis samples (n=47) and a negative linear relationship was seen. Since miR-21 has been shown to be a negative regulator of TAp63 (Papagiannakopoulos *et al.*, 2008), further studies are needed to investigate whether p63 is under regulation

of miR-21 in normal epidermis and what role this potential pathway has in development of psoriasis.

Paper III

In the third study, we wanted to find out whether TRAF4, a p53 regulated gene which is over-expressed in many human carcinomas, was also under the regulation of p63. FaDu cells originating from a human SCCHN of the hypopharynx were transfected with siRNA targeting all p63 isoforms. Down-regulation of p63 mRNA and protein was seen in siRNA transfected cells and the expression of TRAF4 mRNA and protein was also reduced moderately. A clear binding of ΔNp63 proteins to the TRAF4 promoter was seen *in vivo* and a putative p53 response element in the human TRAF4 promoter was found. Furthermore, results from reporter assays showed that TRAF4 was transcriptionally activated by p53 family members. TAp63β and TAp63γ were the most potent transactivators causing 60 to 70-fold activation when ectopically expressed. The ΔNp63 isoforms had very low transcriptional effect on the TRAF4 promoter in wild-type p53 expressing U2OS cells, whereas no effect was seen in p53 negative Saos−2 cells, indicating a p53−dependency for ΔNp63 regulated TRAF4 transactivation. TRAF4 is the most distinct member of the TRAF family. The functions of TRAF4 and its role in cancer are unclear, though over-expression of TRAF4 has been seen in human carcinomas. Since TRAF4 was also transactivated by p53 and TAp73, and as p53 family members can interact physically and functionally, the *in vivo* regulation of TRAF4 expression could be highly complex and tissue specific.

Our further studies showed that TRAF4 localization was associated with SCCHN differentiation. In normal buccal mucosa, well and moderately differentiated tumors, TRAF4 staining was confined to the nucleus. In contrast, nuclear as well as cytoplasmic staining was seen in all cases of poorly differentiated tumors. This differential localization is most apparent when examining different areas of the same tumor, where areas with well/moderate differentiation showed nuclear staining only, compared to the additional cytoplasmic staining seen in areas with poor differentiation. Similarly, a previous study found some tumors to contain only nuclear TRAF4 whereas most contained cytoplasmic staining, but no link was made with differential localization and tumor differentiation (Camilleri-Broet *et al.*, 2007). These data imply that localization of TRAF4 is regulated differently in different tumors and that localization may influence the differentiation processes of malignant squamous epithelial cells.

Paper IV

In this study, we investigated the role(s) of p63 in SCCHN by using microarray technology to simultaneously identify p63 regulated genes in FaDu cells. Results showed that down-regulation of p63 resulted in increased expression of 64 genes and decreased expression of 63 genes. These genes code for proteins involved in diverse cellular functions. Interestingly, more than 20% of the identified genes are related to migration and adhesion. Quantitative RT-PCR for 14 selected genes and Western blotting for 5 genes confirmed that silencing of p63 alters the expression of each of these genes in FaDu cells. *In vivo* binding of ΔNp63 to putative gene promoters were demonstrated in 4 genes: AQP3, IGFBP3, PAI-1 and SERPINI1. Further experiments showed that the PAI-1 promoter could be activated by all p63 isoforms, preferably TAp63γ, whereas for the AQP3 promoter, only TAp63β and TAP63γ isoforms promoted transcriptional activity.

High local recurrence rate after therapy and distant metastasis are major causes of death in patients with SCCHN, calling for molecular biomarkers that could predict recurrence and metastasis. It is obvious that even though various studies have demonstrated abnormalities in p63 expression in SCCHN, the role(s) of p63 in SCCHN are still unclear and the prognostic value of p63 in SCCHN is controversial. We have previously shown that inhibiting p63 expression in FaDu cells did not affect cell proliferation or expression of epithelial differentiation markers, but resulted in significant decreased survival of these cells (Thurfjell *et al.*, 2005). Now in this study, we found that a major set of genes identified were involved in cellular migration or adhesion, similar to reports from other groups (Barbieri *et al.*, 2006; Carroll *et al.*, 2006; Yang *et al.*, 2006). Therefore, decreased survival in cell culture might be the consequence of decreased adhesiveness when endogenous p63 was knocked down.

It is well known that altered expression of integrins and other cell adhesion molecules, matrix metalloproteases (MMPs), other proteases and protease inhibitors are closely linked to tumor growth and are major determinants of metastasis (Hanahan and Weinberg, 2000). Therefore, by a delicate regulation of multiple adhesion and motility related molecules, over-expression of p63 in some SCCHN cases might favor cancer cell survival and could confer them with invasiveness of local host tissue stroma, entry into lymphatics and blood vessels and even survival within newly colonized microenvironment.

General discussion

Psoriasis is a complex disease with abnormalities in both KC biology and immune system, histologically manifested as KC hyper-proliferation and severe inflammation. Involvements of numerous growth-, differentiation-, and immune response-related factors have been suggested for development of psoriasis. However, it is far from understood which factors that are the initial cause of psoriasis, leading to disturbed homeostasis of epidermis. By transcriptional regulation of numerous down-stream targets, p63 is involved in several aspects important for epidermal homeostasis. Epithelial KCs exert the front-line defense of the body from the outside environment and their intrinsic defects might trigger an exaggerated inflammatory response. We found that TAp63 mRNA levels in normal skin of psoriasis patients was not normal, in accordance with the finding that distant uninvolved skin has a pre-psoriatic phenotype (Korver *et al.*, 2006). NB-UVB phototherapy significantly affected expression of some psoriasis-related molecules, such as p53, miR-21 and miR-125b, whereas p63 mRNA levels in treated psoriatic epidermis were still not normal. Therefore, we concluded that p63 might play an important role in psoriasis. Based on our finding that accumulation of p63 proteins was similar under different skin conditions studied, it is necessary to further investigate whether p63 in psoriasis exhibits altered post-translational modifications and if so, what effect that has on different p63 interacting proteins.

Identification of genes directly regulated by p63 under physiological context will provide valuable information to the biological functions of p63 and the mechanism by which it promotes disease development. As p63 over-expression is a common feature of SCCHN, we evaluated the effect of p63 over-expression on SCCHN cells, by looking for p63 regulated genes in SCCHN cells. Microarray analysis identified 127 genes whose expression relies on over-expression of p63. More than 20% of these genes were involved in cell motility. Thus, p63 modulated multiple aspects of cell adhesion and migration in SCCHN. Though the role(s) of p63 in SCCHN are still unclear and the prognostic value of p63 in SCCHN is controversial, our data provide a molecular framework for understanding the role of p63 in human cancer and for explaining the associations of high level ΔNp63α expression in SCCHN tumors with aggressive behavior and poor prognosis *in vivo*.

TRAF4, the most distinct member of the TRAF family, is a mysterious protein with controversial reports on biological function and cellular location.

Intriguingly, we found that TRAF4 was a common transcriptional target of the p53 family, and that TAp63 isoforms were highly potent in TRAF4 transactivation as demonstrated by reporter assay. However, interactions among p53 family members, diverse protein stability and existence of various transcriptional cofactors under physiological conditions, makes it difficult to interpret which p63 isoforms are actually responsible for TRAF4 transactivation. Our additional finding that localization of TRAF4 is associated with differentiation of SCCHN cells, makes TRAF4 an interesting molecule in SCCHN. Further studies should aim at understanding its behavior during cell differentiation. We also examined TRAF4 expression in 12 psoriatic epidermises compared to healthy controls, without finding any difference. These results indicate that different p63 status in SCCHN and psoriasis, together with cell-type specific factors, results in completely different outcomes through different signaling pathways.

In summary, our results provide additional mechanisms of how p63 contributes to epithelial homeostasis. The common KC hyper-proliferation seen in benign and malignant conditions like psoriasis and SCCHN respectively, is not likely a direct effect of aberrant p63 expression. Abnormalities in p63 regulation might, however, be one of the key factors contributing to the exaggerated immune response seen in psoriasis. In SCCHN, over-expressed p63 isoforms might increase cell survival potential and enhance the invasive and metastasising capacity of SCCHN cells. Invasive growth is the hallmark of cancer and metastasis is the principal cause of death. Therefore, in-depth understanding of p63-dependent cell invasion and metastasis is important in cancer research.

Immune surveillance is also an important issue in cancer studies. Cancer cells can tolerate or resist immune surveillance, and produce a range of growth factors and proteases and alter their adjacent stroma to form a supportive environment for cancer progression (Mueller and Fusenig, 2004; Pardoll, 2003). However in psoriasis, the misguided immune response is exaggerated and leads to cell hyper-proliferation. It is well accepted that chronic inflammation could lead to carcinogenesis, however in psoriasis, conversion of a psoriatic plaque to squamous cell carcinoma is exceedingly rare (Nickoloff, 2001). An excess incidence of cancers, mainly esophageal, skin, upper aerodigestive tract, lung, stomach and liver, and also non-Hodgkin lymphoma has been reported in hospitalized patients with psoriasis, which may be attributable to anti-psoriatic treatment and/or the increased use of alcohol and tobacco in these patients (Ji *et al.*, 2009). Therefore, understanding the interplay of immune status and

epithelial cell biology might be fundamental for understanding the pathogenesis of cancer and psoriasis. The NFκB signaling pathway is involved in immune homeostasis and has been implicated in the pathogenesis of many inflammatory related diseases (Pasparakis, 2009). Interestingly, it was recently found that TAp63 was a transcriptional target of NFκB (Wu *et al*., 2010). IKKβ, an activator of NFκB, could phosphorylate and stabilize TAp63γ protein (MacPartlin *et al*., 2008) but promotes ubiquitin-mediated proteasomal degradation of ΔNp63α (Chatterjee *et al*., 2010). Future studies focusing on p63 and immune response are needed in order to increase our knowledge of epithelial homeostasis.

Conclusions

I. Dysregulation of p63 isoforms was seen in psoriasis lesions, a benign skin condition with disturbed epidermal homeostasis. Reduced TAp63 mRNA levels were already seen in patients' normal skin compared to age and sex-matched healthy controls, indicating existence of early molecular changes.

II. Down-regulation of p63 isoforms seen in psoriatic epidermis were not rescued by NB-UVB phototherapy. Thus, clinically normal epidermis after treatment still harbors molecular abnormalities in p63. Therefore, p63 might be one of the key factors contributing to psoriasis.

III. TRAF4 was a common transcriptional target of p53 family members. Localization of the TRAF4 protein was associated with histologic differentiation of SCCHN. p63 cooperating with TRAF4 might be involved in KC differentiation pathways and might be a tumor-specific phenomenon.

IV. Over-expression of p63 in SCCHN influences cell adhesion and motility at multiple points. p63 might contribute to development and/or maintenance of distinct pathological conditions by participating in diverse cellular pathways at different progress stages.

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