p63 – The Master Key to Epithelial Biology

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ABSTRACT

p63 gene is a key regulator of cell differentiation as well as cell proliferation in epithelial cells. It is involved in multiple functions in stratified squamous epithelium. It is seen to be overexpressed in more than 80% of the epithelial tumours. p63 is involved in regulating a number of critical processes including development, stem cell properties, tumourigenesis, apoptosis and senescence. Functions of p63 are not only varied, but also show antagonistic roles with respect to the isoforms. Its 2 isoforms, TAp63 and ΔNp63 have complex multi-dimensional functions. TAp63 acts purely as a tumor suppressor gene similar to p53, while ΔNp63 has major oncogenic functions. Newer studies are elucidating the role of p63 in a more clear light. Deciphering the multiple facets of p63 in its entirety could be the key to developing tailor-made molecular therapies in epithelial malignancies. This review article is an effort to present a holistic view of p63, its isoforms and its varied interactions with other genes.

Keywords: Apoptosis, Cancer, Epithelial-Mesenchymal Transition, Metastasis, Oncogene, p53 Family, p63 Gene, Stem Cells, Tumor Suppressor

INTRODUCTION

p63 gene is a member of the p53 family comprising of p53,p63 and p73 genes. Although phylogenetically, it is the most ancient and conserved member of the family, it is also the most recently discovered entity.[1] It appeared very early in evolution in the metazoan sea-anemone more than a billion years ago.[2] p63 was first identified in C.elegans as a p53 homolog with a Sterile Alpha Motif (SAM). Originally, it was isolated from rat tissue as KET in 1997.[3] Initially, it was referred with varied names by different investigators including p51, KET, p40, p73L, p53CP, and NBP.

Role of p63 is intriguing and confusing because of its different isoforms, atleast 6 isoforms have been identified. The p63 gene locus accounts for 2 distinct isoforms, namely the transactivation domains(TA) and the Delta N(ΔN) isoforms. 47) ΔNp63 isoforms consist of the truncated version and lack the NH2 – transactivation domain(TA-1)and were initially thought to function as " dominant negative" proteins, that is, blocking the function of the full-length protein. Later on, it was discovered that ΔNp63 isotypes can also act as bona fide transactivating factors on their own. The presence of the second transactivating domain (TA-2) is important in this regard.

In addition to the NH2 differences, alternate splicing at the COOH end leads to the formation of 3 additional variants, α, β and ϒ forms. Only ΔNp63 and TAp63 possess a SAM domain, typical of developmental proteins and responsible for the protein-protein interactions. p63 protein is targeted for ubiquitin-mediated degradation by the E3 ligase ITCH, which belongs to the HECT family and SUMO-1.[1,3]

TAp63 and ΔNp63 have distinct and overlapping functions in normal and cancer tissue. ΔNp63 isoforms are considered to promote cell survival while TAp63 isoforms induce cell death.[4] But, this is a very simplistic view of a multi dimensional p63 gene, since ΔNp63 has been shown to inhibit metastasis, with an anti-tumor function.

TAp63 contains a transcription domain and can induce cell cycle arrest as well as apoptosis bestowing upon it the role of a tumor suppressor, similar to p53. ΔNp63 on the other hand, has a primary role in epithelial development.[3] p63 null mice exhibit severe defects in all ectoderm-derived tissues and early post-natal lethality. There is a complete lack of all stratified epithelium, and ectodermal appendages, including hair, mammary gland and teeth, in addition to craniofacial defects and limb truncation.[4]

p63 expression has been studied by various methods including immunohistochemistry, tissue microarrays, studies on knock-out mice, numerous studies on cancer cell lines, Gene expression microarrays, Western blot assays, In situ hybridization, Small interfering RNA(siRNA) transfection, Cell proliferation assay, Wound healing assay, Matrigel invasion Assay, isoform-specific reverse transcriptase-PCR, Real time-PCR, Bioinformatics, ChIP(Chromatin Immunoprecipitation) Assay, Luciferase assay and DNA flow cytometry among others. The analysis of p63 protein expression is highly influenced by the sensitivity and specificity of antibodies and the technique used and the

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precise expression pattern of various isoforms.[5]

Immunohistochemical studies have demonstrated that p63 expression is restricted to the nucleus, with a nucleoplasmic pattern and the expression is restricted to the epithelial cells of stratified epithelia like skin, tonsil, cervix, oesophagus, bladder, specific subpopulation of basal cells in prostate and mammary gland and also, bronchi. Thymomas showed high levels of p63, while Hodgkins lymphomas specifically expressed TA isoform of p63. Soft tissue sarcomas, melanomas, endocrine neoplasms and germ cell tumors are negative for p63.[6] Thus, p63 can be used to diagnostically differentiate spindle cell carcinomas and undifferentiated carcinomas from spindle cell mesenchymal tumours and melanomas.

p63 regulates an impressive array of genes, the target genes include more than 200 transcription factors, a large number of adhesion molecules and a functionally diverse set of signaling molecules, apart from several microRNAs. Thus, p63 may directly alter nearly 7% of the coding genes in the genome.[3]

The gene locus for p63 is at chromosome 3q28 p63 is crucial in the development of all epithelial tissues. It shows a high expression pattern in the ectodermal surfaces of the limb buds, branchial arches and epidermal appendages. It is involved in the genetic patterning and direct morphogenetic patterning of the underlying mesoderm. p63 also plays a pivotal role in the regulation of a wide range of targets controlling cell cycle, stress response, development and signal transduction.[1] p63 has complex roles in various metabolic and developmental cell processes which is discussed.

Role in Development and Differentiation

p63 has a controlling influence on the Notch pathway. Notch signaling is critical for cell fate determination and influences craniofacial development and limb organogenesis. Jagged 1(JAG-1) encodes a ligand for Notch Receptors and in turn, is directly regulated by p63. Pigment epithelium derived factor (PEDF) and Vitamin D Receptor (VDR) and other genes are also targeted by p63. p63 gene family regulates many more targets like TGF-β regulators, HOXC4, FGF and Sonic Hedgehog (Shh) pathways[1]

p63 and Stemness

p63 is a marker of keratinocyte stem cells There are several pathways connected to the stem cell phenotype which involves p63. These pathways include Notch signaling, mTOR signaling, Wnt pathway, SHH pathway and IHH pathway. ΔNp63 plays a crucial role in stem cell maintenance in the epidermis by controlling the number of asymmetric doubling in the compartment. Organization of the basal layer requires a tight regulation of the asymmetric divisions. ΔNp63 controls the proliferative potential and the clock of the number of divisions.[3]

Role in cell Adhesion

Ablation of all p63 isoforms in mammary epithelial MCF-10A cells causes a major down regulation of cell to matrix adhesive molecules, particularly of integrins, β1, β4 and α6, and of the protein Laminin ϒ2, resulting in death by anoikis. This effect can be prevented by expression of a shRNA insensitive ΔNp63α, but not by TAp63ϒ.

The transmembrane Perp facilitates cell to cell adhesion by maintaining desmosome structure and function. Perp is also a direct p63 target in skin development. It is observed that Perp expression is absent in p63 null mice embryos and p63 ablation in mouse keratinocytes downregulates Perp expression.[4]

ΔNp63, the most frequently expressed isoform has been demonstrated as a constant marker of keratinocyte stem cells in the epidermis. p63 is found to be critical for cell adhesion by maintaining Integrin 4β.Therefore, the p63 family, especially ΔNp63α, are considered necessary for epidermal mesenchymal interactions controlling the fate of the stem cells.[1] ΔNp63α has been postulated to play a role in epithelial cell adhesion and survival of basal cells. p63 depletion in organotypic cultures leads to decrease in levels of β1 and β4 integrins. p63 knockout keratinocytes show increased motility and mesenchymal phenotype.[7]

Genetic studies have shown that p63 is crucial to maintain the regenerative proliferative structures within the epithelium. Disruption of p63 function indicates an early step in differentiation.

Role in Cancer

p63 has been detected in multiple epithelial cancers including oral cancer, cancers of the prostate, bladder, lung, breast and also oral precancer. Unlike p53, mutations of p63 are extremely rare. Rather, p63 is seen to be over-expressed in a number of human cancers or shows amplification. More than 80% of primary head and neck squamous cell carcinomas retain p63 expression, with overexpression and, amplification. This gene amplification is important for maintaining the immortality of the cancer cells.[1,3]

p63 is a key regulator of epithelial tissues and acts as a modulator of selective growth advantage in epithelial cancer cells. Epithelial Growth Factor Receptor (EGFR) depletion leads to marked downregulation of ΔNp63α expression, while TAp63ϒ represses EGFR, a growth modulator.[4]

p53/p63/p73 Axis

During tumorigenesis, ΔNp63α blocks the p53-dependent transactivation, thus blocking the apoptotic pathway. ΔNp63α also promotes survival of HNSCC cells by suppressing a p73 dependent proapoptotic transcriptional pathway.[8,9]

TAp63 as a Tumor Suppressor

In contrast to the role of ΔNp63α in promoting cell survival, TPp63α promotes cell cycle arrest, senescence and apoptosis. TAp63 is required for protecting the female germline from genotoxic stress during meiotic arrest. Therefore, TAp63 is considered 'Guardian of the Germline'.[4]

Role in Apoptosis

TAp63 and ΔNp63 have antagonistic role regarding apoptosis. TAp63 can upregulate genes involved in apoptosis. Following DNA damage, TAp63ϒ accumulates and induces apoptosis via Bax, and cell cycle arrest via p21WAF/CEP1. It is observed that combined loss of p63 and p73 results in a failure of cells to undergo apoptosis in response to DNA

damage, even in the presence of a functional p53. ΔNp63 can inhibit the transcriptional activity of TAp63 by competing with the same responsive elements, by sequestration, or by inactivation through formation of hetero- tetramers.[1,3]

RNAi studies using HNSCC cell lines have shown that specific inhibition of p63 triggers apoptotic cell death in the tumour cells with the induction of pro-apoptotic genes Puma and Noxa. The cell death is accompanied by cleavage of the enzyme PARP-1, a specific hallmark of apoptotic cell death, thus confirming the anti –apoptotic function of ΔNp63.[8] ΔNp63α also acts as a transcriptional suppressor of the pro-apoptotic gene IGFBP3 through promoter binding. Expression of IGFBP3 and ΔNp63α are inversely correlated in normal squamous epithelium and in squamous cell carcinoma.[7]

p63 and Epithelial -Mesenchymal Transition(EMT)

EMT is a biological process characterized by biochemical and morphological changes that enable epithelial cells to acquire a mesenchymal cell phenotype during regulated events such as embryonic development and wound healing. However, this process is hijacked by the malignant tumor cells which use it to invade and metastasize to distant sites. Studies have shown that EMT is accompanied with ΔNp63 downregulation at the invasive front of OSCC while reexpression of ΔNp63β by stable cDNAtransfection in OSCC cell lines reverts the EMT phenotypes.[10]

ΔNp63 is involved in the regulation of crucial players of EMT including SNAIL 1/2/3, NOTCH and Jagged which are involved in epithelial asymmetric division, tissue polarity and adhesion.[3] Higashikawa et al demonstrated that Snail downregulated ΔNp63α, thereby promoting EMT. It is postulated that ΔNp63β might inhibit EMT by promoting KLK6-PAR-2 signalling and inhibiting PAR1 expression during malignant transformation.[10]

Olsen et al studied the role of p63 in EMT and its reversal, Mesenchymal to Epithelial Transition (MET). They concluded that p63 is important to maintain the epithelial phenotype in prostate epithelial cells and overexpression of ΔNp63 isoforms can induce a partial mesenchymal to epithelial transition in these cells.[11]

p63 and Metastases

Sun et al demonstrated that TAp63 suppresses metastasis by regulating microRNA processing complex.[5] Studies have shown that TGFβ-dependent cell migration, invasion and metastases are empowered by mutant p53 and opposed by p63. TGFβ acts along with oncogenic Ras and mutant p53 to induce the assembly of a mutant p53/p63 protein complex in which Smads serve as a scaffold for the interaction. Within this ternary complex, p63 functions are antagonized. Inactivation of p63 transforms non-invasive cells into malignant tumors. Thus, TGFβ and p63 play opposite roles in metastatic cells. Thus, loss of p63 function is critical to TGFβ mediated metastatic potential. Targeting of the mutant p53/Smad/p63 complex could represent a potential option for therapeutic intervention. Loss of p63 potently inhibits the expression of Sharp-1 and Cyclin-G2 which act as metastasis suppressors.[12,13] ΔNp63α, at the same time, upregulates anti apoptotic Hsp70, which demonstrates proliferative and anti apoptotic properties and leads to increased metastases.[7]

Role in Epigenetics

p63 also mediates with the epigenetic regulatory machinery. p63 directly regulates expression of several genes encoding the genome organizer and AT-rich-binding protein Satb-1,as well as ATP-dependent chromatin remodelers Lsh and Brg-1 in epidermal keratinocytes. Both Brg-1 and Satb-1 play useful roles in mediating p63 regulated programme of higher order chromatin remodeling within the epidermal differentiation complex (EDC) locus. Lsh regulates chromatin remodeling and mediates ΔNp63 dependent proliferation and survival of Keratin-15 positive stem cells in the skin.[2]

p63 and MicroRNAs

MicroRNAs are a class of small noncoding RNAs that suppress the expression of protein coding genes by repressing protein translation by interacting with messenger RNAs. The complex network between microRNAs and p63 regulates the fate of epidermal cells.[14]

The p63 dependent transcription process is also regulated by certain microRNAs. At the same time, p63 targets a class of distinct miRNAs in basal keratinocytes. TAp63 directly regulates microRNAs through transactivation of *Dicer* promoter, whereas ΔNp63 acts on DGCR8 (DiGeorge syundrome critical region 8) promoter.[2] Experiments using knockout embryonic stem cells have shown that DGCR8, the RNA binding protein that assists Drosha in miR processing, is essential for downregulating the expression of pluripotency markers and promoting Embryonic Stem cell colony differentiation. ΔNp63 regulates DGCR8 through transcription. Recent experiments show that the ablation of ΔNp63 induces epidermal cells to become multipotent. ΔNp63 $^{-/-}$ null epidermal cells that expressed DGCR8 were able to differentiate into skin, liver, muscle and brain cells in vivo.[14] Also, p63 negatively regulates several microRNAs of the miR-34 family. p63 has an inhibitory action on miR-138, miR-181a/b, and miR-130b. On the contrary, miR-203, miR-574-39, and miR-720 regulate p63 expression by restricting its presence to basal cells.[2]

Many studies reinforce the importance of miR-p63 loops in regulating epidermal stratification. miR-203 is rapidly unregulated when primary keratinocytes exit the cell cycle and are induced to differentiate. miR-203 targets p63 and directly represses ΔNp63 expression in the suprabasal layers. miR-203 also has an important role as tumor suppressor, it acts as a metastasis suppressor in epithelial cancers and is able to induce a mesenchymal to epithelial transition, thus inhibiting proliferation, migration and invasion.

An interesting autoregulatory feedback pathway involving iASPP/p63/miRs has been demonstrated which maintains homeostasis in the epithelium. MicroRNAs associated with senescence, miR 130a, miR-181a, miR181b, miR138 target p63 and SIRT-1 and result in their downregulation.[14]

Dynamic interaction between ΔNp63 and miR-138-5p promotes OSCC progression by regulation of cell growth,

metastasis and stemness. OSCC (Oral Squamous Cell Carcinoma) patients with increased ΔNp63 expression but reduced miR-138-5p expression showed a poor prognosis.

p63, Aging and Senescence

Studies on transgenic mice show that overexpression of ΔNp63 along with downregulation of Sirt 1, lead to an accelerated aging phenotype in the skin showing defects in wound healing, decrease in the skin thickness and subcutaneous fat tissue, hair loss and decreased cell proliferation. p63 is thus implicated as a key player in the aging phenomenon.[1] TAp63 induces senescence in cells independent of p53, p19ARF and p16INK4A, but requires p21WAF/CIP1 and Rb. Mechanism of senescence inhibits the progression of cancer, thus emphasizing the tumor suppressor effect of TAp63. However, ΔNp63 has an anti-senescence effect, driving in vivo tumor progression, as a part of its stemness effect. [3]

p63 and Healing

p63 isomers, TAp63 and ΔNp63 show unique and non overlapping functions in wound healing. Usually, ΔNp63 is expressed uniformly in the basal layer of the keratinocytes, in contrast, TAp63 is expressed as a stress response, especially during wound healing. Effects on healing have been elucidated using genetically engineered knockout mice. It is observed that TAp63 is critical to maintain dermal derived stem cells in quiescence through regulation of cyclin dependent kinase inhibitor $p57^{KIP2}$. TAp63 deficient mice show hyperproliferation and premature depletion leading to impairment of wound healing. On the other hand, ΔNp63 functions by transactivating two key genes, Fras-1, required for basement integrity and IKK-α, necessary for terminal differentiation and spinous layer formation.[2]

Response to UV radiation

ΔNp63 is downregulated on exposure to UV radiation, which allows p53, TAp63 and TAp73 to be freed from inhibitory transcriptional complexes with ΔNp63. This further results in activation of downstream genes that protect the cell from further damage.

Therapeutic Considerations

It is recently demonstrated that the abl specific kinase inhibitor Gleevec (Imatinib) reduces Tap63/ ΔNp63 expression in a dose-dependent manner in tumours of the head and neck (HNSCC) and overrides p63 protein induction by DNA damaging agents.[1]

Gressner et al found that endogenous TAp63 is induced by many chemotherapeutic agents like Bleomycin, Doxorubicin and Mitroxantrone in Hepatocellular carcinoma cell lines.[15]

Also, ΔNp63 expression is directly correlated with the clinical response to cisplatin in patients with HNSCC.[16] There is a downregulation of ΔNp63 and upregulation of TAp63 levels after DNA damage. Both p63 (ΔNp63) and p73 are critical mediators of cell death following chemotherapy in HNSCC.[15] In HNSCC, after cisplatin treatment, ΔNp63 is phosphorylated, exported from the nucleus into the cytoplasm and targeted by RACK 1 for proteasome degradation.Thus, in HNSCC, patients with high levels of ΔNp63 are associated with a good response to platinum-based chemotherapy.[5] There is a similar mechanism regulating tumor cisplatin sensitivity in triple negative breast cancer.[17] Cisplatin is most effective in ΔNp63-positive tumors because it targets ΔNp63 and thereby removes a critical survival factor in addition to acting as a genotoxic agent.

Identification of a specific degradation pathway involving E3 ligase Itch is of clinical importance for p63/p73 biology as Itch may be an ideal pharmacological target.[15]

Chemosensitivity is determined by the interactions between the different isoforms of the p53 family members, defining the exact genetic makeup in the given tumor entity is needed for an accurate role in tumor suppression/tumor progression. More novel approaches for targeting and inhibiting p63 function would be to use oligonucleotide based inhibitors of sequence- specific DNA binding or peptidomimetic inhibitors of p63-specific interactions with transactivation machinery[1].

CONCLUSION

In the last two decades, there are a number of studies elucidating the role of p63 gene. p63 has myriad functions including stem cell maintenance, epithelial homeostasis, wound healing, tumorigenesis, apoptosis, EMT and metastasis. This is further complicated by the presence of different isoforms, TAp63 and ΔNp63, with often antagonistic roles

Performing chromatin immunoprecipitation using antibodies for the various isoforms of the p53 family coupled with the next generation sequencing (Chip-Seq) in discrete stem cell compartments within the skin and during development, or after various stresses will be key to understanding transcriptional regulation by this family of genes.[2]

As more and more accurate, pinpoint and sophisticated methods are used for detecting and deciphering p63, the wealth of knowledge about p63 and its isoforms is increasing. Intricacies of p63 functioning, among its various isoforms, role of p63 within the p53/p63/p73 family, as also the vast number of upstream and downstream targets are being better understood. However, many more secrets regarding p63 are still to be unraveled. The ultimate goal would be to achieve specific, tailor- made treatment modalities for various epithelial cancers depending on p63 expression.

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