REVIEW

p63: molecular complexity in development and cancer

Matthew D. Westfall and Jennifer A. Pietenpol

Department of Biochemistry, Center in Molecular Toxicology, The Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

1To whom correspondence should be addressed
Email: jennifer.pietenpol@vanderbilt.edu

Discovery of the p53 homologs p63 and p73 has brought new excitement to the p53 field. Identification of homologous genes coding for several proteins with similar and antagonistic properties towards p53 has been both intriguing and perplexing. A multitude of properties have been attributed to these new homologs and this review will focus on the biochemical and biological aspects of one family member, p63. Although the most ancient member of the p53 family, p63 is the most recently discovered and the least known about this family member. Unlike p53, whose protein expression is not readily detectable in epithelial cells unless they are exposed to various stress conditions, p63 is expressed in select epithelial cells at high levels under normal conditions. p63 is highly expressed in embryonic ectoderm and in the nuclei of basal regenerative cells of many epithelial tissues in the adult including skin, breast myoepithelium, oral epithelium, prostate and urothelia. In contrast to the tumor suppressive function of p53, over-expression of select p63 splice variants is observed in many squamous carcinomas suggesting that p63 can act as an oncogene. Undoubtedly, the biochemical and biological activities attributed to p63 over the next several years will be diverse and regulation of the p63 gene and its several protein products complex. The use of various model systems and the study of human diseases should continue to lead to rapid advances in our understanding of the role of p63 in development, epithelial cell maintenance and tumorigenesis.

Introduction

Many tumor suppressor genes such as pRb and p16INK4A belong to families with several members. However, despite the central role of p53 in tumorigenesis, and a concerted search by many laboratories, related genes were not identified for almost two decades. This changed in 1997 and 1998 with the discoveries of the p53 homologs, p73 and p63, respectively (1–6). Studies over the past several years have revealed a multitude of possible functions for these new homologs in areas such as neurogenesis and stem cell biology. This review focuses on our current knowledge of p63 and specifically on its role in epithelial biology and carcinogenesis. Because p63 was cloned independently by several groups, this led to a rather confusing nomenclature: KET, p51A and p51B, p40, and p73L. However, for the purposes of this review, the newly identified homolog will be referred to as p63. Studies of p63 suggest that it functions primarily in ectodermal differentiation during development and stratified epithelial progenitor-cell maintenance. In contrast to the tumor suppressive function of p53, over-expression of select p63 splice variants is observed in many squamous carcinomas suggesting that p63 can act as an oncogene. How p63 regulates these processes is not well understood, but data from in vitro and in vivo experiments is beginning to provide insight to p63 function. Future studies are required to link p63 biochemical activities to biology, and to determine the interplay of p63 with other signaling pathways including those regulated by other p53 family members.

p63 biochemical activity and regulation

p63 structure

The p63 gene exhibits a high sequence and structural homology to p53 (Figure 1) leading to the early speculation that p63 proteins would function as tumor suppressors similar to p53. Like p53, the p63 gene encodes an N-terminal transactivation domain (in human TAp63, amino acids 1–59), a core DNA-binding domain (in human TAp63, amino acids 142–321) and a carboxy-oligomerization domain (in human TAp63, amino acids 353–397). However, there are significant differences between this homolog and p53. Whereas p53 has a single promoter, the p63 gene contains two transcriptional start sites that are used to generate transcripts encoding proteins with or without an N-terminal transactivation domain. Proteins with the transactivation domain are termed TAp63 and proteins lacking the transactivation domain are termed ΔNp63. Additionally, both genes can be alternatively spliced to generate proteins with different C-termini. For example, six variants can be generated from the two promoters of the p63 gene. However, for the purposes of this review, the newly identified homolog will be referred to as p63. Studies of p63 suggest that it functions primarily in ectodermal differentiation during development and stratified epithelial progenitor-cell maintenance. In contrast to the tumor suppressive function of p53, over-expression of select p63 splice variants is observed in many squamous carcinomas suggesting that p63 can act as an oncogene. How p63 regulates these processes is not well understood, but data from in vitro and in vivo experiments is beginning to provide insight to p63 function. Future studies are required to link p63 biochemical activities to biology, and to determine the interplay of p63 with other signaling pathways including those regulated by other p53 family members.

Abbreviations: AEC, ankyloblepharon ectodermal dysplasia; EEC, ectrodactyly-ectodermal dysplasia clefting; LMS, limb-mammary syndrome; MEFs, mouse embryo fibroblasts.
Role of p63 as a transcription factor

Due to the numerous variants that can be generated from the p63 gene, intensive studies have focused on determining: the variants that are expressed, the signaling pathways regulated by proteins encoded by p63 variants and if p63 activates or represses genes transcription. Since the most significant degree of homology between p53 and p63 is in the DNA-binding domain, and the critical residues for the proper folding of the entire domain as well as for the binding to the target DNA sequences, are completely conserved (3,20), initial studies were performed to determine if p63 could regulate p53-responsive genes.

Transient transfection assays showed that p63 activated or repressed transcription of a reporter gene downstream of an optimal p53 DNA-binding site (3). As predicted from their structures, the TA variants can transactivate p53 target genes, whereas the ΔN variants are believed to act in a dominant-negative manner (3). The same effect of p63 variants was seen using the luciferase reporter gene downstream of the genomic p21\textsuperscript{Warf/Cip1} promoter sequence, known to be regulated by p53 (21,22). Transcriptional activation by TA variants is most likely due to interaction of the transactivating domain with co-activators such as the high mobility group one-like protein SSRP1 or the apoptosis stimulating protein of p53 family members, ASPP1 and ASPP2 (23,24). Recent studies have identified a transcriptional inhibitory domain between the SAM domain and the C-terminus believed to mediate the repressive effects of p63α variants (22,25,26). How the C-terminus functions to repress transcription is not clearly understood but it is proposed that the C-terminus interacts with the TA domain of p63, in a region homologous to the MDM2-binding site in p53, to prevent binding of co-activator proteins (26). Supporting this idea, activity studies comparing TA-variants show the TAp63\textsuperscript{α} variant stimulates transcription as well as wild-type p53 whereas TAp63\textsuperscript{α} shows little or no transactivating activity (3). In contrast, ΔNp63\textsuperscript{α} represses p53- or TAp63\textsuperscript{γ}-mediated transcription, but ΔNp63\textsuperscript{γ} is only able to repress p53-mediated transcription (3).

In addition to reports of p63 regulation of p53 or novel target genes, biochemical evidence of direct DNA binding was necessary to cement the role of p63 as a bona fide sequence-specific transcription factor. In vitro DNA-binding assays using the known p53-binding sites in the p21\textsuperscript{Warf/Cip1} and 14-3-3\textsuperscript{β} promoters demonstrated that ΔNp63\textsuperscript{α} binds both p53 response elements in the p21\textsuperscript{Warf/Cip1} and 14-3-3\textsuperscript{β} promoters (22). Further, chromatin immunoprecipitation (ChIP) assays in primary human epidermal keratinocytes showed p63 binding in vivo to the p53 target genes p21\textsuperscript{Warf/Cip1} and 14-3-3\textsuperscript{β} (22). These findings demonstrate that p63 binds p53 response elements in vivo and suggest p63, depending on the variant, can positively or negatively regulate p53 target genes involved in growth regulation. Additional ChIP experiments with E1A-expressing mouse embryo fibroblasts (MEFs) revealed in vivo p63 binding to the p53 target genes mdm2, bax, PERP and NOXA indicating possible p63 involvement in regulating the stability of p53 and its apoptotic functions (27).

Role of p63 in cell cycle regulation and apoptosis

Initial studies of p63 biochemical activities found that transient transfection or adenoviral infection using TA-containing versions of p63 variants could induce both cell cycle arrest and apoptosis (3,6,28). Interestingly, these same studies showed that the TAp63\textsuperscript{γ} variant had the greatest transactivation activity and TAp63\textsuperscript{α} lesser or minimal activity. Similar assays were performed with the ΔNp63\textsuperscript{α} variants and opposite effects on cell cycle regulation or apoptosis were observed as compared with those generated with TAp63\textsuperscript{α} variants (3). Adding more complexity to the story, transient transfection of TA or ΔNp63\textsuperscript{α} into p53\textsuperscript{-/-} cells caused both cell cycle arrest and apoptosis (29) with up-regulation of specific p53 target genes.
However, in vitro transcriptional assays clearly demonstrate a transcriptional repressive role for AN variants (3,22). Further, a striking example of ΔNp63α countering p53 or p53-like activities in squamous epithelial cells in vivo was provided by Liefer et al. in a study showing that targeted ectopic expression of ΔNp63α to the epidermis of the mouse (via the loricin promoter) could counter ultraviolet radiation-mediated apoptosis (30) that has been shown to be p53-dependent (31).

Additional clues to p63 function were provided by experiments with E1A-expressing MEFs. Loss of p63 in the E1A-expressing MEFs resulted in failure of cells containing functional p53 to undergo apoptosis in response to DNA damage (27). While induction of p21 and MDM2 occurred normally in p63+/−, p73+/−, and double-null E1A-expressing MEFs, loss of p63 and p73 severely affected the induction of bax, NOXA and PERP, genes thought to mediate p53-dependent apoptosis. These results suggest that p53 target genes are differentially affected by the loss of p63 and p73 and that apoptosis-related targets may be specifically regulated within the entire p53 family. However, the lack of developmental epithelial disorders in p53+/− mice (a hallmark phenotype of p63−/− mice) argues that p53 does not play an integral role in p63-mediated signaling in basal epithelial cells. Although the results of this recent study are provocative, it is difficult to fully interpret given that the MEFs used were engineered to express E1A. It is also not evident that any p63 variants, let alone the TA versions, are expressed in MEFs without E1A expression. It is also not evident that any p63 variants, let alone the TA versions, are expressed in MEFs without E1A expression. It is also not evident that any p63 variants, let alone the TA versions, are expressed in MEFs without E1A expression.

Role of p63 in development and epithelial tissues

p63 model systems

Whereas p53−/− mice are developmentally normal but prone to neoplastic disease (32), the p63−/− mice have severe developmental abnormalities. Specifically, the p63−/− mice are born, but die shortly after birth, and are deficient in the development of limbs and several epithelial tissues such as skin, prostate, mammary gland and urothelia (33,34). Two different groups generated p63-deficient mice and although the phenotypes reported were identical, the conclusions drawn from examination of the murine tissues varied. One study found that the embryonic epidermis of p63−/− mice undergoes non-regenerative differentiation (34) whereas the other study found that skin from p63−/− mice does not progress past an early developmental stage and does not express differentiation markers (33). Regardless, the results from the genetically deficient mice are invaluable for future experiments to determine p63 function.

To directly address the role and necessity of the different N- and C-terminal variants of p63 during development and in adult tissue, investigators need to generate variant-specific knockouts in select model systems. Along these lines, using zebrafish as a model system, two groups have investigated the role of the ΔNp63 variants in embryogenesis. Using morpholino antisense oligonucleotides directed against the ΔNp63 variants, Lee et al. demonstrated that ΔNp63 forms of p63 are required for the development of epithelial cells in zebrafish, as the resulting embryos lacked epidermal structures and fins (35). The epidermal cells of embryos blocked for ΔNp63 function failed to undergo proliferation 20 h after fertilization, suggesting that ΔNp63 keeps epidermal cells in a proliferative state (35). Further, the ΔNp63-regulated epidermal proliferation was due to transcriptional inhibition of p53 target genes in vivo (35).

In addition to the obvious importance of p63 in development, p63 is hypothesized to play an important role in epithelial development and subsequent formation of squamous epithelial tissues in humans is a complex biological process. Studies in epidermal development alone have highlighted the involvement of signaling cascades such as the Wnt, Notch and Bmp pathways, as well as the importance of cell-cell and ectoderm-mesenchymal cross-talk (45). However, there are still undoubtedly many components to be identified that are involved in this epidermal developmental. As stated in the previous section, studies using the p63−/− mouse suggest a central role for the p63 protein during squamous epithelial development. Onset of p63 expression in the single-layered ectoderm coincides with initiation of epidermal development at embryonic day 9.5 (33,34). High p63 expression is observed in the apical ectodermal ridge. The functional integrity of the apical ectodermal ridge is necessary for limb outgrowth that is absent in the p63−/− mouse as described above (33,34). p63 expression is also detectable at early stages of embryogenesis on ectodermal surfaces of the branchial arches that are important for morphogenesis of craniofacial skeletal and soft-tissue structures (46), both of which are aberrant in the p63−/− mouse (33,34). A recent study by Koster et al. suggests that the TaP63 variants are required for initiation of an epithelial stratification program and the ΔNp63 variants are required for maintenance of the mature epidermis (47). Further, the authors suggest that the TaP63 variants are necessary to inhibit terminal differentiation during early development (47).
in maintaining the epidermal stem cell population. Immunohistochemical analyses show p63 protein localization and expression in the basal/progenitor cells of several epithelial tissues such as the epidermis, hair follicles, sweat glands, cervix, tongue, esophagus, mammary glands, prostate and urogenital tract with ΔNp63α being the predominant, if not only, p63 variant expressed (3,22,43,44). p63 expression is lost as these cells migrate from the basal layer and become terminally differentiated cells. Furthermore, the tissues listed above are absent in the p63−/− mouse as described earlier (33,34). Although correlative, these observations suggest a role for p63 in maintaining the epidermal stem cell population. Three additional lines of evidence support this line of reasoning. First, application of retinoic acid to keratinocytes grown in culture, which stimulates keratinocyte proliferation in vivo (48), prevents reduction of ΔNp63α protein and blocks progression into differentiation after growth factor removal (42). Secondly, clonal analysis of keratinocytes shows expression of p63 in holoclones that are thought to represent the stem cell compartment of the epidermis (49,50). Thirdly, a decrease in p63 is associated with a reduced proliferative potential and subsequent terminal differentiation of skin keratinocytes in culture (22,43,49). These observations support the hypothesis that p63 plays an essential role in maintaining the proliferative capacity of epithelial stem cells.

Several experiments using transient expression of different TAp63 variants have also given insight to the role of p63 in epithelial cell biology. Sasaki et al. show that TAp63γ can induce expression of Jagged-1, a Notch receptor ligand (51). Notch receptors are ligand-activated transmembrane receptors involved in cell fate decisions throughout development (52,53). In contrast, TAp63γ down-regulated the expression of epidermal growth factor receptor through interaction with the basal transcription factor Sp1 (54). Down-regulation of the epidermal growth factor receptor is associated with epithelial differentiation. Additionally, the TAp63α variant induced expression of the EphA2 receptor tyrosine kinase (55). The EphA2 kinase is found predominantly in adult epithelium and evidence suggests activated EphA2 can negatively regulate cell adhesion and proliferation by disrupting focal adhesions and disruption of the Ras/MAPK pathway (56–58). While these results suggest several potential targets for p63, it is not evident that any of the variants used are expressed in embryonic or adult epithelial tissue. Since ΔNp63α, a known transcriptional repressor is expressed in epithelial cells it is distinctly possible that the genes listed above are bona fide p63 targets but the biological implications would be reversed with ΔNp63α expression.

p63 in human disease

Role of p63 in autosomal dominant disorders

Unlike the case with p53, where germline mutations lead to human cancer predisposition, p63 germline mutations in humans lead to congenital abnormalities characterized by abnormal limb development and/or ectodermal dysplasia, phenotypes that are similar to p63−/− mice. Linkage analysis of ectodactyly-ectodermal dysplasia clefting (EEC) syndrome identified a locus on chromosome 3q27 coinciding with the localization of p63 (20). Subsequent analysis revealed nine unrelated patients with EEC to have heterozygous mutations in the p63 gene (20). Since the identification of p63 mutations in EEC, mutations have been identified in several related developmental disorders such as ankyloblepharon-ectodermal dysplasia and clefting (AEC or Hay-Wells syndrome) (59), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT) (60), limb-mammary syndrome (LMS) (61) and split hand/split foot malformation (SHFM) (62). Of note, different mutations have been identified throughout the p63 gene for these syndromes. For example, EEC (20) and ADULT (60) syndrome result from missense mutations in the DNA-binding domain of p63, whereas AEC (59) and LMS (61) are caused by missense mutations in or near the SAM domain, respectively. In contrast, SHFM results from mutations found throughout the gene (62). The diverse range of mutations suggests many different mechanisms for altering p63 function. While mutations of the DNA-binding domain obviously disrupt or reduce DNA binding similar to what is seen with p53 in human tumors, the effect of other mutations are less obvious. The mutations found in AEC in the SAM domain likely disrupt the structural integrity of the globular domain or interfere with protein–protein interactions (63). A recent study by Fomenkov et al. supports disruption of protein–protein interactions as a mechanism for causing AEC. The study found that the p63α C-terminus (including the SAM domain) interacts with the apobec-1-binding protein-1 that leads to a shift of fibroblast growth factor receptor-2 splicing to its K-SAM variant that is essential for epithelial differentiation (64). AEC mutations disrupt this p63-apobec-1-binding protein-1 interaction and may lead to aberrant epithelial differentiation subsequently causing the AEC phenotype. Similar to AEC missense mutations, LMS mutations may disrupt the SAM domain whereas nonsense LMS mutations result in truncation of the protein and likely loss of a proposed transcriptional inhibitory domain (26). Clearly, it is critical to understand the physiologic role of p63 in ectoderm and epidermal development to better understand the functional implications of these p63 mutations.

Role of p63 in cancer

The initial findings by Yang et al. identified ΔNp63α as the primary p63 variant expressed in squamous epithelial tissues and more importantly determined that ΔNp63α can act antagonistically toward p53 (3). Subsequent studies found deregulated expression of p63, sometimes in conjunction with amplification of its genomic region at 3q27-28, to be a frequent occurrence in a subset of human epithelial cancers (Table 1) (65–69). Amplification of the p63 gene frequently results in over-expression of the ΔNp63α variant (66). Overexpression of the ΔNp63 variant p40 in Rat 1a cells leads to increased growth of these cells in soft agar and as xenograft tumors (66). Of note, Hagiwara et al. and Osada et al. analyzed the sequence of p63 isolated from various human tumors and numerous human cancer cell lines and found p63 to be rarely, if ever, mutated (6,70). Collectively these data suggest that p63 does not function as a tumor suppressor but rather as an oncogene. However, it is important to note that over-expression of p63 and alteration of p53 signaling are not mutually exclusive events (44,66), which would be predicted if the only role of p63 was to counter p53 activity. In advanced cervical carcinomas where the p65 locus has been found amplified (71), HPV is present suggesting that there was selective pressure during tumorgenesis for both loss of p53 function (via HPV E6-mediated degradation; ref. 72) and presumably increased p63 oncogenic activity due to over-expression (71,73).
To better understand the role of p63 in carcinogenesis it was important to determine the variant expressed in the various squamous carcinomas. As mentioned previously, the ΔNp63α variant is the predominant, if not only, p63 variant definitively shown to be expressed at the protein level in the basal cell layer of normal adult epithelium (3,22,43,44). Recently, a comprehensive analysis of p63 expression, in normal and neoplastic tissue, was performed using three serial sections of a multi-tumor tissue microarray (74). The study examined 51 normal human tissues, 350 carcinomas, 25 malignant melanomas and 25 glioblastomas. p63 expression was not detected in basal epithelial tissues (74). However, p63 was expressed in 93% of squamous cell carcinomas of the lung, 10% of ductal carcinomas of the breast and 25% of endometrioid carcinomas of the ovary (74) (Table I). p63 expression was rarely detected in adenocarcinomas of the breast, lung or prostate, which is not surprising as these latter tumors lack the basal cells where p63 is expressed in these tissue types (74). Although it is tempting to speculate that p63 expression is a contributing factor in squamous cell tumor development, until there is a better understanding of the target genes regulated by p63, it is not possible to differentiate between a causative role for p63 versus ΔNp63 expression as a consequence of tumor development. Along these lines, amplification of the 3q26 region is PIK3CA, a positive regulator of the phosphoinositide-3-kinase pathway downstream of the epidermal growth factor receptor possibly explaining p63 overexpression in select squamous cell carcinomas (76).

Despite the lack of information on p63 target genes, identification of p63 interacting proteins may provide insight to how p63 functions in tumor cells. In one study, ectopic expression of ΔNp63α resulted in increased half-life of the hypoxia-inducible factor 1α (77). Stabilization of hypoxia-inducible factor 1α caused up-regulation of the vascular endothelial growth factor that was dependent upon a hypoxia-inducible factor 1-binding site within the vascular endothelial growth factor promoter (77). Other studies have identified ΔNp63α interaction with the B56α regulatory subunit of the protein phosphatase 2A and glycogen synthase kinase 3β proteins (78,79). These interactions are proposed to inhibit the adenomatous polyposis coli destruction complex (78,79). Inhibition of the destruction complex causes decreased β-catenin phosphorylation, subsequent nuclear accumulation, and transcriptional activation of matrix metalloproteinases (78,79). These findings highlight the myriad of biochemical activities that p63 may carry out, ranging from sequence-specific transcriptional activator and repressor to ‘co-factor’ with proteins such as hypoxia-inducible factor 1 or the adenomatous polyposis coli complex.

Conclusions

Identification of the p53 homolog, p63, has opened a new chapter in developmental and cancer biology. While new p53 target genes and functions are published on a monthly basis, the regulation and function of p63 is still in the early stages of discovery. Differences in p53−/− and p63−/− mouse phenotypes alone suggest that p63 regulates signaling pathways that differ from p53. Studies of human tumors and human genetic syndromes have shed some light on p63 function, but a better biochemical understanding of p63 will undoubtedly be required to understand the role of p63 in tumorigenesis and development. While current data suggest a rudimentary understanding of the variant(s) expressed in adult tissue and the functional differences between the variants, numerous questions remain to be addressed by future research: What are the primary variants expressed during development? How do the p63 variants differ in regulation? What are upstream signaling pathways that regulate p63? What are the p63 target genes? Do target genes regulated during development differ from those regulated in adult tissues? Do p63 and p53 regulate distinct and/or overlapping sets of genes? Answers to these questions will advance our understanding of developmental and tumor biology and also shed light on the interplay amongst the p53 family members. Ultimately, p63 research may lead to novel therapeutic approaches for the developmental syndromes and tumor types that are linked to aberrant p63 expression.

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