Adenovirus-mediated p53 tumor suppressor gene therapy of osteosarcoma

Vladimir V Ternovoi[1], David T Curiel[1], Bruce F Smith[2] and Gene P Siegal[3]

[1]*Division of Human Gene Therapy, Departments of Medicine, Obstetrics and Gynecology, Pathology and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL, USA;* [2]*Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL, USA and* [3]*Departments of Pathology, Cell Biology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL, USA*

The clinical outcome for osteosarcoma (OS) remains discouraging despite efforts to optimize treatment using conventional modalities including surgery, radiotherapy and chemotherapy. Novel therapeutic approaches based on our expanding understanding of the mechanisms of tumor cell killing have the potential to alter this situation. Tumor suppressor gene therapy aims to restore the function of a tumor suppressor gene lost or functionally inactivated in cancer cells. One such molecule, the p53 tumor suppressor gene plays a critical role in safeguarding the integrity of the genome and preventing tumorigenesis. Introduction of wild-type (wt) p53 into transformed cells has been shown to be lethal for most cancer cells *in vitro*, but clinical trials of p53 gene replacement have had limited success. Analysis of these clinical trials highlighted the insufficient efficacy of current vectors and low proapoptotic activity of wt p53 as a single agent *in vivo*. In this review, a contemporary summarization of the current status of adenovirus-mediated p53 gene therapy of OS is presented. Advancement in our understanding of p53 tumor suppressor activity, the molecular biology of chemoresistant OS, and recent advances in tumor targeting with adenoviral vectors are also addressed. Based on these parameters, prospects for future investigations are proposed.
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Osteosarcoma (OS) is among the commonest of primary tumors of bone. It represents one of the most morphologically heterogeneous tumors known in human pathology and appears in distinct clinical forms with different degrees of malignancy.[1] Most variants of OS are extremely aggressive as characterized by their rapid growth and early development of distant metastasis, mostly to lung and bones. Death from OS is usually the result of respiratory failure due to progressive pulmonary destruction from metastasis.[2,3]

Standard treatment involves the use of 'up-front' multiagent chemotherapy, definitive surgery of the primary tumor, and postoperative chemotherapy. Introduction of adjuvant and neoadjuvant cytotoxic treatment and improvements in surgical procedures have increased 5-year survival to over 60% compared to < 20% when only surgery and/or radiation therapy had been used.[4,5] Despite attempts to further increase the disease-free survival for poorly responding patients through administration of more intensified therapy, no survival benefit has been convincingly shown.[6–8]

Gene therapy can be defined as the treatment of disease by the introduction of a therapeutic gene or the manipulation of a disease-related gene such as abrogation of an activated oncogene. Tumor suppressor gene therapy is that part of gene therapy which aims to restore the function of a tumor suppressor gene lost or functionally inactivated in cancer cells. In other words, tumor suppressor genes can be defined as genes for which loss-of-function mutations are oncogenic. Most commonly they regulate diverse cellular activities; cell-cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation, migration and tumor angiogenesis.[9] Many cancers have been shown to have inactivated tumor suppressor genes. The concept of tumor suppressor gene therapy is thus based on the following assumptions: (1) restoration of tumor suppressor

Correspondence: Dr GP Siegal, MD, PhD, Division of Anatomic Pathology, Department of Pathology, University of Alabama at Birmingham, KB 506, 619 19th Street South, Birmingham, AL 35233, USA.
E-mail: gsiegal@path.uab.edu

function through gene transfer and expression would suppress the tumor phenotype of a cell and (2) normal nontransformed cells with functional endogenous tumor suppressor genes should tolerate the introduction of low to moderate levels of tumor suppressor genes.[10] Indeed, reintroduction of known tumor suppressors into tumor cells *in vitro* has been shown to cause an acute change in cell physiology and gene expression, resulting in growth arrest or cell death.[11] Despite the response of tumor suppressor gene introduction, the genotype of cancer cells remain markedly different from the putative normal counterparts from which they were derived due to quick acquisition of additional mutations after inactivation of tumor suppressor genes (genetic instability). Introduction of these genes in this context, in fact, cannot reverse the tumorigenic properties of such cells. Importantly, growth-arrested cancer cells are still metabolically active and can promote growth of other tumor cells in their vicinity.[12,13] Furthermore, some of these cells can also resume growing.[14] Therefore, only those tumor suppressor genes which have the capacity to efficiently induce tumor cell death should be considered as good candidates for tumor suppressor gene therapy.

The p53 gene has an attractive apoptotic tumor suppressor profile as a gene therapy agent. Introduction of wild-type (wt) p53 causes death specifically in tumor cells, sparing normal cells. Additionally, p53 can promote death of nearby nontransduced tumor cells via its ability to transactivate genes whose products inhibit angiogenesis[15] and via induction of an immune response against tumor cells,[16] that is p53 causes a bystander effect. Results of preclinical studies on more than 100 cell lines and tissues have shown that introduction of wt p53 into neoplastic cells *in vitro* was lethal for most cells with p53^{mut} or p53^{null} genotypes.[17] Unfortunately, clinical trials based on wt p53 gene replacement have had limited success.[18–20] Furthermore, p53 gene therapy of metastatic disease did not result in improvement in patient status and survival.[21] Analysis of these clinical trials revealed the insufficient efficacy of that generation of Ad vectors and low proapoptotic activity of wt p53 *in vivo*.[22–24]

This review presents a contemporary summarization of the current status of adenovirus-mediated p53 gene therapy of OS. We will first describe the recent advances in our understanding of the mechanisms of tumor suppressor activity of p53 and then focus our discussion on the use of Ad vector expressing p53 in the context of gene therapy of OS.

An overview of p53 tumor suppressor pathway

The particular contribution of p53 to certain functional decisions depends not only on p53. Depend-ing on the plethora of intrinsic and extrinsic factors (the nature and intensity of stress, cellular and tissue contexts), activation of p53 results in different cells' fate.[25,26] Acting primarily as a transcription factor, p53 plays a critical role in regulating the cell cycle and maintaining genomic integrity by inducing growth arrest or apoptosis in response to a variety of stresses[26–29] (see Figure 1).

The structure and function of p53: The p53 tumor suppressor gene encodes a 393 amino-acid modular protein with several interdependent functional domains (see Figure 2).[30,31] The acidic N-terminal domain (amino acids 1–63) is important for transactivation; it contains residues interacting with components of the basal transcriptional machinery.[32] It is also critically involved in regulating the stability and activity of p53 protein via interactions with Mdm2.[33,34] A region in this domain was identified (amino acids 43–63) as being required for p53-dependent apoptosis.[35,36] The proline-rich region (amino acids 64–91) is also necessary for apoptosis.[37–39] The sequence-specific DNA-binding domain within residues 100–300 serves as the core of the p53 protein. The majority of missense mutations found in tumor samples occurs in this region of the gene and result in disruption of the ability of p53 to specifically bind DNA. The oligomerization domain of p53 (amino acids 324–355) participates in the formation of p53 tetramers. The C-terminal basic domain (amino acids 356–393) is an important regulatory domain. One of its functions is to keep p53 in an inactive form until modifications including phosphorylation and acetylation can serve to activate the protein allosterically.[30]

In unstressed cells, p53 is present at low levels and exists in a latent form that requires modification to become active. Various forms of stress (DNA damage, oncogenic stimuli, redox stress and hypoxia) rapidly induce a transient increase in p53 protein via enhanced translation of p53 mRNA and stabilization of p53 protein.[40] Also, a series of post-translational modifications occurs in p53 including phosphorylation, glycosylation and acetylation, that affect a myriad of activities including protein–protein interactions, alternative splicing, homo- and hetero-oligomerization, C- and N-terminal truncations, and DNA-binding capabilities.[30,40] Activated p53 initiates a transcriptional program that reflects the nature of the stress signal, resulting in protein modifications and alternative proteins being associated with p53 protein.[41]

The function of p53 in cancers can be lost by various mechanisms, including lesions that prevent activation of p53, mutations within the TP53 gene (which encodes p53) itself and mutations of downstream mediators of p53 function. Most TP53 mutations found in tumors are point mutations which usually result in the expression of mutant p53 protein. Importantly, these proteins are often more stable than wt p53 and some p53 mutants can
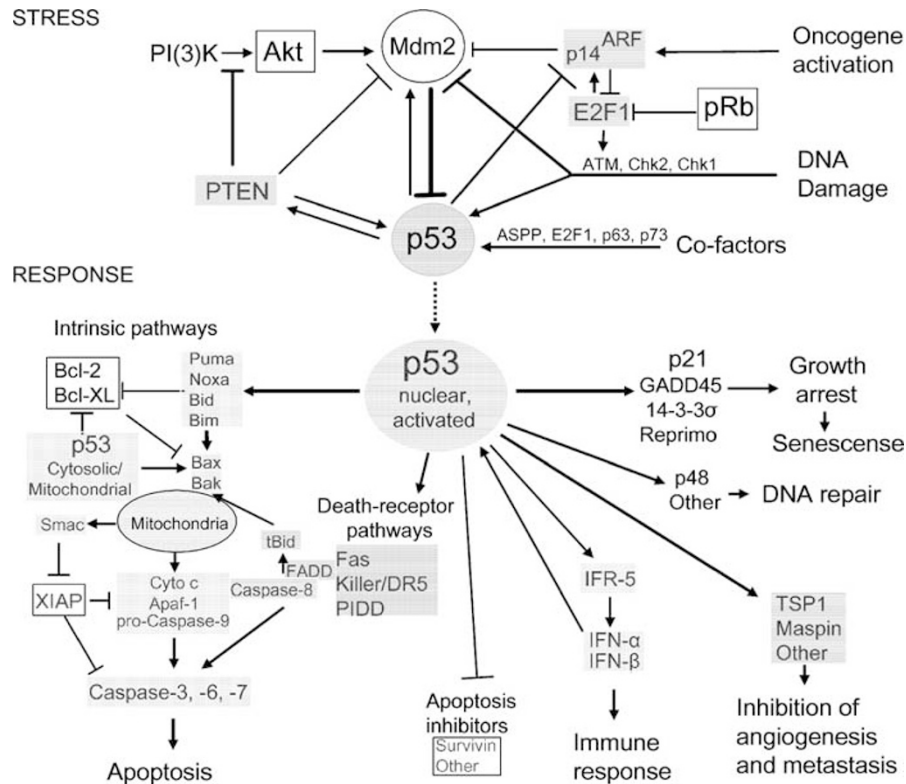
Figure 1 p53-mediated response to stress. A schematic diagram demonstrating some of the known components of the p53 functional circuit. Shown are key components of the apoptotic program as well as some its key regulators. Components in white squares inhibit apoptosis while those in gray squares promote apoptosis. See text for details.
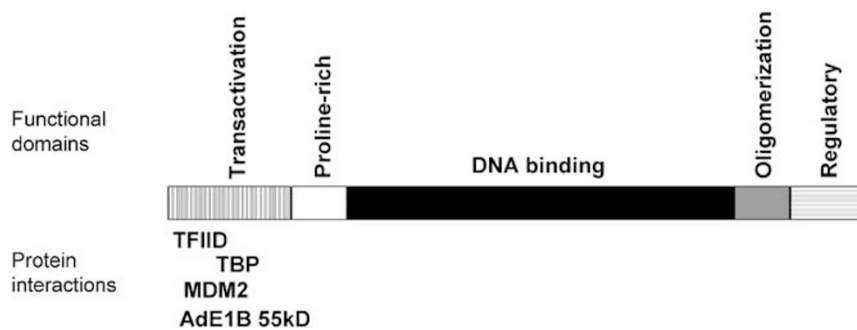

Figure 2 Functional domains of p53 protein. The human p53 protein consists of 393 amino acids and has several functional domains (indicated by the shaded regions) including an acidic or transactivation domain, a proline-rich domain, a sequence-specific DNA-binding domain, an oligomerization domain, and a C-terminal regulatory domain. See text for details.

act as dominant-negative inhibitors of wt p53.[42,43] Certain missense mutations not only result in abrogation of wt p53 activity, but the expressed p53 mutant proteins also tend to gain oncogenic function, such as interference with wt p53-independent apoptosis.[44] Importantly, such p53 mutants contribute to the resistance of tumor cells to commonly used chemotherapeutic agents.[45] Many tumors with a dysfunctional p53 pathway retain wt protein. Defects of the tumor suppressor pathway in these cases results from functional abnormalities in upstream regulators of p53 activity or from effectors of apoptotic activity of p53[46] (see Figure 1).

Mdm2

Mdm2 is a master regulator of p53.[47] It controls p53 function through inhibition of p53-mediated transcriptional activity[48,49] and by promoting p53 degradation.[33,34] Binding of Mdm2 to p53 is essential for this effect. Importantly, the mdm2 gene is a target gene for p53. p53 binds to p53-responsive elements located within the mdm2 gene, and promotes the production of mdm2 transcripts.[50,51] Consequently, an autoregulatory negative feedback loop exists, wherein p53 induces Mdm2 expression and Mdm2 represses p53 activity. This serves as an

important mechanism for restraining p53 function in the absence of stress. Cellular stress induces modifications of both p53 and Mdm2 proteins, which results in reduced avidity of p53 for Mdm2 and thereby, inhibition of ubiquitination and degradation of p53 protein. An inappropriate excess of Mdm2 may result in exaggerated silencing of p53, abrogating its tumor suppressor effects, as it was found that overexpression of Mdm2 in mice resulted in the production of tumors with as little as a four-fold increase in Mdm2 levels giving rise to tumors in 100% of the mice.[52]

Multiple mechanisms may modulate Mdm2 activity and its ability to access p53 for ubiquitination. For example, p14[ARF], human ribosomal protein L11, and cyclin G can act as negative regulators of Mdm2's ability to downregulate p53 levels.[53–55] Other factors recently shown to positively regulate Mdm2 include YY1, gankyrin and KAP1.[56–58] Mdm2 and mdmx prevent ASPP1 and ASPP2 from stimulating the apoptotic function of p53 by binding and inhibiting the transcriptional activity of p53.[59]

Proapoptotic activity of p53

P53 can induce apoptosis in several ways.[60–63] It can act as a transcriptional factor that directly activates the expression of genes known to promote apoptosis including Bax,[64] Bak,[65] PUMA,[66,67] Noxa[68] and Bid.[69] The net effect of induction of these genes is an increasing of the ratio of pro- to antiapoptotic Bcl-2 proteins, consequently, favoring the release of apoptogenic proteins from the mitochondria, caspase activation and apoptosis. In addition, p53 can also transactivate several effectors of apoptosis acting downstream of mitochondria such as Apaf-1 and caspase-6.[70,71] p53 also regulates the extrinsic apoptotic pathway, activating expression of Fas/CD95, DR5 and RDL1 genes[72,73] and is capable of counteracting with antiapoptotic pathways inducing genes including PTEN and IGF-BP3, which inhibit survival signaling.[74,75]

P53 also possesses a proapoptotic function that is independent of its transcriptional activity.[76–78] In response to stress, p53 accumulates in the cytoplasm, where it directly or indirectly activates the proapoptotic proteins to promote mitochondrial outer-membrane permeabilization.[79–82] It seems likely that coordination of the nuclear, cytoplasmic, and mitochondrial activities of p53 cooperate to ultimately cause cell death. In addition to transactivation functions, p53 has also transrepression capabilities that may contribute to apoptosis.[83–85]

To explain how p53 can induce apoptosis or cell-cycle arrest, different models have been proposed. One quantitative model assumes the existence of p53-responsive genes with differing binding affinities. According to this model, a subset of promoters should be activated only when the expression level of p53 reaches a certain threshold. Low levels of p53 protein will result in cell-cycle arrest because

promoters of cell-cycle arrest genes (p21, mdm2) have higher affinity binding sites for p53 than promoters of genes responsive for apoptosis (Bax, IGF-BP3).[36] Not all data support this model. For example, the binding affinity of p53 to the PUMA promoter, which regulates a proapoptotic gene, is similar to the p21 and mdm2 promoters. Other qualitative models proposed that induction of apoptosis or cell-cycle arrest by p53 depends on p53-binding proteins, which are able to modulate the selection of target genes. Importantly, some of these factors have a specific and selective role in enabling the expression of the apoptotic target genes. For example, JMY cooperates with p300 to enhance the ability of p53 to induce the expression of BAX, but does not significantly influence the induction of the p21 gene.[86] The ASPP1 and ASPP2 genes have been shown to interact with p53 and enhance the interaction with the promoter of the BAX gene.[87] Inhibition of ASPP expression blocks the apoptotic response to p53. Other important modulators of p53 apoptotic induction are p63 and p73 proteins. It was shown that p53 is not able to bind promoters of apoptotic targets in p63/p73 double-null cells.[88]

E2F

The E2F family of transcription factors are downstream effectors of the retinoblastoma (Rb) protein pathway. Since their discovery, E2Fs have been viewed as positive regulators of genes required for DNA synthesis.[89,90] Recent findings have changed this view.[91–93] It has been found that depending on the context, E2Fs function as activators or repressors of transcription. Specifically, there is a bifurcation of E2F family members with respect to their transcriptional roles in cell-cycle control; E2F1–3 function mainly as activators of transcription while E2F4 and E2F5 act primarily as repressors. These, new findings considerably broadened our understanding of E2F transcriptional control beyond S phase, E2F also regulates genes involved in DNA repair and recombination, apoptosis, differentiation and development, as well as other genes of still unknown function.

E2F can induce apoptosis in a p53-dependent manner as well as in a p53-independent manner.[92,94] E2F1 transcriptionally regulates the expression of the DNA damage-responsive kinases ATM and Chk2 that phosphorylate p53,[95,96] and induces expression of the proapoptotic cofactors of p53, ASPP1, ASPP2, JMY and TP53INP1.[97,98] E2F also directly activates the p53 proapoptotic target genes PUMA, Noxa, Bim, Hrk/DR5,[99] Apaf1[70] and SIVA,[100] and down-regulates the expression of Mcl-1, an antiapoptotic member of the Bcl-2 family.[92]

E2F factors have been suggested to potentially be involved in acquired resistance to chemotherapeutic drugs in OS cells. Recently, it has been found that E2F1-mediated activation of the p73 promoter

induces apoptosis in human OS cells.[101] Cinti and colleagues have also demonstrated that doxorubicin treatment of chemosensitive human OS cells (HOS) results in increased levels of p73, overexpression of bax, and downregulation of bcl-2. In performing a chromatin immunoprecipitation assay, they detected a multimolecular complex formed by E2F1-pRb2/p130–p300 bound to the p73 promoter.[102] In contrast, in chemoresistant cells (HOSDXR150) a multimolecular complex formed by E2F4-pRb2/p130-histone deacetylase complex 1 was bound to the same p73 promoter region. In another study, significant correlations between E2F and dihydrofolate reductase and thymidilate synthase (TS) in OS patient samples were observed, suggesting the potential involvement of the E2F pathway in methotrexat resistance.[103]

PTEN

In recent years, it has been demonstrated that multiple levels of crosstalk exist between PTEN (phosphates and tensin homolog deleted from chromosome 10) and p53, and that functional PTEN is important for proapoptotic activity of p53. The PTEN tumor suppressor gene is located on chromosome 10q23 and is known to be the most highly mutated tumor suppressor gene after p53.[104] The PTEN encodes a dual specificity protein–lipid phosphatase that plays an important role in regulating proliferation, migration, survival, cell invasion and tumor angiogenesis.[105,106] The lipid phosphatase activity of PTEN is crucial for its tumor suppressive activity. The main PTEN substrate is phosphatydilinositol(3,4,5)-triphosphate (PIP-3). PIP-3 levels are increased upon stimulation by growth factors through activation of PI3 kinase. Accumulation of PIP3 at the membrane allows recruitment of the proto-oncogene serine/threonine kinase Akt/PKB which is then activated by phosphorylation. Activated Akt is a well-known survival factor. Thus, by keeping the levels of PIP-3 low, PTEN prevents prosurvival activity of Akt. Akt also phosphorylates serine residues in the Mdm2 domain that contains a nuclear localization motif. These phosphorylations are important for translocation of Mdm2 from the cytoplasm into the nucleus where it can interact with p53 and inhibit its transcriptional activity.[107,108] Consequently, PTEN protects p53 from survival signaling induced by growth factors via the PI3K-Akt pathway and nuclear entry of Mdm2. Recently Wu and colleagues have shown that by antagonizing the PI3-kinase pathway, PTEN also negatively regulates the P1 promoter on the 5′ end of the mdm2 gene, leading to decreased L-Mdm2 expression and reduced p90[MDM2] isoform production.[109]

p53 is capable of binding directly to a site in the PTEN promoter and inducing PTEN mRNA expression in response to DNA damage.[75] In turn, a p53-binding domain has been identified in PTEN and

evidence exists that physical interaction between PTEN and p53 is important for modulation of p53 function *in vivo*.[110,111] It was further demonstrated that PTEN can control p53 half-life independent of PI3K/Akt/Mdm2 via a currently unknown mechanism. Finally, Donner and colleagues have recently demonstrated that intensive stress induces the phosphorylation of p53 protein which results in a switch of gene promoter targeting; from the promoter for mdm2 to the promoter for PTEN. Formation of the mdm2-p53 autoregulatory feedback loop is thus blocked and p53 together with PTEN form an apoptotic amplification cycle that induces cell death.[107] These findings show the importance of cooperation of PTEN with p53 for efficient action as a tumor suppressor and provide a strong rationale for combined p53 + PTEN cancer gene therapy.

Despite an overall high incidence of PTEN mutations in humans, the majority of them have been identified in epithelial cell-derived tumors.[112] To date, PTEN mutations have not been associated with OS tumorigenesis, despite the fact that the chromosomal loss of 10q has been documented in nearly 30% of human OS tumor samples analyzed.[113]

Prosurvival (antiapoptotic) signaling in tumor cells

Survival signaling provided by the tumor microenvironment significantly modulates cellular response to p53 activation. Interactions of cancer cells with each other and with stromal cells, various growth factors and with components of the extracellular matrix can drastically affect the apoptosis sensitivity of these cells.[114,115]

One of the most important molecules responsible for rescuing cells from p53-induced apoptosis is Akt kinase.[116] The activation of Akt by survival signals is achieved mainly through a kinase cascade involving PI3 kinase. The mechanism by which Akt protects cells from death is multifactorial.[117] Activated Akt kinase can phosphorylate and downregulate expression of PUMA, an important mediator of the p53 apoptotic response and also can inhibit catalytic activity of caspase-9.[118] Akt-mediated phosphorylation of FKHR—a member of the Forkhead family of transcription factors, prevents its nuclear translocation and activation of FKHR target genes, which include the proapoptotic proteins BIM and Fas ligand.[119] Also, the apoptosis-inducing protein Bad after phosphorylation by Akt loses the ability to bind antiapoptotic members of the Bcl-2 family, specifically Bcl-2 and Bcl-XL, thus releasing them for a cell survival response.[120] As was alluded to previously, Akt-mediated phosphorylation of Mdm2 results in inactivation of p53.

Experiments conducted using three-dimensional (3D) *in vitro* models suggest that additional levels of apoptosis signaling regulation likely exist in tissues.[121–123] Furthermore, it has been shown that

fundamental differences exist between degrees of survival of tumor cells incorporated into 3D spheroids *vs* tumors grown in monolayer in response to exposure to death stimuli.[124] Cells in 3D spheroids rapidly acquire multidrug resistance and this phenotype appears to be linked to cell adhesion.

Inhibition of tumor angiogenesis and metastases

The p53 protein stimulates the expression of genes that prevent the growth of new blood vessels in the tumor's vicinity and inhibits tumor invasion and metastasis. Among known p53's target genes with antiangiogenic and antimetastatic activities are thrombospondin-1 (TSP1), KAI1, BAI1 and maspin.[125–128] Reintroduction of wt p53 into tumor cells upregulated the expression of antiangiogenic factors which leads to downregulation of proangiogenic vascular endothelial growth factor and inhibition of neovascularization.[129–133] Suppression of angiogenesis contributed to suppression of both primary tumors and their metastasis.[134]

Role of the immune system in p53 tumor suppressor gene therapy

Despite the fact that the immune system has a huge antitumor potential, tumors are able to escape from immunosurveillance. Rational modulation of the patient's immune response could significantly augment the antitumor efficacy of gene therapy strategies. Interestingly, a link was recently discovered between p53 and the interferon system in modulating tumor suppression and antiviral immunity. The interferon system can upregulate expression of the p53 gene which in the context of other stimuli leads to p53 activation and enhancement of its proapoptotic activity.[16,135] In turn, p53 protein can transactivate expression of the interferon regulatory factor 5 gene,[136] whose product contributes to IFN-α and IFN-β induction.[137] The type I interferons exhibit a wide range of biological activities including antiviral, antiproliferative and antiangiogenic effects.[138,139] They activate cells in the immune system, and help in presentation of cancer cells to the immune system by stimulating production of cell surface molecules (costimulatory molecules, MHC antigens and tumor-associated antigens).

When p53 was overexpressed in the context of insertion of a viral vector in tumor cells, it was seen to induce an antitumor immune response.[140–143] This response was mediated by CD4$^+$ Th cells, CD8$^+$ cytolytic T cells and NK cells and resulted not only in tumor cell rejection but also in establishing long-term immune memory.[144] Importantly, rejection of poorly immunogenic tumors and well-established progressing tumors has been demonstrated when introduction of p53 was combined with inhibition of immunosuppressive activity of Treg cells and activation of innate immunity.[145] Particularly important, this tumor rejection occurred in the absence of autoreactivity to normal tissues.[145,146]

Thus, p53 inhibits tumor progression not only via induction of apoptotic cell death (an 'intrinsic' tumor suppressor mechanism), but also via stimulation of antitumor immunity (an 'extrinsic' tumor suppressor mechanism). This is important because reintroduction of p53 into every cell in a solid tumor is not currently possible. Induction of an efficient antitumor immune response should help to eliminate uninfected tumor cells and prevent relapse. Nevertheless, as a high ratio of activated immune cells to tumor cells is required for immunologic tumor destruction,[147] it is important to eliminate (kill) as many tumor cells as possible by other means. In this regard the oncolytic capabilities of a conditionally replicating adenovirus (CRAd) agent could be advantageous for this purpose.

p53 in development and differentiation

In addition to its role as a tumor suppressor and cell-cycle checkpoint control protein, p53 has been implicated as an important protein in development and differentiation.[148–151] Particularly interesting are the findings regarding p53's role in stem cell self-renewal and differentiation. It has been shown that p53 induces differentiation of mouse embryonic stem cells (ESC).[152] This role of p53 in cellular differentiation represents an alternative mechanism to maintaining genetic stability in ESCs, which are resistant to p53-dependent cell-cycle arrest and apoptosis after DNA damage.[153] Induction of the differentiation of ESCs into cell types that can undergo efficient p53-dependent cell-cycle arrest or apoptosis will result in the elimination of genetically abnormal cells. p53 also regulates asymmetric self-renewal and immortal DNA strand cosegregation—two unique attributes of adult stem cells (ASC), which have been proposed to restrict their carcinogenic potential.[154] Asymmetric self-renewal is characterized by divisions that produce a new ASC and a nonstem cell sister. Immortal DNA strand cosegregation means that at each cell division, stem cells continuously nonrandomly segregate to themselves the set of chromosomes with the oldest template DNA strands (ie, immortal DNA strands). This molecular maneuver allows ASC to significantly reduce the number of mutations which arise from unrepaired or misrepaired replication errors. Using engineered cultured cells that exhibit asymmetric self-renewal and immortal DNA strand cosegregation, Sherley and colleagues have shown that both ASC-specific functions are regulated by the p53 gene.[154] Recently, two groups demonstrated increased self-renewal of neural stem cells in mice lacking p53.[155,156] Prenatal exposure of these mice to mutagens lead to the formation of glioblastoma-like tumor in the adult supraventricular zone of the cerebrum.[155]

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Role of p53 in the development of bone and in OS pathogenesis

Bone malformations (failures in skull growth and delayed longitudinal bone growth) and a high incidence of OSs in p53 null or mutant mice suggest that p53's function may be critical during skeletogenesis.[155,157,158] Abnormalities of the p53 gene in OS occur with frequencies approaching 50% of all cases.[159] In *in vitro* studies it has been shown that p53 loss of function mutations often lead to a lack of terminal differentiation for the osteogenic lineage, and this property is regained after stable transfection of the cells with wt p53.[160] Analysis of clonal mesenchymal stem cell (MSC) populations derived from p53$^{-/-}$ mice have revealed that Runx2 and osteopontin, early and intermediate osteogenic markers, were upregulated in p53$^{-/-}$ MSC compared to wt cells during osteogenesis.[161] Interestingly, the terminal osteogenic marker gene, osteocalcin was lower in p53$^{-/-}$ MSC. Jones and colleagues have also found that p53-null osteoprogenitor cells have increased expression of Runx2. In contrast, expression of Runx2 has been lost in osteoblasts with hyperactive p53.[162] Wang *et al* have reported that osterix, another transcription factor regulating bone formation and osteoblast differentiation,[163] was upregulated in calvarial bones of p53$^{-/-}$ mice.[164] Thus, it seems, that the lack of p53 function results in enhanced early osteogenesis in MSC, but prevents terminal differentiation toward a mature osteocyte phenotype.

Particularly interesting are findings that p53-null osteoprogenitor cells have increased proliferation, increased expression of Runx2, and increased tumorigenic potential, as mice specifically p53 deleted develop OS.[162] Runx2 belongs to the RUNX (Runt-related transcription factor) family of genes which encode transcription factors that can bind and recruit a range of coactivators or corepressors (CoR) and thereby serve as orchestrators of transcription at target promoter sites.[165,166] Runx2 promotes skeletal cell differentiation from multi-potential mesenchymal cells,[167–169] and also contributes to cell growth control of osteogenic cells.[170,171] Its expression is tightly regulated by a broad spectrum of factors including (1, 25)-dihydroxyvitamin D3, ascorbic acid, bone morphogenetic protein-2 (BMP-2), transforming growth factor-β, tumor necrosis factor-α, fibroblast growth factor-2, retinoic acid and parathyroid hormone.[172] p53 functionally interact with many of these factors and lack of p53 might explain the fact that the control of Runx2 levels and its function is consistently disrupted in OS cells.[171,173] For example, p53-deficient cells display an impaired cytostatic response to TGF-β signals.[174] Also, it has been shown that Runx2 induces the growth arrest of osteoblastic cells through induction of p27^{KIP1}.[173] This function of Runx2 might be inefficient in p53-null cells, because p27 acts as a haploinsufficient tumor

suppressor,[175] but it has been shown that down-regulation of p27 is a common feature in p53$^{-/-}$ tumors.[176] From the point of view of OS pathogenesis, particularly interesting is the realization that Runx2 has a survival function,[177] and due to this ability, probably collaborates with other oncogenes in tumor development.[178] As will be discussed below, deregulation of Wnt signaling pathway in p53$^{-/-}$ osteoblasts appears to result in the upregulation of *c-myc*.

Presence of functional p53 is important for regulated expression of other factors involved in osteogenesis especially osteocalcin[179] and BMP-2.[180] Noted too are the findings about interactions between p53 and the Wnt/β-catenin signaling pathway in osteoblasts[181] in light of the emerging role of the Wnt pathway in controlling osteoblast differentiation.[182–186] The Wnt/β-catenin signaling pathway is transduced through stabilizing β-catenin protein by inhibiting glycogen synthase kinase-3-mediated β-catenin phosphorylation.[187] Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus where it displaces CoR from the Lef1/Tcf transcription factor. This, in turn, directly interacts with Lef1/Tcf and recruits transcriptional coactivators to stimulate expression of many genes, including c-*myc* and cyclin D1. Approximately 70% of OS samples displayed deregulation of β-catenin.[188]

Recently, it has been demonstrated that the inactivation of p53 in OS directly by mutation *vs* indirectly by human HDM2 amplification may have different cellular consequences with respect to the stability of the genome; mutations of p53 correlated significantly with the presence of high levels of genomic instability while inhibition of tumor suppressive properties of p53, via amplification and overexpression of HDM2, did not displayed similar high levels.[189] Also, mice bearing certain 'knock-in' p53 mutants have a tendency for development of metastasizing OS.[190,191] Studying the properties of such TP53 mutants has demonstrated their ability to bind and inhibit the function of p63 and p73. Notably, these data indicate *in vivo* differences between loss of p53 or presence of a p53 mutation in tumor development and have significant implications for therapy.[191]

Molecular profiling of chemoresistant OS

Gene expression profiling of chemotherapy-resistant OS has been recently explored using microarray technology.[192–194] These studies revealed that the chemoresistance signature includes genes related to 'bone metabolism,' 'cancer biology' and 'drug resistance'. Particularly important are the findings regarding dysregulation of TWIST1 overexpression in chemoresistant OS cells.[192] TWIST1 has been shown to affect the expression and DNA-binding ability of Runx2.[195,196] In addition, TWIST1 has been found to cooperate with MYC and MYCN to

suppress the p53-dependent apoptosis pathway,[197,198] and is involved in Taxol resistance and metastasis.[199,200] Importantly, overexpression of TWIST1 is associated with characteristics of osteoprogenitor cells that have decreased proliferation and a less mature phenotype.[201] As all of these are properties of cancer stem cells,[202,203] Lau and colleagues have suggested that chemoresistant OS cells overexpressing TWIST1 may arise from putative cancer stem cells.[192] Cells with stem cell qualities have been identified in malignancies of hematopoietic origin and in some solid tumors.[202] According to current understanding, cancer stem cells are likely to share many of the properties of normal stem cells, including relative quiescence, resistance to drug and toxins through the expression of ABC transporters, capacity for DNA repair, and resistance to apoptosis.[202] These features combined with the ability for unlimited self-renewal might explain the failure of current therapies to eradicate solid tumors. Thus, in order to cure cancer, anticancer agents must be able to kill cancer stem cells.

p53 tumor suppressor gene therapy of OS

Up until very recently only one cell line, Saos-2, or its derivatives were used in tumor suppressor gene therapy studies of OS.[204–208] This cell line has a homozygous deletion of the p53 gene and also lacks expression of the Rb gene. Reintroduction of wt p53 in Saos-2 induces cell death by apoptosis.[205] Importantly, when delivered and expressed efficiently using Ad vectors, wt p53 alone induces apoptotic death of Saos-2 cells without additional stimuli. Sensitivity to p53 proapoptotic activity therefore makes Saos-2 cells a suitable system for the analysis of certain aspects of tumor suppressor gene therapy. For example, Densmore *et al* have advantageously employed the Saos-2 cell system to study the potential of polyethyleneimine as a p53 gene delivery vehicle to OS lung metastases. Using the Saos-LM6 cell line, a metastatic derivative of the Saos-2 line, they demonstrated that aerosol delivery of plasmid DNA/polyethylenemine complexes containing either wt p53 gene or a constitutively active p53-CD(1–366) to mice with established lung micrometastases resulted in significant reduction in the numbers and size of tumors.[209] In another study Song and Boyce used Saos-2 cells to explore the use of an insect baculovirus as a vector for gene delivery.[210] They showed that infection with this vector expressing wt p53 (BV-p53) at a MOI of 100 (at which nearly 100% of cells were transduced) resulted in apoptotic cell death in about 50% of cells. When the same dose of BV-p53 was used in combination with adriamycin (a DNA-damaging chemotherapeutic drug), >95% of Saos-2 cells were killed.

Despite demonstrated suitability of Saos-2 cells for studying certain questions of OS biology, a wide panel of OS tumor cell lines would be preferable to provide more reliable information regarding the feasibility of tumor suppressor gene therapy. In this respect, in recent studies by Ganjavi *et al*[211] and Hellwinkel *et al*[212] attempts were made to explore the antitumor efficacy of tumor suppressor gene reintroduction using a panel of different human OS cell lines. Ganjavi and associates used HOS, KHOS/NP and MNNG cell lines in addition to Saos-2 cells. It should be noted that KHOS/NP and MNNG are derivatives of HOS cells. In these studies infection with Ad-p53wt at an MOI of 8 p.f.u./cell (16 p.f.u./cell for KHOS/NP) induced apoptosis in approximately half the cell population in each cell line. Introduction of p53 in these cell lines dramatically enhanced their sensitivity to DNA damaging drugs cisplatin and doxorubicin.

Hellwinkel *et al* used a panel of five human OS cell lines. Results of these studies demonstrated substantial cell death only in two of five tumor cell lines tested. Again, Saos-2 cells demonstrated highest sensitivity to wt p53; only 14% of these cells were alive after infection with Ad-p53wt at an MOI of 25. U2OS cells have also demonstrated sensitivity to wt p53 reintroduction, but a much higher MOI was required to induce cell death. Approximately 70% of U2OS cells were alive after infection with Ad-p53wt at an MOI of 25, and MOIs of 100 and 200 were required to achieve 90% cell killing. It is important to note that Ad5-based adenoviral vector transduces U2OS cells more efficiently than Saos-2 cells. The lower response of U2OS cells to wt p53 introduction might be explained by inhibition of p53 activity by Mdm2 which is overexpressed in U2OS cells. Three other OS cell lines studied (K-HOS, MG-63 and SJSA) have demonstrated diminished cell proliferation in response to Ad-p53 infection but not cell death. The results with SJSA and MG-63 cells can be explained by low efficiency of Ad transduction of these cells and overexpression of Mdm2.[213–216] The reasons for the very low apoptotic response of K-HOS cells on p53 introduction remain unknown.

To explore whether genotoxic stress could augment the response of studied OS cells to p53 introduction, Hellwinkel *et al* combined Ad-p53 infection with treatment by cisplatin or doxorubicin. Except for U2OS and Saos-2 cells, the drugs did not multiply the effects of p53 introduction. Further results by this group demonstrated that efficient induction of apoptosis in OS cells, via introduction of a wt p53 tumor suppressor gene using an Ad5 based adenoviral vector, can be achieved only in cases where OS cells express high levels of Ad5 receptors and do not express p53 inhibitors.

About 30% of OS tumors overexpress Mdm2 due to mdm2 gene amplification,[213] and, consequently, introduction of wt p53 is unlikely to be efficacious. To overcome the inhibition of p53 by Mdm2, modified p53 genes have been constructed. Specifically, Conseiller *et al*[217] generated a chimeric tumor suppressor 1 (CTS1) containing the p53 core domain

(amino acids 75–325), with the VP16 activation domain ligated to the 5′-end. They further optimized the leucine-zipper domain that was ligated to the 3′-end of the p53 core domain. This CTS1 demonstrated resistance to inactivation by Mdm2 and by the p53 mutant, H175. In a transient transfection assay with Saos-2 cells, CTS1 has shown the ability to induce apoptosis faster than wt p53 protein. To explore the antitumor potential of CTS1 in the context of Mdm2 overexpression, Bougeret et al[218] constructed an Ad vector expressing CTS1 under control of the CMV promoter (AV.CTS1). Using the human OS cell line SA1 (also known as Osa-CL) which overexpresses endogenous Mdm2, they found that AV.CTS1 induced apoptosis six-fold more efficiently than Ad-expressing wt p53 (Ad-p53) in vitro. Furthermore, induction of apoptosis was significantly higher in AV.CTS1-transduced pre-established SA1 tumors in nude mice compared to Ad-p53-treated tumors. In contrast, AV.CTS1 induced cell-cycle arrest and cell death less efficiently than Ad-p53 in tumor cells harboring nonfunctional p53. Results of studies with CTS1 have shown that modified p53 can be more efficient than wt p53 in induction of apoptosis in cells with high levels of Mdm2.

A different approach was used by Tang et al[219] to overcome the inhibition of p53 by Mdm2. Previous work had established that the hydrophobic residues Leu-14 and Phe-19 of p53 were crucial for the interaction between p53 and Hdm2.[32] To study proapoptotic properties of p53 with amino-acid substitutions at Leu-14 and Phe-19, Lin and colleagues infected cells with different levels of Mdm2 with an adenoviral vector expressing a modified p53 gene (Ad-p53 14/19).[219] They found that in HT1080 cells (low Mdm2), Ad-p53 14/19 induced apoptosis at a level similar to Ad-wtp53. In human OS SJSA cells (high Mdm2) Ad-p53 14/19 induced dramatically more apoptosis than Ad-wtp53 (44 vs 11%). Also, infection of SJSA cells with Ad-p53 14/19 sensitized them to doxorubicin and cisplatin. After exposure of SJSA cells to 0.1 μg ml^{-1} of doxorubicin or 5 μg ml^{-1} cisplatin only 4 and 24%, respectively, were apoptotic. Combined treatment of SJSA cells with Ad-wtp53 plus doxorubicin or cisplatin at the same doses induced apoptotic cell death only in 15 or 33% of cells, respectively, demonstrating continued resistance. In contrast, Ad-p53 14/19 plus doxorubicin or cisplatin generated apoptosis in 63 or 80% of SJSA, respectively. Thus, the studies of Bougeret et al and Tang et al demonstrated that abrogation of the Mdm2 binding site in p53 results in augmentation of its proapoptotic activity in OS cells with a high level of Mdm2 expression comparing to wt p53.

p53 in the context of CRAd agents (armed CRAds)

The anticancer potency of p53 in the context of a nonreplicative Ad vector is limited because the vector infects only a small fraction of the cells within a tumor. Employment of a replication-competent Ad vector has the potential of transgene delivery to significantly more cells via repeated cycles of infection, cell lysis, and virus spread to neighboring cells.[220,221] Also, replication-competent Ad vectors have the potential to increase transgene expression up to 1000-fold in each infected cell, compared with a nonreplicating Ad. However, p53 expression might be deleterious for viral replication due to induction of preliminary apoptosis. Also, spreading of the Ad vector through the tumoral mass might be blocked due to induction of the interferon system by p53.[16] Alternatively, several Ad5 early gene products oppose p53 function. For example, adenoviral E1B-55-kDa protein binds and inactivates p53.[222] The E1B-55-kDa protein and another Ad protein E4orf6 together mediate the ubiquitination and degradation of p53.[223–226] Other Ad proteins can indirectly interfere with p53 activity. The E1A gene products antagonize p53 activity and this correlates with its ability to interact with p300 and the p400/TRRAP protein complex.[227–229] The E1B-19-kDa protein inhibits the downstream effects of p53 by blocking induction of apoptosis.[230,231] In contrast, the Ad5 E3 region encodes the adenoviral death protein which is cytotoxic but, owing to strong expression only at very late stages of viral infection, is not deleterious for viral production and promotes lysis of infected cells.[232]

Therefore, expression of p53 at late stages of the viral life cycle might be not deleterious to virion progeny production. To explore this strategy, Southoff et al[233] constructed a replicating Ad vector (Adp53rc) that contains the p53 cDNA inserted into the adenoviral fiber transcription unit, using an internal ribosomal entry site and demonstrated that the expression pattern of the p53 protein correlated with the late expression pattern of the adenoviral fiber protein. Adp53rc demonstrated improved oncolytic properties compared to the control Ad vector (Ad-co) which had the same genetic backbone, but lacked the p53 gene. Despite this, Adp53rc induced apoptotic cell death only in 10% of infected cells, compared with 5% induced by infection with Ad-co. Also, expression of the p53 target genes p21 and bax were not detected despite strong nuclear expression of p53. Thus, p53 caused no obstacles to viral replication but p53's properties as a transcriptional activator were significantly inhibited by Ad.

It is possible that despite the Ad genes which oppose p53 functions (which are strongly expressed at early stages of viral replication) low concentrations of these proteins might be sufficient to inhibit p53-dependent transactivation at late stages of infection. The E1B-55-kDA protein deserves special attention in this regard. It has several activities that inhibit p53 function.[234] According to this model E1B-55K inhibits activation by p53 by binding a repression domain to it. Importantly, E1B-55K increases p53 stability by binding to the same region

in the amino-terminal domain of p53 bound by Mdm2.[32] Also, p53 bound to E1B-55K has a 10-fold higher affinity for its binding site than free p53.[235] As a result, p53 is converted from an activator into a constitutive repressor of genes with p53-binding sites. The stabilization of p53 by E1B-55-kDa protein in the absence of E4orf6 protein leads to a significant increase in p53 concentration.[236] Thus, E1B-55K will significantly limit p53 tumor suppressor activity in the context of replication competent Ad vector. To prevent such inhibition, modified p53 might be used instead of wt p53. For example, Dobbelstein and colleagues found that replacement of the five amino acid residues at position 24–28 by the homologous residues from p73 resulted in complete resistance of chimeric p53 (p53mt24–28) to E1B-55K- and E4orf6-mediated degradation.[225] This chimeric p53 when expressed in the context of a first-generation Ad vector demonstrated resistance to degradation by Ad proteins in cells coinfected with wt Ad.[237] Importantly, chimeric p53 was capable of inducing such p53 target genes as *p21/CDKN1A*, *mdm2* and *bax*, regardless of the infection with replicative viruses.[237,238] Interesting, even strong overexpression of p53mt24–28 allowed the virus to replicate as efficiently as when it was absent. These findings demonstrate that employment of modified p53 in the context of a replication-competent Ad vector is a feasible approach.

The efficiency of p53 in the context of CRAd agents on OS cell lines has been studied by Gerritsen and colleagues.[215,239] To restrict Ad replication to tumor cells, amino acids 122–129 in the conserved region 2 domain of E1A (necessary for binding to the Rb protein) were deleted. Using a crystal violet assay, van Beusechem *et al* found that a CRAd agent expressing wt p53 (AdΔ24-p53) exhibited enhanced oncolytic capacity compared with a control vector (AdΔ24) in killing OS cells with low levels of Mdm2. AdΔ24-p53 was ∼100-fold more potent than AdΔ24 against U2OS cells and 10-fold more potent against Saos-2 cells, but had no advantage when examined in MG-63 and MNNG-HOS cells, which express high levels of Mdm2. In contrast, a CRAd agent expressing a p53 variant resistant to Mdm2-mediated degradation (AdΔ24-p53(14/19)) was ∼10 times more effective than either AdΔ24 or AdΔ24-p53 in killing Mdm2-high cells. However, AdΔ24-p53 (14/19) was less effective than AdΔ24-p53 against Mdm2-low cells. It should be noted that the low Coxsackie and Adenovirus Receptor (CAR) and high-Mdm2 MG-63 OS cell line was highly resistant to all CRAd agents tested and required 100 p.f.u./cell or more of AdΔ24-p53 (14/19) to be eradicated. Thus, significant improvements in CRAd agents are needed to efficiently eliminate OS tumors with high levels of Mdm2. In this regard, it seems that a p53 variant resistant only to Mdm2 is not an optimal alternative to p53 in a CRAd context. As E1B-55-kDa protein binds residues 23–27 of p53,[32] p53(14/19) is still a candidate construct for inhibition and degradation. It is tempting to speculate that substitution of amino-acid residues 14 and 19 of p53 combined with replacement of residues at position 24–28 by the homologous residues from p73 might result in resistance of chimeric p53 to degradation by Mdm2 and Ad proteins. Such modified proteins should still retain the capability to transactivate p53-responsive genes. Also, capsid modification of Ad vector to target OS tumors expressing low levels of CAR receptors should enhance the oncolytic potential of CRAd agents.

Enhancement of transduction efficiency of OS cells with Ad vectors

Adenoviral vectors often transduce tumor cells inefficiently due to low levels of adenovirus receptor molecules on these cells. The adenovirus enters cells in two separate steps. First, the knob domain of the adenovirus fiber binds the primary attachment receptor. Specifically, the Ad5 and Ad2 viruses whose genetic backbone is mostly used for adenoviral vector construction bind to the CAR.[240] The second step is internalization into the target cell which is mediated by an interaction of the Ad virion with a_vb_3 and a_vb_5 integrins.[241] The transduction efficiency of Ad is known to correlate with levels of primary and secondary receptors on the cell surface.[242,243] It has thus been recognized that improved tumor cell transduction could be achieved via redirection of the adenoviral vector to non-CAR cellular entry pathways in CAR-deficient neoplastic cells.[244,245] This understanding has resulted in the generation of Ad vectors possessing augmented tumor cell transduction properties.[246–249]

A substantial fraction of OS tumors contain cells expressing low levels of CAR and have thus demonstrated resistance to conventional Ad vectors.[250,251] Witlox *et al*[252] observed that primary OS tumor cells express high levels of integrins a_vb_3 and a_vb_5, and generated an adenoviral vector containing an Arg-Gly-Asp (RGD-4C) integrin-targeting motif (AdCMVlucRGD) in the fiber knob. They showed that this retargeted Ad vector transduces OS cells about 100-fold more efficiently than unmodified Ad5. Testing a panel of Ad vectors recently generated in our lab, we found that replacement of the human knob domain of the Ad5 fiber with knob domains of canine adenoviruses CAV1 and CAV2 resulted in an augmentation of transduction efficiency of human OS cells (V Ternovoi, unpublished data, 2005).[253,254] Particularly interesting are results with a fiber mosaic Ad5 vector encoding two fibers: the $\sigma 1$ and the wt Ad5 fiber.[255] This mosaic transduces some primary OS cell lines even more efficiently than AdCMVlucRGD (V Ternovoi, unpublished data, 2005).

Recently, selective targeting of cognate antigen has been achieved via genetic incorporation of a cytosol stable single chain fragments of antibody (scFv),

known as an intrabody, into a deknobbed Ad fiber.[256] In this regard, a scFv derived from the monoclonal antibody 8H9 (MAb 8H9) could be an excellent candidate for specific targeting of OS cells. The antigen epitope recognized by the 8H9 mouse antibody is a putative cell surface glycoprotein expressed on cell membranes in a broad spectrum of tumors of neuroectodermal, mesenchymal and epithelial origin, with restricted distribution in normal tissues.[257] Importantly, 28 of 29 human OS have tested positive with 8H9 by immunohistochemistry. Recombinant immunotoxin containing the 8H9 scFv selectively killed cells that react with the MAb 8H9 and produced regression of human cancers growing in severe combined immunodeficient mice that express the 8H9 antigen.[258] Particularly important, the dose that caused significant regression in mice was well tolerated in monkeys.

Animal models

The immune system may play an important role in the realization of p53's overall antitumor potential. As has been discussed above, p53 overexpression in the context of viral vectors can induce a powerful antitumor response. This response can even result in the rejection of well-established tumors, but only in the context of immunomodulation strategies aimed at overcoming the tolerogenic activity of the tumor microenvironment. These results are encouraging, but they have been achieved using animals with experimentally established tumors. Spontaneous tumors present a significantly more complex challenge for induction of an efficient immune response.[259] They are engaged in the process of successfully fighting against host defense systems and are comprised of a population of heterogenous tumor cells and recruited host stromal cells. Stromal cells, including fibroblasts, inflammatory cells, and vascular cells, generate a microenvironment that actively fosters tumor growth and prevents an immune response against these same tumor cells.[260–266] Together, the heterogenous tumor cell populations, shaped by the immune system in the process of multistep progression,[267,268] and considering the tumor microenvironment which actively induces the status of immune tolerance,[269] allow exceptional establishment of spontaneous tumors, which experimentally established tumors cannot reproduce. Thus, (1) generation of an efficient antitumor immune response will be much harder to achieve with naturally occurring tumors and (2) to get clinically relevant information regarding therapeutic efficacy of p53 tumor suppressor gene therapy, its preclinical evaluation should be carried out using immunocompetent animals with spontaneously developed OS.

An immunocompetent animal model could also be helpful in finding a solution for the problem that results from high immunogenicity of Ad vectors. Adenoviruses induce a potent immune response which significantly reduces efficiency of Ad-based vectors.[270,271] Ablation of immune response have been shown to facilitate oncolytic virus intratumoral propagation.[249,272,273] Consequently, immunosuppression would be beneficial for CRAd intratumoral spreading and expression of p53 in this context should result in a significantly more potent tumor cell killing via apoptogenic activity of p53. In contradistinction, a realization exists that the tumor suppressor effect of p53 relies on the induction of an antitumor immune response. Thus immunosuppression should diminish antitumor potential. One of the strategies to solve this problem might be induction of temporal immunoablation in the first phase of treatment followed by immunostimulation to achieve an active Th1 immune response against tumor antigens. In this scenario, the Ad vector will get opportunity to achieve tumor penetration and spread, kill a significant fraction of tumor cells, and subsequent administration of immunopotentiating agents with Th1-polarizing activity would augment an antitumor response.

Canine OS bears striking resemblance to human OS,[274,275] and in our hands and others appears to be a useful animal model for testing gene therapy approaches because dogs share many physiological features with humans.[276] Greatly accelerated kinetics of tumor growth and progression in dogs relative to humans creates the opportunity to assess treatment outcomes in a reasonable time frame. To study host–vector interactions and to develop strategies facilitating Ad vector intratumoral replication in the face of an active immune system, we generated a CRAd agent based on the canine adenovirus type 2 genome.[277] Interestingly, we have also found that human adenovirus type 5 can productively infect canine tumor cells of different tissue origins. Furthermore, we provided evidence of Ad5 lateral spreading in primary canine OS cells.[278] These findings raise the probability of exploiting canine OS as a model for preclinical analysis of candidate Ad5 based vectors designed for human OS gene therapy.

Concluding remarks and future directions

OS is often resistant to conventional multimodal therapy for malignant disease. Expression profiling of chemoresistant OS has results in partial unmasking of its resistance signature. The gene expression profile and other properties of resistance to therapy closely resemble properties of cancer stem cells. Stem cell populations have been identified in a range of hematopoietic and solid tumors, and the capacity of these cells for unlimited self-renewal and intrinsic resistance to cell death is now recognized as important mechanisms of tumor resistance to current therapy.

Adenovirus-mediated p53 tumor suppressor gene therapy is a newer treatment modality with high antitumor potential. In fact, this is a combined

modality which employes several antitumor mechanisms: p53 'intrinsic' and 'extrinsic' tumor suppressor mechanisms and cytolytic activity of replication competent Ad. However, analysis of the peer-reviewed literature revealed that wt p53 and currently used Ad vectors may not be efficient tools for OS treatment because OS cells generally overexpress inhibitors of wt p53 and express low levels of the CAR receptor. Recent advances in understanding the molecular mechanisms of p53's proapoptotic action suggest that cointroduction of a modified p53 gene resistant to inhibitors (Mdm2 and adenoviral protein E1B-55K) with the PTEN gene into tumor cells should result in efficient apoptosis induction in these tumor cells with a high apoptotic threshold. The clinical relevance of this therapeutic approach will be higher if infection of a significant fraction of the tumor cells can be achieved. Temporal immunoablation to facilitate CRAd agent tumor targeting and intratumoral spreading with subsequent immunopotentiation to overcome the immunosuppressive activity of the tumor microenvironment, while boosting the immune response against tumor antigens, presents one possible solution to this problem.

The complexity of factors influencing the final outcome of this new modality and its reliance on immune mechanisms requires employment of an adequate model, and canine OS may represent the ideal preclinical model for validation of adenovirus-mediated p53 tumor suppressor gene therapy. Further insights into the mechanisms of tumor progression, tumor–stroma interactions and tumor–immune system relationships, along with p53's biology, host–vector and virus-cell signal transduction interplay will result in more rationally designed therapeutic strategies with increased clinical benefits for OS patients.

Acknowledgements

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