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Mechanisms of Prostate Cancer Cell Survival After Inhibition of AR Expression

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ABSTRACT

Recent reports have shown that the AR is the key determinant of the molecular changes required for driving prostate cancer cells from an androgen-dependent to an androgen-independent or androgen depletion-independent (ADI) state. Several recent publications suggest that down-regulation of AR expression should therefore be considered the principal strategy for the treatment of ADI prostate cancer. However, no valid data is available about how androgen-dependent prostate cancer cells respond to apoptosis-inducing drugs after knocking down AR expression and whether prostate cancer cells escape apoptosis after inhibition of AR expression. This review will focus on mechanisms of prostate cancer cell survival after inhibition of AR activity mediated either by androgen depletion or by targeting the expression of AR by siRNA. We have shown that knocking down AR expression by siRNA induced PI3K-independent activation of Akt, which was mediated by calcium/calmodulin-dependent kinase II (CaMKII). We also showed that the expression of *CaMKII* genes is under AR control: active AR in the presence of androgens inhibits *CaMKII* gene expression whereas inhibition of AR activity results in an elevated level of kinase activity and in enhanced expression of *CaMKII* genes. This in turn activates the anti-apoptotic PI3K/Akt pathways. CaMKII also express anti-apoptotic activity that is independent from the Akt pathway. This may therefore be an important mechanism by which prostate cancer cells escape apoptosis after androgen depletion or knocking down AR expression. In addition, we have found that there is another way to escape cell death after AR inhibition: DNA damaging agents cannot fully activate p53 in the absence of AR and as a result p53 down stream targets, for example, microRNA-34, cannot be activated and induce apoptosis. This implies that there may be a need for re-evaluation of the therapeutic approaches to human prostate cancer. J. Cell. Biochem. 106: 363–371, 2009. © 2008 Wiley-Liss, Inc.

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Several reviews have recently been published that analyze the molecular mechanisms of apoptotic signaling in cancer [Plati et al., 2008], molecular regulation of androgen action in prostate cancer [Dehm and Tindall, 2006], and death receptor-induced cell death in prostate cancer [Guseva et al., 2004]. Therefore, we will not describe here the apoptotic cascade (e.g., extrinsic and intrinsic pathways, pro- and anti-apoptotic factors, etc.), the role of AR and its multiple co-regulators in prostate cancer cell survival, or the progression of prostate cancer from an androgen-dependent to an androgen depletion-independent (ADI) state. This review will focus on mechanisms of prostate cancer cell survival after inhibition of AR activity, mediated either by androgen depletion or by targeting the expression of AR by siRNA. These mechanisms involve PI3K-independent activation of Akt and inhibition of the p53/microRNA-34 pathway.

APOPTOSIS, ANDROGEN REGULATION, AND PROSTATE CANCER

Apoptosis or programmed cell death is a cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues and to eliminate unneeded or aging cells as an organism develops. Apoptosis is physiologically normal and an important process for multicellular organisms [Ellis et al., 1991]. However, inappropriate apoptosis is implicated in many human diseases, including cancer [Evan and Vousden, 2001].

Many different factors contribute to the development of prostate cancer including somatic mutations of AR or AR amplification. Some mutations in the AR result in altered ligand specificity of the AR permitting activation by non-androgenic steroid hormones or

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even by anti-androgens. AR amplification with concomitant over-expression of AR can increase the sensitivity of prostate cancer cells to low levels of androgens that eventually result in the development of androgen-independent prostate cancer [Linja et al., 2001]. Recent reports have shown that the AR is the key determinant of the molecular changes required for driving prostate cancer cells from an androgen-dependent to an androgen-independent or ADI state [Chen et al., 2004; Isaacs and Isaacs, 2004; Roy-Burman et al., 2005]. There are also other mechanisms for developing an ADI state including cross-talk between the AR and other signal transduction pathways, alterations in the expression of steroid co-activators and co-repressors, and androgen-independent mechanisms [Isaacs and Isaacs, 2004]. Several recent publications suggest that down-regulation of AR expression should be considered the principal strategy for the treatment of ADI prostate cancer. In three separate studies, knocking down AR by shRNA or siRNA in AR-positive cell lines resulted in apoptotic death [Liao et al., 2005; Yang et al., 2005; Cheng et al., 2006]. These data show that inhibition of AR expression is in itself sufficient to induce cell death in AR-positive cells.

As has been reviewed by Litvinov et al. [2003] androgen ablation therapy is not curative because of the accumulation of molecular changes inducing gain of function in the AR signaling pathway that results in activation of novel AR-dependent signaling pathway but without requiring androgen ligand binding. It has also been recently shown that AR possesses an intrinsic androgen-independent transcriptional activity [Huang et al., 2002] and this ligand-independent activity was observed when AR was highly expressed. The novel mechanism to generate a constitutively active AR that mediates prostate cancer therapy resistance was recently discovered by Dehm et al. [2008]. The authors found that three AR isoforms are expressed in androgen-independent 22Rv1 cell line: a full-length version with duplicated exon 3 and two truncated versions lacking the COOH-terminal domain (CTD). These CTD-truncated isoforms were found to be encoded by mRNAs that have a novel exon 2b at their 3′ end. These isoforms are constitutively active and promote the expression of AR-dependent genes and proliferation of 22Rv1 in a ligand-independent manner. Importantly, AR mRNA containing exon 2b and their protein products are expressed in other prostate cancer cell lines as well as in xenografts of therapy-resistant prostate cancer. Importantly, the authors have identified an effective mechanism by which prostate cancer can synthesize a constitutively active AR.

The role of androgen action in prostate cancer including androgen regulation of proliferation and apoptosis has recently been reviewed by Dehm and Tindall [2006]. We will discuss here only one question related to apoptosis in prostate cancer: the different role of caspase-3 and caspase-7 in prostate cancer apoptosis. Marcelli et al. [1998] showed that caspase-7, not caspase-3, was activated in lovastatin-induced apoptosis of LNCaP. We showed that Fas-mediated apoptosis in prostate cancer cell lines activated caspase-7, but not caspase-3 [Rokhlin et al., 1998]. These data were puzzling for a long-time since the widespread view was that these caspases occupy functionally redundant roles in apoptosis. However, Walsh et al. [2008] has recently shown that the enzymatic activities of caspase-3 and caspase-7 clearly differed toward natural substrates and therefore these caspases occupy non-redundant roles within cell death pathways. The nature of the caspase-7 preferable substrate(s) in prostate cancer cells remains to be investigated.

THE ROLE OF p53 IN PROSTATE CANCER APOPTOSIS

p53 is a regulator of genotoxic stress that plays an important role in DNA damage response, DNA repair, cell cycle regulation, and in triggering apoptosis after cell injury. p53 regulates the expression of a variety of apoptosis-related genes that affect both the intrinsic (Bax, Noxa, Puma, Bid, Bcl-2, Bcl-XL) and extrinsic (Fas, TRAIL-R2, PIDD, DcR1, DcR2) pathways [reviewed by Reed, 2003]. Moreover, p53 is the most commonly mutated gene in human malignancy, which makes p53 a major potential target for gene-specific therapy of cancer [Vogelstein et al., 2000]. A fundamental role of p53 in prostate cancer progression was found in the mouse prostate reconstitution (MPR) model [Thompson et al., 1995]. Prostate cancer was identified in 100% of heterozygous and homozygous p53 mutant MPRs with metastatic deposits in 95% of the mice, but no metastasis were found in wild-type p53 mice. In addition, the authors noted that the pattern of metastasis was remarkably similar to that in human prostate cancer. It is noteworthy that in many instances the lack of p53 mutations does not mean that p53 is functionally active in tumors. For example, in neuroblastomas, p53 is sequestered in the cytosol [Moll et al., 1996], and in sarcomas and cervical carcinomas it is inactivated by Mdm2 and E6 proteins, respectively [Miller et al., 1996; Rapp and Chen, 1998]. It remains unclear in prostate cancer whether p53 is functionally active and how it affects the biology of these tumors.

In our own work, we switched off p53 function by transfection of LNCaP with genetic suppressor element 56 (GSE-56) that resulted in the generation of the LN-56 subline. This subline differs from LNCaP by only a single genetic alteration specifically targeting the function of p53. The established system allowed us to demonstrate the involvement of p53 in TNF-α-induced apoptosis and to investigate the mechanisms of p53-dependent apoptosis [Rokhlin et al., 2000]. LN-56 cells became resistant to TNF-α and this resistance was accompanied by the lack of p21/WAF1 up-regulation that could be responsible for the observed effects of p53 suppression on TNF-α-dependent apoptosis. We also observed p53-dependent elevation of p21/WAF1 levels that was accompanied by caspase-dependent p21/WAF1 proteolysis during TNF-α-mediated apoptosis in LNCaP, but not in LN-56.

It has been reported that 80–85% of tumor-derived alterations in the p53 gene are missense point mutations localized within the DNA-binding domain of the protein [Soussi et al., 2000]. The majority of p53 mutant tumors express a single mutant allele of p53 (see p53 mutation database: http://www.iarc.fr/p53/Index.html). However, p53 mutation databases contain several cases in which two different p53 mutations are co-expressed in one tumor. The biological significance of co-expression of two different mutants in one tumor has not been analyzed and became the subject of our work [Gurova et al., 2003]. As a model, we chose the prostate cancer cell line DU145 that is resistant to Fas-mediated apoptosis [Rokhlin

et al., 1997] and carries different mutations in the two alleles of p53, resulting in the co-expression of two mutant proteins with different amino acid substitutions (Pro to Leu in 223, and Val to Phe in 274 codons [Issacs et al., 1991]. We characterized the biological properties of each of the DU145-derived mutants in comparison to wild-type p53 after transduction into several p53-deficient cell lines, separately and in combination, so we could investigate whether there is an isoform of co-operation between the mutants creating additional selective advantages to cancer cells. Both mutants showed some properties that closely resemble wild-type p53. However, these relatively ''weak'' mutants create a p53 protein with new structural and functional properties when co-expressed in one cell. Synergism of the DU145-derived mutants was revealed in their effect on cell sensitivity to Fas-mediated apoptosis, the most unusual property of these mutants. Whereas each of them alone had some, albeit weak, suppressive effect on the Fas sensitivity of transduced prostatic cell line PC3, that is itself p53-negative and Fas-sensitive, the combination of p53-274(Phe) and p53-223(Leu) caused a strong anti-Fas effect possibly acting by down-regulating Fas receptor (FasR) expression. Two major conclusions of this work are the generation of p53 protein with new properties from a combination of two rather weak mutants and the strong inhibition of Fas-mediated apoptosis by the resulting p53 protein, providing an unusual example of gain-of-function that presumably results from the combination of two mutant p53 subunits in one protein.

In addition to the role of p53 in TNF- and Fas-mediated apoptosis in prostate tumors, p53 apparently regulates the expression of prostate specific antigen (PSA) [Gurova et al., 2002]. By using cDNA microarray gene expression profiling, we found a fourfold increase in PSA mRNA levels in LNCaP after suppression of p53. The p53 pathway was suppressed by a dominant-negative p53 mutant (GSE-56). Consistently, p53 suppression either by GSE-56 or by dominant-negative mutant p53-175His caused a four- to eightfold increase in secretion of PSA protein in culture medium, suggesting that PSA gene expression is under negative-control of p53. Since LNCaP is considered a meaningful in vitro model of hormone-dependent prostate cancer, we can presume that the results obtained in these cells may reflect regulation of PSA in naturally occurring tumors. Thus, it appears that one of the most useful diagnostic prostate tumor markers is, in fact, a tissue specific indicator of p53 inactivation in prostate cells. Being dependent on p53 inactivation, elevated production of PSA may therefore be indicative for the ongoing selection of p53-deficient cell variants with the broken control of apoptosis. In fact, the loss of functional p53 by LNCaP is accompanied not only by elevated PSA secretion but also by acquisition of high tumorigenecity and resistance to TNF-α-mediated apoptosis [Rokhlin et al., 2000].

THE ROLE OF Akt IN PROSTATE CANCER

The serine/threonine kinase Akt is an important regulator of cell proliferation and survival. Akt has a wide-range of cellular targets, and the oncogenicity of Akt arises from activation of both proliferative and anti-apoptotic signaling [Chan et al., 1999]. Furthermore, Akt contributes to tumor progression by promoting cell invasiveness and angiogenesis. These observations establish Akt as an attractive target for cancer therapy [Graff, 2002]. Since inhibition of Akt activity induces apoptosis in a range of mammalian cells, Akt inhibition may be effective, in combination with other anticancer drugs, in the treatment of tumors with other mutations [Hill and Hemmings, 2002]. For example, Chen et al. [2001] showed that modulation of Akt activity by pharmacological or genetic approaches alters the cellular responsiveness to TRAIL. We also observed that TRAIL can transiently activate caspase-8 in LNCaP but does not induce apoptosis. Inhibition of the PI3K/Akt pathway by wortmannin resulted in caspase cascade activation, caspase-dependent proteolysis of p21/WAF1, MDM2, and Akt itself, and led to cell death [Rokhlin et al., 2002].

Akt is activated via the PI3K pathway that has emerged as a critical pathway for cell survival in prostate cells. Expression of all three Akt isoforms has been found in normal prostate and tumors [Zinda et al., 2001]. Androgen withdrawal results in an increase of PI3K/Akt pathway activity, which supports survival after androgen ablation [Murillo et al., 2001]. We have also shown that androgen deprivation increased the levels of Akt protein and phospho-active Akt, and that this could be reversed in the presence of dihydrotestosterone (DHT) [Rokhlin et al., 2002]. These data indicate that the regulation of Akt activity is androgen-dependent. It has been shown that Akt phosphorylates the androgen receptor at Ser-210 and Ser-790 and results in suppression of androgen receptor transactivation [El-Deiry, 2001]. Taken together, these data suggest that there is the cross-talk between these two signaling pathways and that it is finely regulated. Importantly, after androgen deprivation or inhibition of AR expression by siRNA AR, inhibitors of PI3K/Akt pathway are ineffective in inducing apoptosis [Rokhlin et al., 2007], which we will discuss in the next section.

The role of different PI3K isoforms in androgen-induced AR transactivation and gene expression has recently been investigated [Zhu et al., 2008]. Using a gene-specific siRNA approach, the authors determined that the regulatory isoform p85α and the catalytic isoform p110β were required for androgen-stimulated AR trans-activation and cell proliferation in prostate cancer cells. Over-expression of p110β led to androgen-independent AR transactivation whereas silencing of p110β gene abolished tumor growth. Importantly, gene expression analysis of clinical specimens showed that both p85α and p110β were highly expressed in malignant prostate tissues and their expression levels correlated with disease progression. These data indicate that p85α and p110β aberrant expression or activation might play an important role in prostate cancer progression.

MECHANISMS OF PROSTATE CANCER CELL SURVIVAL AFTER AR INHIBITION

It has been reported that the AR is the key determinant for the molecular changes required for apoptosis resistance and driving prostate cancer cells from an androgen-dependent to an ADI state [Dehm and Tindall, 2006]. Several recent publications suggest that down-regulation of AR expression should therefore be considered the main strategy for the treatment of ADI prostate cancer [Chen

et al., 2004; Liao et al., 2005; Yang et al., 2005; Cheng et al., 2006]. However, no valid data is available about how androgen-dependent prostate cells respond to apoptosis-inducing drugs after knocking down AR expression and whether prostate cancer cells escape apoptosis after inhibition of AR expression. We have recently shown that knocking down AR expression by siRNA induced PI3K-independent activation of Akt, which was mediated by calcium/calmodulin-dependent kinase II (CaMKII). We also showed that the expression of *CaMKII* genes is under AR control: active AR in the presence of androgens inhibits *CaMKII* gene expression whereas inhibition of AR activity results in an elevated level of kinase activity and in enhanced expression of *CaMKII* genes [Rokhlin et al., 2007]. This in turn activates the anti-apoptotic PI3K/Akt pathway. CaMKII also express anti-apoptotic activity, which is independent from Akt pathway. This may therefore be a mechanism by which prostate cancer cells escape apoptosis after androgen depletion or knocking down AR expression. In addition, we also found that there is another way to escape cell death after AR inhibition: DNA damaging agents, for example, doxorubicin (DOX) and camptothecin, cannot fully activate p53 in the absence of AR and as a result p53 down stream targets cannot be activated and induce apoptosis [Rokhlin et al., 2008].

ROLE OF CALCIUM/CALMODULIN-DEPENDENT KINASE II (CAMKII) IN PROSTATE CANCER CELL SURVIVAL

We have previously shown that androgen deprivation increased the level of phospho-active Akt and suppressed apoptosis [Rokhlin et al., 2002; Guseva et al., 2004]. However, we did not understand why wortmannin converted LNCaP from apoptosis-resistant to -sensitive in steroid-containing medium (FCS) but does not do so in steroid-free media (SFC). The results of our study [Rokhlin et al., 2007] provide an explanation: when maintained in SFC cells exhibit an increase in CaMKII activity, an important and previously unrecognized mediator of the anti-apoptotic response in prostate cancer. Furthermore, we found that KN-93, an inhibitor of CaMKII, decreased the expression of AR, and the anti-apoptotic Bcl-2 family member Mcl-1 (inhibitors of PI3K/Akt pathway did not change Mcl-1 expression), whereas KN-93 increased the expression of p53 and pro-apoptotic protein PUMA (manuscript in preparation). These data indicate that CaMKII can inhibit apoptosis not only by activating Akt and also by another pathway(s) that does not overlap with Akt activity.

CaMKII is encoded by four separate genes (α, β, γ, and δ). The product of these genes can be alternatively spliced leading to the production of 24 different proteins [reviewed in Rodriguez-Mora et al., 2005]. Every cell type has at least one isoform of CaMKII with some cells expressing multiple isoforms. The general structure of CaM-kinases includes a N-terminal kinase domain, followed by an auto-inhibitory domain, an overlapping CaM-binding domain, and in the case of CaMKII, a C-terminal association domain responsible for multimerization. All members of the CaM-kinase family are activated in response to the binding of calcium to calmodulin. Upon calcium/calmodulin binding, CaMKII undergoes auto-phosphorylation in the pseudosubstrate domain of the kinase: one subunit phosphorylates the neighboring subunit on Thr286 in the auto-inhibitory domain and both subunits must be bound to Ca^{2+}/CaM in order for this phosphorylation to occur. Once phosphorylated in this manner, the pseudosubstrate domain is unable to interact with the kinase domain and CaMKII activity becomes independent of calcium/calmodulin binding. Because the phosphorylation allows CaMKII to be independent of Ca^{2+}/CaM, dephosphorylation of CaMKII must occur to return to an inactive state. Both protein phosphatases 1 and 2A (PP1 and PP2A) appear to play important roles in the dephosphorylation of CaMKII. There are few studies investigating the role of CaMKII in cancer, including in apoptosis and tumor progression. [Tombes and Krystal, 1997; Yang et al., 2003; Xiao et al., 2005; Song et al., 2006]. There is only one article that has a remote link between CaMKII and prostate cancer [Wang et al., 2003].

It has recently been shown that a RNAi approach was effective in knocking down AR expression in prostate cancer cells. This treatment resulted in reduced cell growth and apoptosis [Chen et al., 2004; Liao et al., 2005; Yang et al., 2005; Cheng et al., 2006]. To investigate the mechanism of cell death after treatment with AR siRNA, we designed and synthesized a panel of 14 siRNAs against the human AR gene and selected the most potent siRNA for further studies. We observed that measurable levels of caspase activation and cell death were seen only after seven days of knocking down AR expression whereas treatment of LNCaP with inhibitor of PI3K/Akt pathway, wortmannin, in combination with TNF-α or TRAIL induced high-levels of caspase activity after 24–48 h of treatment. We then showed that knocking down AR expression by siRNA decreased caspase activity after treatment with wortmannin in combination with TRAIL. These data suggest that inhibition of AR activity activates a kinase(s) that can activate Akt by a PI3K-independent pathway. The main point of these experiments was that AR siRNA can itself induce caspase activity and cell death, and that AR siRNA acts as anti-apoptotic agent when LNCaP is treated with wortmannin [Rokhlin et al., 2007]. The explanation of these unusual data came from experiments that showed that inhibition of AR activates CaMKII, which activates (phosphorylates) Akt by a PI3K-independent pathway [Rokhlin et al., 2007].

We have shown that expression of *CaMKII* genes is under AR control. Further, Akt-Thr308 was protected from wortmannin's effect in CaMKII transfectants, and CaMKII activity was increased after treatment with AR siRNA and after culturing of LNCaP in SFC. Importantly, after culturing in SFC the effect of KN-93 in combination with other drugs was more than two times higher compared to wortmannin. Therefore, after androgen deprivation KN-93 was a more potent inducer of cell death than wortmannin when they were used in combination with thapsigargin, TRAIL, and DOX. These data emphasize the importance of CaMKII activity in apoptosis resistance in SFC. Although our data clearly show that expression of *CaMKII* genes are under AR control, we do not yet have a mechanistic understanding of how this is accomplished. One anti-apoptotic pathway utilized by CaMKII is inhibition of caspase expression at the procaspase level and inhibition of caspase activation. Over-expression of CaMKII resulted in inhibition of procaspase-7 and procaspase-8 expression. Both these caspases play

a crucial role in apoptosis in LNCaP. Therefore, the decreased levels of an initiator caspase (8) and an executioner caspase (7) should deprive cells of their ability to execute apoptosis. Further, over-expression of CaMKII prevents or diminishes the activation of caspase-2, -7, and -8. The inhibition of caspase-2 is of special interest. We have previously shown that caspase-2 expression is regulated by AR [Rokhlin et al., 2005]. Caspase-2 is the most unusual member of the caspase family since it has features of both an initiator as well as an executioner caspase. Another distinguishing feature of caspase-2 is its ability to localize in nuclei and participate in the late stages of apoptosis. It has been shown that caspase-2 plays a prominent role in different cell death systems [reviewed by Degterev et al., 2003] including the direct release of apoptogenic factors from mitochondria [Lassus et al., 2002]. Caspase-2 can act upstream of caspase-8 and prime cancer cells to TRAIL-mediated apoptosis by processing procaspase-8 [Shin et al., 2005]. It is interesting that processed caspase-2 can induce mitochondrial-mediated apoptosis independent of its enzymatic activity [Robertson et al., 2004]. The leading role of caspase-2 in apoptosis has been shown in caspase-2-deficient mice: oocytes and B lymphoblasts of these mice were found to be resistant to cell death following exposure to chemotherapeutic drugs, granzyme B, and perforin [Bergeron et al., 1998].

The data described above indicate that expression of *CaMKII* genes and CaMKII activity are under AR control. One remaining question is whether CaMKII activity can affect androgen/AR-dependent signaling, that is, is there cross-talk between AR- and CaMKII-mediated pathways?

It is well known that androgen induces AR activity as a transcription factor that binds specific DNA sequences, recruits RNA polymerase II and a basal transcriptional complex for efficient transcription of cellular genes. The transcriptional activity of AR is mediated by co-activators and co-repressors, which in response to binding of androgen to AR and nuclear translocation, are assembled at AREs [Dehm and Tindall, 2006]. We also investigated the role of CaMKII in AR-dependent signaling and found that CaMKII over-expression increased PSA secretion and that cell growth in SFC was increased by more than 40% in CaMKII transfectants. Taken together, our experiments suggest that there is a cross-talk between androgen/AR- and CaMKII-dependent pathways.

It is well established that androgen ablation therapy, although effective in providing temporary apoptosis enhancement, may also serve in facilitating progression into an androgen-independent phenotype. Our data show that CaMKII could be one of the important proteins in prostate cancer cells escaping apoptosis after androgen ablation, and facilitating the progression of prostate cancer to an androgen-independent state.

DNA DAMAGING AGENTS CANNOT ACTIVATE p53-MICRORNA-34 IN THE ABSENCE OF AR

We extended our analysis of the role of AR in the response to different apoptosis-inducing agents and found that knocking down AR expression resulted in resistance to DNA double-strand break agents, DOX and Camptothecin. In addition to CaMKII activation there is another way to escape cell death after AR inhibition: DNA damaging agents cannot fully activate p53 in the absence of AR and as a result microRNA-34, a p53 down stream target, cannot be activated and induce apoptosis [Rokhlin et al., 2008].

Micro RNAs (miRNAs) are approximately 21-nucleotide long RNA regulators of post-transcriptional gene expression that regulate mRNA translation or stability in the cytoplasm. By base pairing to mRNAs, miRNAs mediate translational repression or mRNA degradation. However, recent findings indicate that miRNA-mediated repression can be reversed, prevented, or even act as translational activators [Filipowicz et al., 2008]. It is extremely interesting that miRNA can repress translation in proliferating cells but induce translation up-regulation upon cell-cycle arrest [Vasudevan et al., 2007]; these authors propose that translation regulation by microRNPs oscillates between repression and activation during the cell cycle. The changes in cellular protein synthesis in response to miRNA induction or knockdown on a proteome-wide scale has also been measured [Selbach et al., 2008]. The results of this study show that a single miRNA can directly down-regulates production of 100 proteins. The authors also show that in addition to the effect on mRNA levels, miRNAs translationally repress hundreds of direct target genes.

To identify the miRNA signature specific for prostate cancer, miRNA expression profiling of prostate cancer cell lines, xenografts, benign prostatic hyperplasia, and prostate carcinoma samples was carried out by using an oligonucleotide array hybridization method [Porkka et al., 2007]. Differential expression of 51 miRNAs between benign tumors and carcinomas was detected, 37 of them showing down-regulation and 14 up-regulation in carcinoma samples. The results of this study also showed that androgens might regulate the expression of some miRNAs. In another study, the involvement of miRNA expression in prostate cancer was evaluated by genome-wide expression of miRNAs in primary prostate tumors compared to non-tumor prostate tissues [Ambs et al., 2008]. The expression of the miR-1, miR-32, and miR-106b-25 cluster in prostate cancer has been reported. In addition, the expression of miR-338, miR-126, miR-181b-1, miR-181c, and miR-221 were found to be under androgen control. Ozen et al. [2008] demonstrated significant down-regulation of several miRNAs in clinically localized prostate cancer when compared to benign peripheral zone tissue.

The most commonly reported modification of p53 is phosphorylation that results in p53 stabilization, accumulation, and transcriptional activation. Seventeen phosphorylation sites have been detected in human cells following DNA damage. Further, the same p53 site can be phosphorylated by several protein kinases and distinct protein kinases can also phosphorylate several distinct sites [Bode and Dong, 2004]. A variety of downstream apoptosis-related genes that affect both the intrinsic and extrinsic pathways are regulated by p53 [Rozan and El-Deiry, 2007]. Recent studies have implicated the miR-34 family of microRNAs in the p53 tumor suppressor network revealing interplay between proteins and non-coding RNAs in this crucial tumor-suppressor pathway [He et al., 2007a]. For example, it has been shown that the expression of miR-34a, miR-34b, and miR-34c is induced by DNA damaging agents in a p53-dependent manner [He et al., 2007b; Corney et al., 2007; Tarasov et al., 2007; Raver-Shapira et al., 2007; Chang et al., 2007].

There are at least three kinases that are under AR control that can potentially modulate p53 activation after treatment with DNA damage agents: SPAK, MDC1, and CaMKII. SPAK, originally termed Ste20/SPS1-related proline- and alanine-rich kinase [Johnston et al., 2000] is under AR control in LNCaP [Qi et al., 2001]. Our own data confirm this observation. However, decreased expression of SPAK did not effect the activation of p53 after DOX treatment. These results suggest that SPAK does not participate in attenuating p53 activity after knocking down AR expression. MDC1/NFBD1 (nuclear factor with BRCT domain/1 mediator of DNA damage checkpoint protein) is a nuclear protein that is involved in early cellular responses to genotoxic stress and after DNA damage, co-operating with gH2AX to recruit DNA repair proteins to the sites of DNA damage [Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003; Stucki and Jackson, 2004]. Suppression of MDC1 expression results in reduced apoptosis in response to DNA damage in A549 cells and phosphorylation of p53 at Ser20 is decreased after irradiation in cells transfected with siRNA MDC1 [Lou et al., 2003]. However, when the p53-negative cell line H1299 was transfected with siRNA MDC1 and with a p53 expression plasmid the down-regulation of the MDC1 resulted in a significant increase in the phosphorylation levels of exogenously expressed p53 at Ser15 even in the absence of DNA-damaging agents [Nakanishi et al., 2005]. This apparent discrepancy might be due to the cell type-specific effects as well as to different experimental conditions. We have shown for the first time that the expression of MDC1 in prostate cells is under control of AR; expression of MDC1 is sharply decreased in siRNA AR LNCaP cells as well as in LNCaP cultured in SFC. The role of MDC1 in p53 phosphorylation became evident when MDC1 was knocked down and then treated with DOX. In these cells the level of p53-Ser15 was reduced more than threefold compared to control cells. As discussed above, knocking down AR induces CaMKII activity and our results show that over-expression of CaMKII partially prevents p53 inactivation. Thus, AR sends contradictory signals to kinases that act upstream of p53 pathway.

Our own data indicate that the level of miR-34a increased threefold after DOX but did not change in si-AR treated cells; the level of miR-34c increased 27-fold after DOX but only 2-fold in si-AR cells. Apparently, AR-dependent inhibition of p53 resulted in suppression of miR-34a and -34c, which in turn inhibits DOX-mediated apoptosis. We also investigated whether AR-dependent DOX-mediated miR-34 inhibition was prostate cell specific. We observed that DOX induced miR-34c expression to much lower extent in MCF-7 compared to LNCaP and no substantial changes were noted in miR-34 levels after DOX treatment in si-AR cells. A robust miR-34c induction after DOX treatment was not observed in AR-negative prostate cell lines DU145 and PC3, or in LNCaP cultured in SFC. These data suggest that miR-34c induction is mediated by AR-dependent p53 activity.

It is of interest that DOX-mediated induction of miR-34c in LNCaP is about 10-fold higher compared to miR-34a. He et al. [2007c] also found that DOX treatment of TOV21G cells induced miR-34c expression 47-fold but miR-34a—only 13-fold. In addition, the copy number of miR-34a is much higher compared to miR-34c in all cell lines investigated. Apparently, it is not the absolute number of miR-34 copies that is important but the actual phenotypic output of miR-34 activation that may depend on the spectrum of its target that are available for repression (activation). The relatively week effect of miR-34a on apoptosis was noted by Raver-Shapira et al. [2007]. They found that over-expression of miR-34a resulted in very mild increase of apoptosis in H1299 cells, which indicated that miR-34a is necessary, but not sufficient, for apoptosis, at least in this setting. As these authors suggest, miR-34a co-operates with additional modulators (other miRNAs, other p53-regulated genes) to facilitate apoptosis. Our data also suggest that miR-34a plays a minor or auxiliary role in DOX-mediated apoptosis. We showed that inhibition of individual miR-34, either 34a or 34c, or forced expression of individual miR-34a or miR-34c did not modulate DOX-mediated apoptosis. Only simultaneous inhibition or forced expression of both miR-34 resulted in inhibition or induction of DOX-mediated apoptosis. Taken together, our data indicate that co-operation between miR-34a and 34c plays an important role in AR-dependent p53-mediated apoptosis in prostate cancer.

One remarkable finding in our study is the elevated level of miR-34c in DU145 (13,750 copies in DU145, vs. 50–250 copies in four other prostate cancer cell lines). The low number of miR-34c copies was also found in other studies [He et al., 2007c; Chang et al., 2007]. We speculate that the reason for this high level of expression is that DU145 that this cell line is the only one so far investigated that expresses two distinct mutant p53 alleles (223Leu, 274Phe). We have previously found that the biological effect of these mutants was dramatically different when of each mutant was expressed alone [Gurova et al., 2003]. Whether these p53 mutants are indeed responsible for elevated level of miR-34c expression remains to be investigated.

CONCLUSION

We have investigated whether AR-positive prostate cancer cells can escape apoptosis after inhibition of AR expression. We discovered that inhibition of AR expression induced PI3K-independent activation of anti-apoptotic protein Akt, which was mediated by CaMKII. Moreover, CaMKII can affect apoptotic response of prostate cancer cells by Akt-independent mechanism: KN-93, CaMKII inhibitor, sharply decreased the level of anti-apoptotic protein Mcl-1 whereas different inhibitors of PI3K/Akt pathway did not change the Mcl-1 expression. Moreover, KN-93 induces p53 expression and p53-dependent pro-apoptotic protein PUMA. CaMKII-mediated pathways may be one of the mechanism by which prostate cancer cells escape cell death after inhibition of AR activity. We than found that there is another mechanism: after inhibition of AR expression DNA damaging agents cannot fully activate p53 and as a result p53 down stream targets cannot be activated and induce apoptosis. The different mechanisms that mediate inhibition of apoptosis after suppression of AR activity are summarized in Figure 1. This implies that there may be a need for re-evaluation of the therapeutic approaches to human prostate cancer. A comprehensive genetic analysis of 24 pancreatic cancers has recently been performed [Jones et al., 2008]. Authors found that pancreatic cancers contain an average of 63 genetic alterations which defined a core set of 12 signaling pathways. The pathway

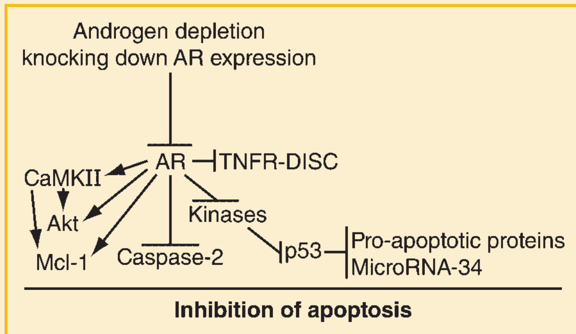

Fig. 1. Different mechanisms mediate inhibition of apoptosis after suppression of AR activity. Inhibition of AR activity results in: activation of the anti-apoptotic CaMKII/Akt pathways; increased expression of the anti-apoptotic protein Mcl-1; inhibition of tumor necrosis factor receptors (TNFR) DISC formation; inhibition of kinases that activate p53, which results in suppression of p53 and its down stream targets; and inhibition of caspase-2.

components that are altered in any individual tumor vary widely but many of them are likely to impact these pathway(s). Therefore, pancreatic cancers result from genetic alteration of a large number of genes that function through a relatively small number of pathways and processes. Authors concluded "that the best hope for therapeutic development may lie in the discovery of agents that target the physiological effects of the altered pathways and processes rather than their individual gene components." From this point of view, KN-93 may be an excellent candidate for treatment of ADI prostate cancer: KN-93 inhibits CaMKII/Akt pathways, inhibits AR activity and expression of anti-apoptotic protein Mcl-1 and induces expression of pro-apoptotic proteins p53 and PUMA. Therefore, KN-93 targets several metabolic pathways, which may lead to therapeutic treatment of prostate cancer.

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