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## Targeting prostate cancer based on signal transduction and cell cycle pathways

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### Abstract

Prostate cancer remains a leading cause of death in men despite increased capacity to diagnose at earlier stages. After prostate cancer has become hormone independent, which often occurs after hormonal ablation therapies, it is difficult to effectively treat. Prostate cancer may arise from mutations and dysregulation of various genes involved in regulation signal transduction (e.g., PTEN, Akt, etc.) and the cell cycle (e.g., p53, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, Rb, etc.). This review focuses on the aberrant interactions of signal transduction and cell cycle genes products and how they can contribute to prostate cancer and alter therapeutic effectiveness.

### Keywords

radiosensitization; prostate cancer; p53; MDM-2; MDM-2; antagonists; senescence; PTEN; Akt

### Prostate Cancer: The Most Prevalent Cancer in Men

Due to increasingly reliable methods of detection, carcinoma of the prostate (CaP) has become the most commonly-diagnosed cancer in men in the United States. This year alone, approximately 230,000 American males will be diagnosed with CaP and nearly 30,000 will die from this deadly disease (source: American Cancer Society). Despite increased public awareness and improved procedures for surgical intervention of CaP, there remains no effective cure for patients with advanced disease. Moreover, treatment regimens are limited to radiation and/or chemotherapy, which primarily provide palliative benefits while offering little in the form of increased life expectancy. Consequently, studies designed to better understand the etiology of this disease should lead to more effective treatments in the clinic.

Over the past decade, scientists have discovered that early detection drastically reduces death due to CaP. Consequently, during this time a vast majority of the research that has focused on identifying prognostic indicators of advanced disease versus those markers associated with

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earlier stages of CaP. However, much less has been discovered during this time regarding the development and natural progression of the disease.

There are a number of factors that have been correlated to the development of CaP including age (increased diagnosis after 60 years; 1 in 7 men), environmental factors (race, diet, etc.), familial inheritance (10% of CaP are associated with hereditary factors), and steroid hormone signaling (decreased reliance upon testosterone), among others.<sup>1</sup> While each of these factors appears to be intimately associated with the development of disease, one must comprehend what occurs on the molecular level to develop better treatment strategies and possible cures.<sup>2,3</sup>

The prostate gland surrounds the urethra at the base of the bladder and functions by producing secretory proteins for semen. There are at least three different cell types that comprise the prostatic epithelium: (1) secretory luminal epithelial cells—a well-differentiated androgen-dependent cell that functions to secrete prostatic proteins, (2) basal cells—situated between the secretory luminal cell layer and the underlying basement membrane, (3) neuroendocrine cells— androgen-independent cells that are located in the same stratum as the basal cells and are believed to provide paracrine signals to the adjacent luminal cells, and (4) prostate stem cells—not proven yet, but anticipated to exist.

Prostatic intraepithelial neoplasia (PIN) are preneoplastic lesions that precede the advent of prostate cancer. PIN lesions are characterized by loss of basal cells and invasion of luminal cells into the periphery. The exact causes surrounding the onset of PIN are unknown; however, a prominent theory is that loss of particular chromosomes may be major contributory factors in this process. Chromosomes 8p, 10q and 13q are all frequently lost in CaP; there are a number of important tumor suppressor genes found on these chromosomes including NKX3.1,<sup>4-6</sup> PTEN/MMAC1,<sup>7-12</sup> and Rb,<sup>13-16</sup> respectively.

## Central Role of PI3K/PTEN/Akt/mTOR Pathway in Prostate Cancer

Chromosome 10q is frequently lost (50-80%) in prostatic lesions. PTEN is located within this locus (10q23) and is a well-known negative regulator of the PI3K/Akt signal transduction cascade. The PI3K/Akt signaling pathway is known for its role in mediating cell survival, as well as cell cycle progression and neoplastic transformation.<sup>17</sup> Consequently, it would stand to reason that mutation of PTEN would initiate heightened activation of this cascade, which could then confer a number of cancer-like properties to the cell. Many of these, particularly cellular survival, are hallmark features of CaP tumor cells.

Phosphatidylinositol-3-kinase (PI3K) is a heterodimeric protein with an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. PI3K serves to phosphorylate a series of membrane phospholipids including PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>, catalyzing the transfer of ATP-derived phosphate to the D-3 position of the inositol ring of membrane phosphoinositides, thereby forming the second messenger lipids PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. Most often, PI3K is activated via the binding of a ligand to its cognate receptor, whereby p85 associates with phosphorylated tyrosine residues on the receptor via an SH2 (Src-homology 2) domain. After association with the receptor, the p110 catalytic subunit then transfers phosphate groups to the aforementioned membrane phospholipids. It is these lipids, specifically PtdIns(3,4,5)P<sub>3</sub>, that attract a series of kinases to the plasma membrane thereby initiating the signaling cascade.<sup>18</sup> An overview of the PI3K/PTEN/Akt/mTOR pathway is presented in Figure 1.

Downstream of PI3K is the primary effector molecule of the PI3K signaling cascade, Akt/PKB (protein kinase B). Akt was originally discovered as the cellular homologue of the transforming retrovirus AKT8 and as a kinase with properties similar to protein kinases A and C.<sup>19,20</sup> Akt contains an amino-terminal pleckstrin homology (PH) domain that serves to target the protein

to the membrane for activation.<sup>21-23</sup> Within its central region, Akt has a large kinase domain and is flanked on the carboxy-terminus by hydrophobic and proline-rich regions.<sup>24,25</sup> Akt is activated via phosphorylation of two residues: T308, S473. While it is generally agreed upon that maximal activation of Akt requires phosphorylation of both of these residues, there are conflicting reports regarding the relative importance of S473 and T308 for initial activation of Akt. It was originally thought that either of these residues could be phosphorylated to initiate Akt activity and that phosphorylation of one residue was not necessary for phosphorylation of the other to occur.<sup>26</sup> More recent evidence suggests that phosphorylation of T308 is absolutely essential for phosphorylation of S473.<sup>27,28</sup> Each of these studies was carried out in the HEK293 cell line, which further compounds the issue because the differences cannot be attributed to cell type.

The phosphatidylinositol-dependent kinases (PDKs) are responsible for activation of Akt. PDK1 is the kinase responsible for phosphorylation of T308,<sup>29</sup> while the actual kinase that phosphorylates S473 (PDK2) remains controversial. Some report that phosphorylation of T308 triggers autophosphorylation of S473,<sup>30</sup> while others hypothesize that PDK2 is the integrin-linked kinase (ILK).<sup>31</sup> However, it is now believed that ILK may facilitate phosphorylation of S473 indirectly, perhaps by acting as a scaffolding protein.<sup>32</sup> Akt can also be phosphorylated by the mTOR complex called Rictor.<sup>33</sup> Phosphorylation of Akt is complicated as it can be phosphorylated by a complex which lies downstream of itself. Moreover, it can be dephosphorylated by p70<sup>S6K</sup> which also lies downstream of Akt.<sup>34</sup> Once activated on either/both of these residues, Akt leaves the cell membrane to phosphorylate intracellular substrates.

After activation, Akt is able to translocate to the nucleus<sup>35</sup> where it affects the activity of a number of transcriptional regulators. Cyclic-AMP response element binding protein (CREB),<sup>36</sup> E2F,<sup>37-39</sup> NFκB (via Iκ-K),<sup>40,41</sup> and the forkhead transcription factors<sup>42-50</sup> are all either direct or indirect substrates of Akt and each can promote either cellular proliferation or survival. Aside from transcription factors, Akt is able to target a number of other molecules to affect the survival state of the cell including caspase-9,<sup>51</sup> the pro-apoptotic molecule BAD,<sup>52,53</sup> and glycogen-synthase kinase-3β (GSK-3β).<sup>54</sup> When these targets are phosphorylated by Akt, they may either be activated or inactivated but the end result is to promote survival of the cell.

Negative regulation of the PI3K pathway is primarily accomplished through the action of the PTEN tumor suppressor protein. *PTEN* encodes a lipid and protein phosphatase whose primary lipid substrate is PtdIns(3,4,5)P<sub>3</sub>. The protein substrate(s) of *PTEN* are more varied, including focal adhesion kinase (FAK), the Shc exchange protein and the transcriptional regulators ETS-2,<sup>55</sup> and Sp1.<sup>56</sup> *PTEN* also may negatively regulate the activation of the platelet-derived growth factor (PDGF) receptor.<sup>57</sup>

*PTEN* has four primary structural domains. On the amino terminus is the lipid and protein phosphatase domain, which is flanked adjacent to the C2 domain that is responsible for lipid binding and membrane localization. Next are two PEST domains which regulate protein stability. Lastly, *PTEN* has a PDZ domain, which helps facilitate protein-protein interactions. Mutations within the phosphatase domain have been reported to nullify the endogenous function of *PTEN*.<sup>58-60</sup>

As previously mentioned, loss of *PTEN* function in advanced CaP is quite common (50-80% of patients).<sup>61,62</sup> As a consequence, this results in an overabundance of lipid second messengers [PtdIns(3,4,5)P<sub>3</sub>], which can cause constitutive activation of PH domain-containing proteins including Akt. It is for this reason that Akt is found to be highly-activated in advanced cases of CaP.<sup>63,64</sup> Aberrant Akt activation is able to elicit the pro-survival properties observed in CaP cells through a number of mechanisms, described hereafter. Hence inhibiting Akt or restoring *PTEN* activities are potential therapeutic targets in prostate cancer.

p27<sup>Kip1</sup> is a central mediator of cell cycle progression whose role as a cyclin-dependent kinase (CDK) inhibitor is lost in many cases of advanced CaPs.<sup>65-69</sup> The role of p27<sup>Kip1</sup> in preventing prostatic disease is bolstered by evidence which shows that p27<sup>Kip1</sup>-null mice quickly develop prostatic hyperplasia.<sup>70</sup> Akt appears to affect p27<sup>Kip1</sup> expression via the intermediate molecule, FKHR. FKHR (FOXO1) is a member of the forkhead/FoxO family of transcription factors, which are known to stimulate transcription of p27<sup>Kip1</sup>.<sup>71</sup> Upon activation, Akt phosphorylates FKHR causing its inactivation and subsequent downregulation of p27<sup>Kip1</sup>. Further evidence has shown that constitutively-active Akt may be able to decrease the half-life of p27<sup>Kip1</sup> as well.<sup>72,73</sup> This series of events leads to a phenotype whereby CaP cells have less restriction on cell cycle progression, thereby promoting unregulated cell division. Supporting these observations are other studies which show that as CaP progresses from an androgen-dependent to -independent state, p27<sup>Kip1</sup> levels are drastically diminished.<sup>74,75</sup>

Akt regulates the apoptotic response to a variety of stimuli via its ability to interact with a number of key players in the apoptotic process. First, Akt can directly phosphorylate BAD on S136,<sup>76</sup> causing its inactivation and inability to interact with anti-apoptotic members of the Bcl-2 family of proteins (Bcl-2, Bcl-X<sub>L</sub>).<sup>77</sup> Next, activated Akt can independently inhibit the release of cytochrome c from the mitochondria, which is a potent activator of the apoptotic caspase cascade.<sup>65</sup> Akt also is capable of phosphorylating procaspase-9, preventing its cleavage into the pro-apoptotic caspase-9 initiator of programmed cell death.<sup>51</sup> Lastly, the Akt target, FKHR is capable of upregulating Fas ligand and Bim, two very important molecules that are potent inducers of apoptosis; however, when inactivated by Akt, FKHR is localized to the cytosol where it is unable to augment expression of these genes.<sup>49,79</sup> Akt can also phosphorylate Bim which inhibits its proapoptotic activity. In concert, these events caused by Akt activation would appear to greatly affect the survival status of the cell.

Akt also plays significant roles in protein translation, particularly by regulating those proteins involved in growth and survival. A specific target is mTOR (mammalian target of rapamycin) which is able to induce phosphorylation of eIF-4E binding protein-1 (4E-BP1). After the appropriate phosphorylation events, 4E-BP1 disassociates from the mRNA cap-binding protein eIF-4E, which allows eIF-4E to interact with the eIF-4E translation initiation complex to initiate protein synthesis. Another target of Akt is p70<sup>S6K</sup> which is a well-known enhancer of protein synthesis.<sup>80-84</sup> mTOR has been shown to be critically important in autophagy,<sup>85-87</sup> a mechanism of cell death which will be discussed below.

Messenger RNAs differ in their ability to be translated; the length and sequence of the 5' UTR largely dictates the efficiency with which an mRNA transcript will be translated. Most mRNAs contain short, unstructured GC-poor 5' UTRs and are efficiently translated. In contrast, protooncogene and growth factor mRNAs are characterized by long, GC-rich sequences in the 5' UTR, which can often hinder the ability of the eIF-4E complex to efficiently scan and initiate translation at the start codon. Consequently, under normal circumstances these mRNAs are not efficiently translated.<sup>88,89</sup> However, upon Akt-mediated activation of mTOR, these latter mRNAs are highly and disproportionately translated; several key proteins that are overexpressed as a consequence of this event include c-myc,<sup>90-96</sup> cyclin D1,<sup>97,98</sup> and VEGF<sup>99</sup> among others.<sup>88,89</sup> Cyclin D1 has been reported to be overexpressed in CaP xenografts and metastases,<sup>100,101</sup> while early stage prostatic lesions possess much lower levels of the protein.<sup>102</sup> A number of reports support the notion that mTOR signaling is a prominent feature of CaP progression, as recurrent tumors have altered expression of a number of molecular targets of rapamycin.<sup>103-107</sup> Hence mTOR inhibitors such as rapamycin may be effective in prostate cancer therapy.

## Role of MAPK Signaling in Prostate Cancer

The PI3K/Akt signaling cascade is primarily associated with cell survival; conversely, the Raf/MEK/ERK pathway is associated with growth, proliferation and differentiation.<sup>108-114</sup> Despite the wealth of information surrounding the role of PI3K-mediated signaling in prostate cancer, much less is known about the function of Raf/MEK/ERK signaling in CaP.

Like PI3K, activation of the Raf/MEK/ERK pathway is initiated by a mitogen-receptor ligation at the cellular surface. Growth factor receptors are comprised of at least two subunits which oligomerize upon binding of the respective mitogen; this event is immediately preceded by increased tyrosine kinase activity on the cytoplasmic portion of the receptor. Most often this is due to autophosphorylation, whereby one subunit of the receptor acts as a tyrosine kinase and another subunit serves as a substrate. The phosphorylated tyrosine residue(s) then serve as docking sites for cytoplasmic proteins that contain SH2 domains.<sup>115</sup> After this event, a complex of proteins is able to initiate a series of phospho-relay events that can ultimately alter gene expression in the nucleus, as described hereafter.

Ras is a 21-kDa membrane-localized, monomeric G-protein that is considered to be the initiator of signaling in the Raf/MEK/ERK pathway. The consensus amongst researchers is that mutations in the Ras gene are relatively uncommon in prostatic malignancies<sup>116-118</sup> as compared to other tumor types (~30%). After receptor ligation and activation, SH2-containing proteins are targeted to the phosphorylated tyrosine residue(s) on the receptor; one such protein is Grb2.<sup>119</sup> Grb2 also contains an SH3 domain whose primary function is to bind to proline-rich regions of other molecules, such as SOS (Son of Sevenless). SOS is a guanine dissociation stimulator of Ras which binds to Ras after receptor activation. After binding to SOS, Ras undergoes a conformational change that causes the dissociation of GDP from Ras, allowing it to then associate with GTP. In the GTP-bound form, Ras is in its active state and able to recruit the Raf kinase to the plasma membrane for activation.<sup>120-123</sup> To negatively regulate this process, there exist a number of GTPase-activating proteins (GAPs) that are able to stimulate the GTPase activity of Ras in order to convert it back to its original GDP-bound state.<sup>124</sup>

Immediately downstream of Ras is the Raf kinase. Currently, there are three known isoforms of Raf: Raf-1 (c-Raf), A-Raf and B-Raf.<sup>125-128</sup> Each of these isoforms has been described to have both overlapping and unique regulatory functions.<sup>128</sup> Activation of Raf (A-Raf and Raf-1 but not B-Raf) is a two-step process—first, active GTP-bound Ras recruits Raf to the cellular membrane, which allows association with additional proteins necessary for activation (i.e., scaffolding proteins, etc.).<sup>129</sup> The first step is necessary but not sufficient for activation of Raf. Consequently, a second step is required whereby Raf (A-Raf and Raf-1 but not B-Raf) is phosphorylated on tyrosine and/or serine/ threonine residues. A member of the Src family of kinases is responsible for this event when it occurs on tyrosine residues,<sup>130</sup> whereas protein kinase C (PKC) is a kinase implicated in activating Raf on serine/threonine residues.<sup>131,132</sup> An overview of the Raf/MEK/ERK pathway is presented in Figure 2.

Regulation of Raf activation, however, is much more complex than simple phosphorylation of a few nominal residues. Dr. Walter Kolch has contributed significantly to this area and has published a number of thorough reviews on the topic.<sup>133-136</sup> Briefly, Raf-1 is maintained in an inactive state when phosphorylated on serines 259 and 621 by binding to 14-3-3 proteins.<sup>136-138</sup> Activation of Raf-1 is initialized when Ras displaces the 14-3-3 protein, allowing phosphatase 2A (PP2A) to access and remove the phosphate group from S259.<sup>139,140</sup> Evidence suggests that nearly 80% of all Raf-1 exists in the phospho-S259 form and that it is impossible to activate the protein while in this state.<sup>138,141</sup> When activated, Raf-1 is phosphorylated on S338; mutation of this residue is sufficient to eradicate Raf-1 activation by mitogenic stimulation.<sup>142,143</sup> For these reasons, the use of phosphospecific antibodies to

detect Raf-1 when phosphorylated on S338 has been used extensively as a qualitative indicator of Raf-1 activity with reliable accuracy.<sup>143</sup>

After activation, Raf targets its downstream substrate(s) MEK1/2. MEK-1 and MEK-2 (hereafter, MEK) are phosphorylated on S218 and S222 by the Raf proteins.<sup>144</sup> Phosphorylation on these two residues has been reported to increase MEK activity over 7000-fold.<sup>145</sup> The kinase suppressor of Ras (KSR) is a scaffolding protein that mediates the interaction between Raf and MEK, allowing for efficient phosphorylation of the MEK protein.<sup>146</sup> Recent reports have suggested that the association of Raf, MEK and KSR is not as simplistic as it was once thought—there appear to be modulators of KSR activity, including MEK partner-1 (MP1)<sup>147</sup> and Raf kinase inhibitor protein (RKIP).<sup>148</sup> The function of MP1 is believed to be another scaffolding protein that enhances the association of MEK and B-Raf, whereas RKIP actually disrupts the binding between Raf-1, MEK and RKIP. Together, each of these proteins plays a unique role in the activation of MEK by Raf although it appears that the molecules involved may be more numerous than just these proteins.<sup>133</sup> RKIP has been postulated to be a tumor suppressor protein. Relatedly, decreased expression of RKIP has been associated with prostate cancer progression.<sup>148-150</sup>

ERK-1 and -2 are 44 and 42 kDa kinases that are directly downstream of the MEKs. Phosphorylation of ERK-2 occurs on residues T183 and Y185; maximal activation is achieved via phosphorylation by MEK on both of these residues.<sup>151,152</sup> After activation, ERK-2 can dimerize with other ERK-2 molecules and induce nuclear translocation.<sup>153</sup> Upon entering the nucleus, ERK-2 dimers are thought to activate an array of nuclear targets, including topoisomerase II- $\alpha$ , suggesting that ERK-2 has a role in chromatin remodeling during mitotic events.<sup>154</sup> A number of cytoplasmic ERK targets exist as well, the primary of which is the p90 ribosomal S6 kinase (p90<sup>RSK</sup>)<sup>155</sup> which is responsible for phosphorylating several transcriptional regulators including CREB,<sup>156</sup> estrogen receptor- $\alpha$ ,<sup>157</sup> I $\kappa$ B/NF $\kappa$ B,<sup>158,159</sup> and c-Fos.<sup>160</sup> In addition to p90<sup>RSK</sup>, ERK is also a known inducer of the activator protein-1 (AP-1) family of transcription factors; these modulators of transcription include c-Jun, c-Fos and ATF-1 and each alters gene expression of individual target genes when activated. Each of the above described phosphorylation promotes an environment that favors proliferation.

MAPK signal transduction has not been extensively studied in the prostate; very few publications exist detailing the involvement of the Raf/MEK/ERK pathway in the advancement of prostatic disease. Within the data that have been reported, there exists some degree of divergence—some reports show that this cascade is involved in CaP development, while others report the opposite. Consequently, the current level of understanding of MAPK activation in CaP is ambiguous.

While aberrant MAPK activity is common in many cancers, there is little evidence to suggest that MAPK activity is increased in CaP. The absence of mutations in Ras may be a factor that contributes to this observation,<sup>117,118</sup> as many cancers with constitutive MAPK activity possess mutations in the Ras G-protein. One study has reported that activation of the MAPK pathway with conditionally activated mutants of Raf resulted in cell cycle arrest and decreased plating efficiency.<sup>161</sup> Moreover, the authors suggested that activation of this pathway may be a therapeutic target for CaP cells. Supporting this notion are other studies that show that treatment of CaP cells with resveratrol or phenylethyl isothiocyanate, two compounds that induce apoptosis in a variety of cancers, can induce apoptosis in cells of prostatic origin in an ERK-dependent manner.<sup>162-164</sup>

The use of ERK activation as a prognostic indicator of disease is quickly becoming a useful tool in determining the stage of progression of CaP. Initially, it was reported that ERK activation was increased in high-grade PIN when compared to tissue derived from the normal

prostate, as measured by immunohistochemical analysis.<sup>165,166</sup> Another study supports this observation; Price et al., show that there was significantly increased activation of ERK in tissue removed from patients who had undergone radical prostatectomies.<sup>167</sup> Contrary to these results, however, are other reports which state that ERK activation is actually diminished as the disease progresses from normal tissue to PIN to invasive carcinoma.<sup>168,169</sup> Taken together, these data are non-conclusive, but they do suggest that ERK activity may be a viable means of determining patient prognosis if accurately ascertained. ERK activity may be increased in certain subsets of prostate cancer patients.

## Roles of Cell Cycle Proteins in Prostate Cancer

In addition to the PI3K/PTEN/Akt and Raf/MEK/ERK and other signaling pathways, lies a complex network of proteins which regulate cell cycle progression. Often some of these cell cycle regulatory proteins (e.g., p53) may serve to regulate the activity of signaling pathways including PI3K/PTEN/Akt and Raf/MEK/ERK.<sup>170-181</sup> An overview of some of these potential interactions is presented in Figure 3. Numerous proteins involved in cell cycle regulation are often mutated in prostate cancer, resulting in activation of oncogenes and loss of tumor suppressor proteins. There is no dominant molecular profile that initiates or maintains prostatic carcinogenesis, but rather a complex mixture of mutations that disrupt cell cycle control and cell death pathways. Cell cycle proteins commonly mutated at early to late stages of prostate cancer progression include Rb, p14, p16, p53 and p27.<sup>182-189</sup> Mutations within these proteins result in defective cell cycle checkpoint control, leading to further chromosomal instability and inactivation of the protective tumor preventative failsafe, cellular senescence.<sup>190-193</sup> The PI3K/PTEN/Akt and Raf/MEK/ERK pathways are also commonly the target of mutations that promote cell proliferation and block apoptosis and elicit some of their effects through cell cycle pathways. Furthermore, and perhaps more importantly mutations at upstream receptor genes feed into these pathways. Combined mutations within and among these pathways may alter the success of radio- and chemotherapies. Knowledge of the pathways altered, prior to initiating therapy, could provide a molecular signature for clinicians to optimize sensitization strategies based upon the individual's profile. The use of small-molecule inhibitors aimed at dysregulated pathways in combination with various therapeutic approaches may improve the success of treatment and prolong survival of the patient.<sup>194-203</sup>

Although the frequency of p53 mutations in early prostate cancer is low, heterozygous loss of function (LOF) mutations often accompany late stage carcinoma.<sup>189,204</sup> Current models of the molecular network coordinating responses to DNA damage place p53 at the crossroads of several stress response pathways critical for maintaining genome integrity; including cell cycle arrest, DNA repair, mitotic catastrophe, apoptosis and cellular senescence.<sup>205-211</sup> The mechanisms by which p53 directs cells down each of these alternative avenues of cell fate determination differs among cells of various origins.<sup>212-214</sup> It is important that each of these distinct forms of p53-dependent cell death be collectively evaluated when investigating new therapeutic strategies for increasing the sensitivity of CaP cells to various therapeutic approaches.

Since p53 function is crucial to coordinating the DNA damage response, multiple upstream pathways control p53 activation. While the list of stress signals that activate p53 continues to grow, including hypoxia, ribosomal stress and loss of cell-cell contacts, the primary pathways appear to be oncogenic stress and DNA damage. Interestingly, various stress signals use different mechanisms for p53 activation. Under resting conditions, the ubiquitin degradation pathway maintains a high rate of p53 turnover that is dependent on the E3 ubiquitin ligase, murine double-minute 2 (MDM2).<sup>215-218</sup> Hypophosphorylated MDM2 binds the p53 C-terminus and targets p53 for degradation.<sup>219</sup> Moreover, the p53 binding domain of MDM2 overlaps with the DNA binding domain of p53, effectively impairing p53 activity.<sup>220</sup> The p53

protein is also regulated by MDMX, a homologue of MDM2, that binds MDM2 through its C-terminal RING domain and stimulates the MDM2-dependent ubiquitination of p53.<sup>215,221,222</sup> Therapy induced DNA damage triggers a phosphorylation cascade involving ataxia telangiectasia mutated (ATM) and ATM-related (ATR) proteins that prevent p53 degradation through multiple phosphorylation events on MDM2, MDMX and p53.<sup>223-228</sup>

Active p53 functions as a transcriptional regulator within a complex network involving the selective transactivation and transrepression of multiple genes involved in cell cycle control and apoptosis. p53 functions as a tetrameric transcription factor. The hetero-oligomerization between a heterozygous mutant allele and wild-type (WT) p53 proteins may be sufficient to alter the function of p53 tetramers. The dominant negative (DN) actions of missense p53 mutations were first demonstrated in experiments with ectopic expression of WT and mutant p53 proteins<sup>204,229</sup> and more recently in thymocytes expressing p53 point mutations at single-copy levels.<sup>230,231</sup> Alternatively, mutant p53 alleles may impart a gain of function (GOF) that could also impact tumor development and the progression of prostate cancer.<sup>230-233</sup>

p53 can activate the Raf/MEK/ERK pathway by the discoidin domain receptor-1 (DDR-1).<sup>234-236</sup> Furthermore, ERK can phosphorylate p53 which results in its stabilization.<sup>237</sup> The PI3K/PTEN/Akt pathway also serves to regulate p53 activity through Akt-mediated phosphorylation of MDM-2 which enhances its activity and destabilizes p53.<sup>238</sup> Furthermore, there are reciprocal interactions between p53 and PTEN which serve to cross regulate each other's expression.<sup>239-242</sup> Thus targeting the p53, PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathway may enhance certain prostate cancer therapies.

Many studies of in vitro prostate cancer sensitivity have used apoptosis assays to assess the efficacy of cell killing and have been limited to a few CaP cell lines that were derived from metastatic lesions containing p53 mutations rarely observed in organ-confined tumors.<sup>233,243-245</sup> The best characterized lines include PC-3 and DU145 cells that harbor pairs of deleted or inactivated p53 alleles and display a complete loss of p53 function, a condition often associated with decreased radiosensitivity.<sup>246-248</sup> However, in cultures of CaP cells retaining a functional p53 protein (LNCaP and 22Rv1) cellular senescence is the dominant form of death. Alternative stress response pathways controlled by this tumor suppressor, including cell cycle arrest, DNA damage repair, mitotic catastrophe and apoptosis, contributed significantly less to radiation-induced death.<sup>249</sup> Although autophagy is an additional form of cell death that has recently been reported to enhance the therapeutic sensitivity of PC-3 and DU145 cells,<sup>249</sup> neither of these cell lines express a functional p53. Thus, in the contemporary setting of earlier detection where complete p53 inactivation is rare, the induction of terminal growth arrest may be a primary mode of clonogenic death in radiation therapy of prostate cancer.<sup>250</sup> This would be consistent with the observation that in some patients complete regression of prostatic tumors can take more than a year after finishing therapy.

Recent evidence indicates that tumor regression after therapy might involve the phagocytosis of senescent cells by macrophages and the autophagy of senescent cells themselves. Pronounced alterations in the endosomal/lysosomal pathway are evident in senescent cells. Lipofuscin accumulation at the level of autophagic vacuoles accounts for the increased granularity observed in the side-scatter profile of senescent cells and is a hallmark of cellular senescence. Autophagy is an ancient pathway for homeostatic turnover of long-lived intracellular components and for nutrient acquisition during starvation and stress. Recently, p53 has been shown to positively modulate damage-regulated autophagy modulator (DRAM)-dependent autophagy.<sup>251</sup> Thus, therapy induced activation of p53, cellular senescence and autophagy may contribute to the tumor regression observed in vivo when investigators have experimentally “turned on” the p53 gene in tumor-bearing mice.<sup>252,253</sup>

The p53, p21 and Rb tumor suppressors are important senescence regulators. p53, p21 and Rb are transiently activated in response to stressors that induce 'premature' senescence in tumor cells. Transactivation of the cyclin-dependent kinase (CDK) inhibitor p21 appears most relevant as a downstream mediator of p53-dependent terminal growth arrest.<sup>254</sup> How p21 and other CDK inhibitors (e.g., p16<sup>Ink4a</sup>) promote senescence is not precisely known, but one well-studied mechanism for p21-induced senescence involves the activation of Rb. Rb family members (Rb, p107, p130) are corepressors of the E2F transcription factors required for cell proliferation. With an accumulation of CDK inhibitors and the onset of senescent arrest, Rb is converted to an active hypophosphorylated form that stably interacts with, and sequesters, E2F and other proteins that influence gene expression and promote cell cycle progression.<sup>255</sup> Notably, the cellular levels of p53, p21 and active Rb do not remain elevated after the onset of senescence, suggesting that molecular interactions required to initiate the senescent state may be dispensable for its maintenance. For example, conditional expression of p53, p21 or p16 causes cellular senescence in many settings, and the cells remain in a senescent state after promoter shutoff.<sup>256-258</sup> Because expression of another CDK inhibitor, p16, rises as p21 goes down, it has been suggested that p16 may be responsible for maintaining a stable growth arrest, while others contend that both p53 and p16 function in the maintenance of growth arrest in senescent cells.<sup>259</sup>

Another recent explanation for the permanence of cellular senescence is that p16 enables Rb to establish heterochromatin changes that maintain E2F-responsive promoters in an irreversibly silenced state and no longer dependent on the presence of p16 or Rb.<sup>255</sup> These unusual foci of heterochromatin are physically associated with tightly packed and trimethylated histone proteins (e.g., H3K9) and the histone methyltransferase Suv39h1.<sup>260,261</sup> Whether these p16/Rb-initiated modifications of heterochromatin are truly irreversible remains to be determined. Moreover, there is evidence that the premature senescence induced by chemotherapeutic drugs and radiation in tumor cells can occur in the absence of p53, p21 as well as p16, indicating that additional genes and pathways may also mediate damage-induced senescence of tumor cells.<sup>262</sup>

*Rb* inactivation is common in prostate cancer and generally precedes somatic alterations affecting the tumor suppressor genes *p53* and *PTEN*.<sup>183,185,263</sup> Interactions among these three major tumor suppressor genes appear to directly influence tumor development in the mouse prostate.<sup>264,265</sup> For instance, conditional deletion of an *Rb* allele specifically in the mouse prostate epithelium results in the development of focal hyperplasia but not tumorigenesis,<sup>266</sup> while acute inactivation of *PTEN* alone induces a p53-dependent cellular senescence.<sup>264</sup> In contrast, when combined with inactivation of the Rb family proteins (Rb/p107/p130) or *p53*, a loss of *PTEN* either elicits an accelerated rate of tumor progression or development of invasive adenocarcinoma in mice, respectively.<sup>264-266</sup> These transgenic studies establish that a p53-mediated senescence response restricts cell proliferation after loss of *PTEN* in the mouse prostate in vivo and that a concomitant loss of either *Rb* or *p53* is sufficient to bypass this impediment to tumor growth.

## Treatment of Prostate Cancer

The primary treatment strategy for non-metastatic CaP is generally either radiation or complete removal of the prostate (radical prostatectomy). Unfortunately, patients often relapse and aberrant growth of the prostate returns (or the neoplasia is not detected in its infantile stages). These patients are most often subjected to androgen ablation therapy in which either chemical or surgical castration is performed in concert with non-steroidal anti-androgens. Furthermore, since prostate cells are heavily reliant upon androgens for growth and/or survival, these cells generally die within a short period of time. Consequently, the prostatic tumor undergoes a complete remission for a period of approximately 18 months.<sup>267</sup>

After androgen ablation therapy, most tumors eventually relapse and begin to grow in the absence of testosterone; this event is known as hormone relapse and patients in this stage are said to possess hormone-refractory prostate cancer (HRPC). The expected lifespan of patients who progress to the point of HRPC is less than 24 months and the prognosis is invariably death.<sup>267</sup> Hormone relapse is almost always accompanied by an increase in prostate-specific antigen (PSA) levels. It is for this reason that measurement of PSA levels has become the primary means of detection for tumorigenicity within the prostate.

After hormone relapse, therapeutic options become more limited. Modern chemotherapy is just beginning to be commonly-utilized in the clinic. Until recently, it was believed that chemotherapy was relatively ineffective against CaP cells,<sup>268,269</sup> however, the development of the PSA test as a qualitative measurement of therapeutic efficacy soon refuted the notion that chemotherapy was ineffective in tumor cells of prostatic origin. Before the PSA test, efficacy of chemotoxic drugs was primarily monitored by more objective means (tumor size and/or volume), however it has now become evident that this method of assessing drug efficacy is both inaccurate and unreliable—reported response rates from the same drug were increased from 20% (tumor size) to nearly 75% (PSA levels) merely by changing the detection method used in these studies.<sup>199</sup> In light of these discoveries, there is a renewed interest in determining the therapeutic value of chemo-therapy in CaP patients.

Most often, CaP patients are administered a variety of chemo-therapeutic compounds to combat the disease.<sup>270-275</sup> Employing the use of these drugs, however, has been met with only modest success due to issues of toxicity and the drug-resistant nature of CaP. Efforts to minimize drug resistance should prove effective in extending the life expectancy of HRPC patients.

Drug resistance is manifested via a multitude of biomolecular events including inhibiting entrance of chemotoxic agents into the cell, loss of enzymatic activities involved in metabolizing the drug, and overexpression of ABC (ATP-Binding Cassette) drug efflux pumps, among others.<sup>276-278</sup> Recently, we and others have shown that aberrant activity of the PI3K/PTEN/Akt/mTOR signaling cascade can augment drug resistance in advanced CaP cells.<sup>279, 280</sup> These reports detail several potential mechanisms responsible for increased chemoresistance including PI3K-induced overexpression of the MRP-1 drug pump as well as activation of the mTOR molecule that is downstream of PI3K/Akt. Consequently, targeted inhibition of the PI3K/Akt signaling pathway is quickly gaining interest with regards to chemotherapeutic intervention in an array of cell types<sup>281,282</sup>

## Chemotherapy of Prostate Cancer

Chemotherapy agents used to combat CaP usually fall into three categories: (1) interchelating agents (2) microtubule stabilizing agents, and (3) alkylating compounds. Anthracyclines are antibiotic compounds derived from the *Streptomyces* species that are used to treat a variety of cancers including CaP. Daunorubicin, doxorubicin and epirubicin are all members of this class of compounds. Anthracyclines possess a number of mechanisms of action including intercalating between base pairs of DNA, creating free radicals that initiate DNA damage, and inhibition of the nuclear enzyme topoisomerase II.<sup>283</sup> By intercalating into DNA, anthracyclines inhibit both DNA and RNA synthesis.<sup>284,285</sup> Inhibition of topoisomerase II, which causes torsional strain on DNA causing strand breakage, was later proven to be an integral event in anthracycline-induced cell death.<sup>286</sup> Together, these modes of action initiate a series of events that can ultimately lead to the demise of a cancer cell

Mitoxantrone is an anthracenedione with a structure similar to doxorubicin but appears to have a better toxicity profile than that of anthracyclines.<sup>287,288</sup> Due to its similar structure to anthracyclines, it is not surprising that the mechanism of action of mitoxantrone is similar as well. Mitoxantrone is thought to induce apoptosis by both DNA intercalation and inhibition of

topoisomerase II activity.<sup>287,288</sup> Mitoxantrone has been investigated for its ability to induce death of CaP cells—these studies have concluded that mitoxantrone is effective in palliation of CaP symptoms, but is unlikely to prolong survival.<sup>287,288</sup>

The central premise behind all neoplasia is the uncontrolled nature of cell division. An accumulation of genetic events (i.e., mutations) that affect the cell cycle can lead to aberrant growth and cell division. Therefore, the process of mitosis whereby the mitotic spindle functions to divide chromatids to each daughter cell is a viable target in many cancers. Microtubules comprise the mitotic spindle and proper polymerization/depolymerization of these microtubules is essential for efficient progression through mitosis and subsequent cell division. Taxanes are a family of chemotherapeutic drugs that have the capacity to bind to microtubules, specifically  $\beta$ -tubulin, thereby preventing depolymerization.<sup>289,290</sup> This event is essential for cell division; as such, the use of taxanes has become prominent in cancer research, particularly CaP.<sup>291-293</sup> There are a growing number of taxanes currently available in the clinic including paclitaxel and docetaxel. Interestingly, these drugs may have an additional modes of action: they appear to induce the phosphorylation and inactivation of the anti-apoptotic molecule, Bcl-2.<sup>294,295</sup>

Estramustine is a stable conjugate of estradiol and nornitrogen mustard<sup>296</sup> that is commonly used in CaP patients. Estramustine was designed as an alkylating agent specifically for treatment in tumors of prostatic origin. The premise of the initial design of the drug was that the estradiol portion of the agent would facilitate uptake by steroid receptors, whereas the nitrogen mustard moiety would perform the alkylation; however, evidence exists to refute the notion that estramustine acts in this manner. It is thought that estramustine inhibits microtubule function and affects structural proteins within the nuclear matrix.<sup>297,298</sup> In high doses, estramustine is not well-tolerated,<sup>299</sup> however it is currently being evaluated in combination with other drugs at less toxic doses and results have been promising.

## Roles of PI3K/PTEN/Akt/mTOR, Raf/MEK/ERK and p53 Pathways in Prostate Cancer Drug Resistance

The PI3K/PTEN/Akt/mTOR signal transduction pathway is well-known for its roles in cell cycle progression, survival and growth of cells. Recent evidence suggests that this pathway may also contribute to the development of drug resistance in a variety of cell types.<sup>300-308</sup> Efforts to identify specific molecules that confer drug resistance will be the next wave of these types of studies. To this point, the data disproportionately implicates the PI3K/PTEN/Akt/mTOR pathway in the development of drug resistance. However, this pathway is capable of inducing such effects in a number of ways; as such, it is necessary to investigate potential means of resistance in these cell types.

Given the involvement of ABC drug transporters in acquired chemoresistance, we monitored the expression of prototypical members of the ABC superfamily of efflux pumps.<sup>281</sup> None of the CaP cell lines tested (LNCaP, 22Rv-1, DU145 or PC3) was positive for *MDR-1* gene expression. Interestingly, CaP cells with constitutive PI3K activity were positive for *MRP-1* expression, whereas PTEN-positive (DU145 wild-type) cells expressed minimal levels of this gene.<sup>281</sup> However, activation of PI3K in DU145 cells potentiated upregulation of *MRP-1*, indicating that the PI3K pathway may modulate expression of the *MRP-1* gene. The observations made on the RNA level were congruent with those reported in a recent publication where the authors showed that *MRP-1*, not *MDR-1*, was the predominant drug pump expressed in advanced CaP cells.<sup>281</sup> These trends were also observed on the protein level when various DN PTEN and constitutively-active PI3K mutants were observed to increase levels of the *MRP-1* gene product.<sup>281</sup> Collectively, the data identify *MRP-1* as a mediator of CaP drug resistance and an indirect mechanism responsible for its upregulation.

Wang and Beck were the first to hypothesize that p53 is a negative regulator of *MRP-1* expression.<sup>309</sup> However, to date, there is no report of any p53-binding motifs located within the *MRP-1* promoter region.<sup>310</sup> Therefore, one might hypothesize that repression of *MRP-1* by p53 occurs by an indirect means rather than an immediate interaction with the *MRP-1* promoter. Beck also reported that expression of the specificity protein-1 (Sp1) transcription factor is a strong activator of *MRP-1* expression.<sup>309</sup> Given that there are three Sp1 binding sites in the *MRP-1* promoter and that Sp1 activity has been reported to be PI3K-dependent,<sup>310</sup> it is plausible to suggest that Sp1 is critical for *MRP-1* expression in advanced CaP cells. p53 (LNCaP: p53-positive, DU145 and PC3: p53-negative) may be the dominant regulatory molecule governing MRP-1 expression, but PI3K activity and subsequent Sp1 activation, may be necessary for maximal expression of *MRP-1*.

To assess the role of MRP-1 in mediating chemoresistance in DU145 and PC3 cells, RNA interference was exploited to inhibit expression of this gene. The results presented in Lee et al., prove that inhibition of MRP-1 function, through downregulation of *MRP-1* expression, can sensitize *MRP-1*-expressing cells to chemo-therapeutic compounds.<sup>281</sup> The importance of these findings is significant because it provides a viable target for future antineoplastic drug formulations—agents synthesized to antagonize the activity of MRP-1 should increase the efficacy of chemotoxic drugs while having minor, if any, effects on the viability of cells not expressing *MRP-1*.

Lastly, it is imperative to consider the context of PI3K signaling in CaP cells. The PI3K/PTEN/Akt/mTOR pathway is most often accepted as a mediator of cellular survival, rather than proliferation (i.e., MAPK signaling). Within the prostate gland, the population of cells that undergo active proliferation is between 1-3%<sup>311,312</sup> and this observation may explain why diagnosis of CaP does not often occur before the latter stages of life. Although proliferation of CaP cells is slow, turnover of CaP cells appears to be slower—the loss of PTEN and upregulation of PI3K activity leads to a pheno-type whereby the cell does not undergo apoptosis when directed. Consequently, pathways that mediate survival, rather than proliferation (i.e., Raf/MEK/ERK signaling), may be more attractive targets for reducing tumor growth in CaP patients.

## MAPK Signaling and Drug Resistance Studies

The Raf/MEK/ERK signal transduction cascade is responsible for a number of cellular processes including proliferation, growth and differentiation. The relationship between Raf/MEK/ERK signaling and drug resistance is also well-documented. There are reports which propose that activation of the Raf/MEK/ERK pathway can increase the probability of survival in chemotoxic agents;<sup>313,314</sup> conversely, there is also an abundance of data which suggests that activation of ERK is a necessity for drug-induced apoptosis.<sup>315-319</sup> In prostatic disease, however, the role of ERK activation in chemoresistance remains controversial. At this time, there does not appear to be a single role for ERK in drug-induced cell death—instead, it seems that cell type and drug selection are two key factors in evaluating the role of ERK activation to drug treatments.

To examine the effects of ERK activity on the development of drug resistance in CaP cells, DU145 cells were stably-transfected with constitutively-activated mutants of both Raf-1 ( $\Delta$ Raf-1) and B-Raf (B-Raf G468A and B-Raf V600E).<sup>320</sup> In doing so, it was possible to ascertain the role(s) of Raf/MEK/ERK activation in altering the response of CaP cells to doxorubicin or paclitaxel treatment. Each of the constitutively-active mutants was able to increase activity of ERK, as measured by a phospho-specific antibody. Thus, it was then possible to move forward with these studies with the knowledge that the cell lines had increased activity of the Raf/MEK/ERK pathway when compared to non-transduced DU145 cells.

The effects of activation of the Raf kinase, via stable transfection of various DNA constructs, were then determined. The data,<sup>320</sup> were somewhat surprising because they showed that activation of the Raf/MEK/ERK pathway did not enhance the chemoresistance profile of DU145 cells. On the contrary, subsequent experiments have indicated that activation of this cascade upon restoration of WT p53, increased the sensitivity of the cells to both radiotherapy and chemotherapy.<sup>250</sup>

To confirm/refute the intriguing results, further experiments were performed. Those data demonstrate that inhibition of MAPK activity does little to enhance the killing capacity of doxorubicin and/or paclitaxel in DU145 cells. Together, these data confirm the data from previous studies and strongly suggest that activation of the Raf/MEK/ERK signaling cascade does not increase drug resistance in CaP cells. In fact activation of Raf/MEK/ERK may actually chemosensitize prostate cancer cells.

Initially, these results were somewhat surprising because our and other laboratories have previously reported<sup>321-323</sup> that activation of the Raf/MEK/ERK cascade provided protection from doxorubicin in the MCF-7 breast carcinoma and other cell lines. However, after a thorough literature search, it appears that activation of the Raf/MEK/ERK pathway has a pro-apoptotic effect when cancer cells are treated with chemotherapeutic agents in the majority of cell types. Interestingly, the pro-apoptotic action of drug-induced ERK activity is reported to be p53-dependent.<sup>324,325</sup> DU145 cells, however, possess a mutated p53 protein product which is insufficient in many of the activities associated with WT p53 activity (i.e., DNA damage-induced cell cycle arrest and p21 regulation, among others).<sup>326</sup> Therefore, it is quite feasible that activation of the Raf/MEK/ERK pathway is unable to induce chemoresistance in DU145 cells because of the absence of WT p53 function in this cell type. When a functional p53 gene was introduced into these cells, their chemo- and radiosensitivity increased dramatically as did the activation of the Raf/MEK/ERK cascade.

Intracellular signaling does not occur in a linear fashion. Instead, signaling between adjacent pathways is known to converge, cross and intersect one another in a complex, interwoven pattern that is generally undefined. Oftentimes, reports on crosstalk between pathways conflict each other, indicating that cell type must be considered when determining potential interactions within the cell. There are very few reports that have investigated the degree of crosstalk that occurs in cells of prostatic origin. Given the importance of the topic of these studies, it was vital to determine if potential crosstalk between the PI3K/PTEN/Akt and Raf/MEK/ERK pathways occurs and, if so, to what degree it affects drug resistance in CaP cells.

Dr. Jeffrey Kreisberg's laboratory (University of Texas Health Science Center, San Antonio) has demonstrated the importance of monitoring phospho-Akt levels as a prognostic tool for CaP patients.<sup>327,328</sup> In addition, they also report that decreased phospho-ERK levels are indicative of a poor prognosis.<sup>327</sup> Taken as a collective whole, the correlative data might suggest that activation of Akt is linked to the inactivation of ERK. This hypothesis was investigated further because preliminary data indicated that it may indeed be true.

It was soon discovered that inhibition of PI3K with LY294002 could cause increased levels of phospho-ERK.<sup>329</sup> This finding was subsequently verified using another inhibitor of PI3K, wortmannin. These results suggested that activated PI3K could mediate downregulation of the Raf/MEK/ERK pathway when active; on the contrary, inhibition of PI3K appears to activate the MAPK pathway.<sup>329</sup>

The complex phosphoregulation of Raf was described earlier; it is generally accepted that phosphorylation of S259 on Raf-1 is an event that inactivates the molecule from signaling downstream (to MEK).<sup>330</sup> To assess Raf activity in CaP cells, both DU145 and PC3 cells were assayed for their phospho-Raf-1<sup>S259</sup> content. Interestingly, PC3 cells displayed much higher

levels of inactivated Raf-1 than the DU145 cell line.<sup>329</sup> This observation supports the hypothesis that activated PI3K/Akt can suppress the Raf/MEK/ERK pathway in a manner that involves Raf-1 inactivation.

To further test the hypothesis that the PI3K/Akt pathway causes inactivation of ERK through phosphorylation of Raf-1, reciprocal co-immunoprecipitation assays demonstrated that Akt and Raf-1 were found in conjunction with one another in PC3 cells; however, this was not as apparent in the DU145 cell type.<sup>329</sup> DU145 and PC3 cells were also subjected to low concentrations of doxorubicin and/or paclitaxel over two months and resistant cells were selected. DU145 cells selected in drug displayed elevated high levels of phospho-ERK, but relative levels of phospho-Akt when compared to wild-type cells.<sup>329</sup> PC3 cells, however, were unchanged in terms of phospho-Akt (high levels) and phospho-ERK (low). Together, these analyses support the notion that the absence of PTEN plays a major role in the inactivation of Raf-1 in PC3 cells, but not DU145 cells. While the evidence presented to prove that activation of ERK to potentiate the anti-apoptotic effects of doxorubicin and paclitaxel is minimal, the literature certainly supports this premise.<sup>318,319,330-333</sup> Consequently, it appears that PI3K activation may have an additional mechanism of action to confer drug resistance to CaP cells—inactivation of the Raf/MEK/ERK kinase cascade.

The significance of the studies performed is several-fold and has implications for not only acquired chemoresistance, but also normal progression of prostatic disease. The loss of *PTEN* expression appears to be a major factor in a series of events that can lead to drug-resistance and HRPC. After *PTEN*-loss and consequent activation of PI3K, resistance to chemotherapeutic compounds can occur in a number of ways. First, activation of PI3K was shown to upregulate expression of the *MRP-1* drug efflux pump gene through an unknown mechanism. Secondly, PI3K can activate Akt which can deactivate Raf by phosphorylation on S259; inactivation of Raf shuts down Raf/MEK/ERK signaling, which has been shown to be essential for chemotoxic drug-induced death. Although there exist alternative mechanisms which the CaP cell employs to become resistant to antineoplastic agents, steps can be taken now to exploit this new knowledge so that better therapies are present in the future.

Activation of Raf-1 and/or B-Raf was not capable of increasing the cellular resistance to either doxorubicin and/or paclitaxel. Initially, this was a troublesome finding because most believe that Raf/MEK/ERK activation leads to an intracellular state that enables the cell to more effectively respond to chemotoxic compounds. However, upon further analysis, it was discovered that this mechanism of action is dependent on the activity of the tumor suppressor protein, p53. Pertinent to these studies is that DU145 cells are p53 mutants (PC3: p53-null). Taken together, this data helps explain why activation of Raf-1 and B-Raf is insufficient to sensitize DU145 cells to doxorubicin and/or paclitaxel. Therefore, chemotherapeutic resistance proves to be a complex process depending on activates of multiple signaling pathways.

## Radiosensitization of Prostate Cancer

Conventional-dose (72 Gy) external beam radiation is elected as a preferred treatment modality by many CaP patients.<sup>334,335</sup> However, there is evidence that conventional-dose radiation often does not provide complete tumor eradication with a resultant radiorecurrent prostate cancer and five year distant metastasis-free survival of less than 80%.<sup>336-338</sup> Given this problem, it is not surprising that radiotherapy dose escalation has been recommended for patients with poor prognostic features (e.g., positive surgical margins);<sup>339</sup> but the toxicity associated with dose-escalated therapy is not trivial and it is recognized that 5-10% of all radiotherapy patients develop severe acute or late effects.<sup>247</sup> Accordingly, there is intense interest in understanding critical determinants in the efficacy of irradiation (IR)-induced CaP cell killing and developing targeted therapeutic strategies for radiosensitization.

Curative radiation therapy aims at preventing tumor regrowth by inducing tumor cell death and loss of reproductive integrity. These processes together are referred to as clonogenic death. Clonogenic cells are defined as tumor cells possessing the capacity to produce a colony of progeny and therefore contribute to the regrowth of the tumor. These cells are also more commonly referred to in other types of cancer as tumor stem cells or cancer-initiating cells.<sup>340-346</sup>

Radiation therapy creates substantial DNA damage that is recognized by p53 and forces most cells to either undergo programmed cell death (e.g., apoptosis), necrosis or enter a state of reproductive death (i.e., cellular senescence). Radiation therapy is conventionally delivered in fractions of 1.8-2.0 Gy doses for 5 days a week until a total of 60-80 Gy is reached. After sequential doses, more than 99% of the malignant cells are typically killed. However, the surviving fraction may amount to more than a million cells per gram of tumor volume.<sup>347</sup> These survivors retain the potential to actively contribute to radiorecurrent prostate cancer and are a major target for radiosensitization. However, the cellular and molecular heterogeneity of CaP make this subpopulation of radioresistant cells a difficult target to strike using a single modality of adjunctive therapy.<sup>349</sup>

### Priming the p53 Pathway for Prostate Cancer Therapy

p53 activity is regulated by the E3 ubiquitin ligase MDM2, which binds and targets p53 for degradation.<sup>350</sup> Overexpression of MDM2 has been reported in prostate cancer cells and may serve to protect those cells from p53-mediated cellular senescence in response to therapy.<sup>350</sup> Thus, targeting MDM2 seems to be a reasonable target for sensitization to both chemotherapy and radiotherapy. Investigators have downregulated MDM2 using antisense and successfully sensitized LNCaP cells.<sup>351,352</sup> While antisense raised against MDM2 demonstrated that regulation of p53 is critical for sensitization, the use of antisense may prove to be difficult in the clinical setting.

Recently, a selective small-molecule inhibitor of MDM2-p53 binding, Nutlin-3 has been identified and characterized. Nutlin-3 disrupts MDM2 binding and p53 in cell culture at a concentration of 5-10  $\mu$ M or inhibits tumor growth when given orally at 200 mg/kg.<sup>353</sup> Nutlin-3 specifically activated the p53 pathway in numerous cancer cells possessing WT p53, inducing cell cycle arrest and apoptosis to varying extents.<sup>354</sup> Since Nutlin-3 uncouples p53 from MDM2-mediated degradation, treatment with the drug prior to therapy may increase steady-state levels of p53, effectively priming the cellular ability to respond maximally to radiation. Investigators first demonstrated that administration of Nutlin-3 prior to therapy can effectively sensitize WT p53 lung cancer cells.<sup>245</sup> We have recently shown that disruption of p53-MDM2 interactions by the small-molecule MDM2 antagonist Nutlin-3 can effectively sensitize prostate cancer cells to a clinically-relevant dose (2 Gy) of IR, provided these CaP cells express WT p53.<sup>250</sup> Moreover, the increase in clonogenic cell death was entirely attributable to an increased induction of p53-dependent cellular senescence.

While Nutlin-3 may radiosensitize some tumors by “priming” the p53 pathway, it seems clear that this strategy will not apply to all prostate tumors. p53 activation requires both MDM2 degradation and MDMX inactivation. Nutlin-3 does not disrupt MDMX binding of MDM2 and MDMX overexpression prevents p53 stabilization by Nutlin-3.<sup>354</sup> Nutlin-3 disrupts MDM2 binding and inactivation of p53 in cell culture models. The observations suggest that further development of small-molecule inhibitors of MDMX, or alternative molecular targets, may be useful in combination with Nutlin-3 to provide a clinically effective radioresponse. Moreover, p53 activators could have an adverse outcome in patients with p53 GOF mutant alleles and it will be essential to determine their p53 status prior to initiating treatment.<sup>355</sup> Because prostate cancer is slow growing and is now often detected while still localized, the

clinician may some day have the opportunity to individualize treatment based on knowledge of the patient's p53 status.

## AKT as an Adjunctive Target to p53 in Sensitization of Prostate Cancer

Small molecule inhibitors of PI3K such as LY294002 have been reported to effectively sensitize LNCaP and other prostate cancer cells.<sup>281,356</sup> Recently the novel Akt inhibitor, Perifosine, has been shown to have some effects on prostate cancer growth.<sup>357</sup> Thus, inactivating mutations of PTEN may limit therapy induced cell killing in prostate tumors due to unrestrained Akt activity. This may be related to the observation that the PI3K-Akt pathway effectively inhibits p53 by increasing its degradation via the MDM2 pathway.<sup>358</sup> More recently, however, it has been reported that activation of PI3K signaling results in the downstream activation of p53 and the p53-dependent senescence pathway.<sup>359-361</sup> This discrepancy may be the result of oncogenic stress stimulating p14, which would inactivate MDM2 regardless of the increased shuttling into the nucleus via Akt phosphorylation. Thus it is unclear whether targeting the Akt pathway in conjunction with p53 activation will synergize or antagonize the radioresponsiveness of prostate cancer cells. Future studies will be directed at resolving this controversy in order to understand how and when these tumor suppressor pathways interact to influence the radiosensitivity of CaP cells.

## Conclusions

The developments of complimentary or alternative treatment strategies to standard chemo- and radiotherapies are invaluable to increase survival in patients with metastatic CaP. After hormone relapse, nearly 100% of patients succumb to this disease within 18 months. During this period, chemotherapy is administered primarily for palliation of symptoms associated with disease. Given the success of chemotherapeutic intervention in other cancer types, it stands to reason that it can be an effective means of treatment for prostatic disease as well. However, defining the mechanisms of chemoresistance within the prostatic tumor will be the caveat that enables this notion to become a reality.

Studies that investigate the mechanistic behavior of androgen-independent CaP cells are of the utmost importance in discovering better treatments with higher levels of efficacy. The genotype of the tumor must be considered when developing treatment regimens for a CaP patient as multiple signaling pathways feed into each other. Fortunately, it appears that clinical oncologists are headed in this direction,<sup>361,362</sup> as many physicians are beginning to push for the development of diagnostic tools that can be utilized to determine specific mutations that are present in a particular person's tumor. Taking into account the research discussed in this review, it appears that genotyping tumors will be an effective means of developing better treatment strategies for patients with HRPc, as the absence of a single gene (i.e., *PTEN*) can make a substantial difference in the response of patients to chemotherapeutic agents. In Figure 4, an overview is presented of the sites of mutation which have been identified in prostate cancer which result in activation of the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways.

In previous publications, we have reported that the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK signaling pathways have synergistic effects which lead to the transformation of hematopoietic cells.<sup>363-369</sup> Recently, we reported that Akt activation can lead to inactivation of the MAPK pathway via Raf-1.<sup>329</sup> The results of these studies have importance in many arenas of prostatic research: disease progression and etiology, therapeutic efficacy and resistance to chemotoxic compounds.

PTEN, the negative regulator of PI3K signaling, is lost in a large percentage of prostatic carcinomas. Consequently, many patients with CaP harbor tumors with high levels of Akt signaling.<sup>370,371</sup> Our previous work demonstrated that aberrant Akt signaling can lead to

heightened levels of MRP-1 expression thereby causing increased resistance to doxorubicin and paclitaxel, two drugs commonly used to treat CaP patients.<sup>281</sup> Furthermore, we also demonstrated the role for Akt in the progression of CaP, namely inactivation of the Raf/MEK/ERK cascade.<sup>329</sup>

Signals transduced by the Raf/MEK/ERK cascade are generally thought to induce cellular growth, differentiation and/or proliferation.<sup>372</sup> Given that chemotoxic drugs target cells which divide rapidly, one would then hypothesize that cells with high levels of ERK activity would be more effectively targeted by these compounds. Conversely, the PI3K/Akt pathway is known for its role in cell survival—as such, cells with heightened Akt activity may not be as affected by treatment with chemotherapeutic drugs. Our previous reports<sup>281,363</sup> support this hypothesis.

Inactivation of ERK has been reported to coincide with higher Gleason grades and poorly differentiated CaP.<sup>373</sup> Due to its role in inducing differentiation, one hypothesis is that, in normal prostatic epithelial cells, ERK acts to induce cellular differentiation; however upon tumorigenesis, ERK is inactivated causing cells to differentiate much less efficiently, which is a hallmark of advanced CaP.<sup>371</sup> The data presented in our recent report support the notion that ERK is inactivated in advanced CaP.<sup>329</sup> Furthermore, evidence in this report<sup>329</sup> indicates that loss of PTEN and subsequent Akt activation is responsible for ERK inactivation via phosphorylation of Raf-1 on S259.<sup>329</sup> Lastly, others have shown that ERK activation is necessary for drug-induced death;<sup>374-376</sup> the findings herein suggest that administration of chemotoxic drugs may have minor deleterious effects in cells with heightened Akt activity (i.e., those with PTEN deletions), as they will serve to downregulate signaling within the Raf/MEK/ERK pathway. Tumor suppressors such as p53, PTEN and Rb play critical roles in prostate cancer growth. Clearly mutation at these tumor suppressors have multiple effects on cellular growth and death pathways. A summary of the effects of PTEN and p53 inactivation on PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK in prostate cancer is presented in Figure 5.

Resistance to chemotherapeutic compounds is a problem that continues to impede the success of modern drug regimens.<sup>377,378</sup> Circumventing this resistance is an area of intense investigation due to the short life-span of patients who are afflicted with HRPC. Previously, we and others have identified the PI3K/Akt pathway as a primary mediator of drug resistance in advanced CaP.<sup>281,379</sup> After loss or mutation of PTEN, Akt becomes constitutively active thereby causing phosphorylation and inactivation of Raf-1 on S259.<sup>329</sup> Since this event is one that inactivates Raf-1, signals ordinarily transduced by the Raf/MEK/ERK cascade are negated. Consequently, the cell loses its ability to further differentiate, while heightened Akt activity confers a survival advantage to the cell. Together, these events may lead to a tumor microenvironment where the individual cells are not only resistant to chemotherapeutic compounds, but also fail to differentiate efficiently. While chemotherapeutics have had minimal success so far, the future for effective treatment may involve combinations with drugs simultaneously targeting signaling pathways (Akt, MAPK, p53). Clearly combining targeted therapy with chemotherapy as well as other therapeutic approaches may have significant potential in prostate cancer therapy as prostate cancer is a disease based in part on mutations in critical signaling and cell cycle regulatory genes which aberrantly regulate prostate cell growth.

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## Abbreviations

AP-1, activator protein-1  
 ATM, ataxia teleangetasia mutated  
 ATR, ATM-related  
 CaP, prostate carcinoma  
 CDK, cyclin-dependent kinase  
 CREB, cyclic-AMP response element binding protein  
 DDR-1, discoidin domain receptor-1  
 DN, dominant negative  
 DSB, double strand break  
 FAK, focal adhesion kinase  
 FKHR (FOXO1), member of the forkhead/FoxO  
 4E-BP1-eIF, 4E binding protein-1  
 GAP, GTPase-activating protein  
 GOF, gain of function  
 GSK-3 $\beta$ , glycogen-synthase kinase-3 $\beta$   
 ILK, integrin-linked kinase  
 IR, ionizing radiation  
 KSR, kinase suppressor of Ras  
 LOF, loss of function  
 MDM2, murine double-minute 2  
 MP1, MEK partner-1  
 mTOR, mammalian target of rapamycin  
 p90<sup>RSK</sup>, p90 ribosomal S6 kinase  
 PDGF, platelet-derived growth factor  
 PDK, phosphatidylinositide-dependent kinases  
 PH, pleckstrin homology  
 PI3K, phosphoinositol-3 kinase  
 PKB, protein kinase B  
 PKC, protein kinase C  
 PP2A, phosphatase 2A  
 PIN, prostatic intraepithelial neoplasia  
 PTEN, phosphatase and tensin homologue deleted on chromosome ten  
 Rictor, mTOR complex  
 RKIP, Raf kinase inhibitor protein  
 SH2, Src-homology 2  
 SOS, son of sevenless  
 WT, wild type

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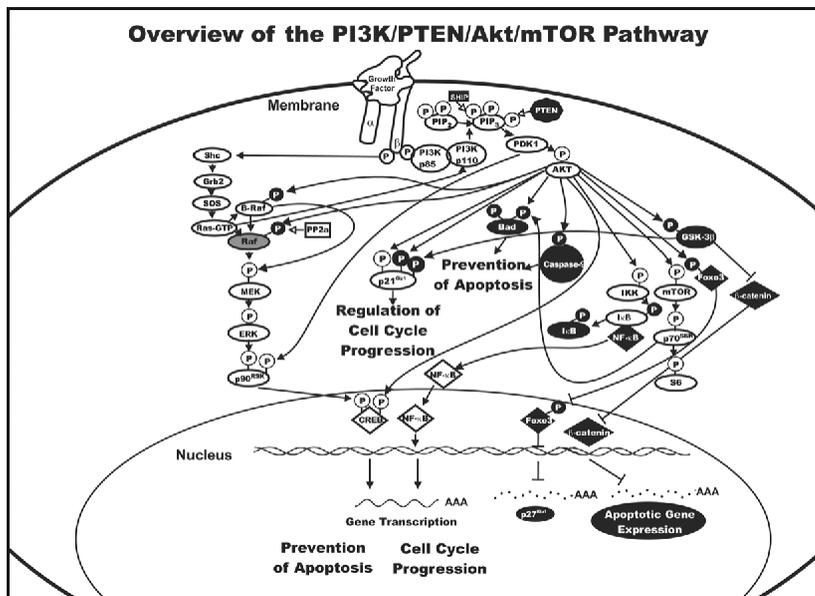
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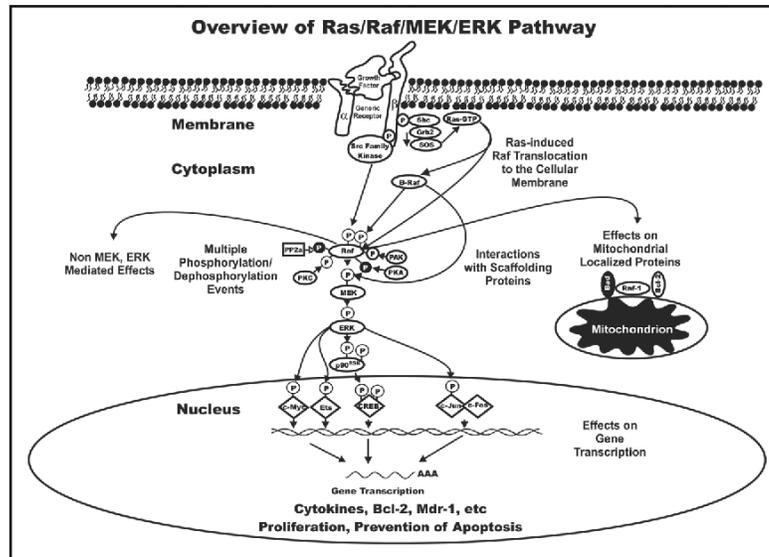
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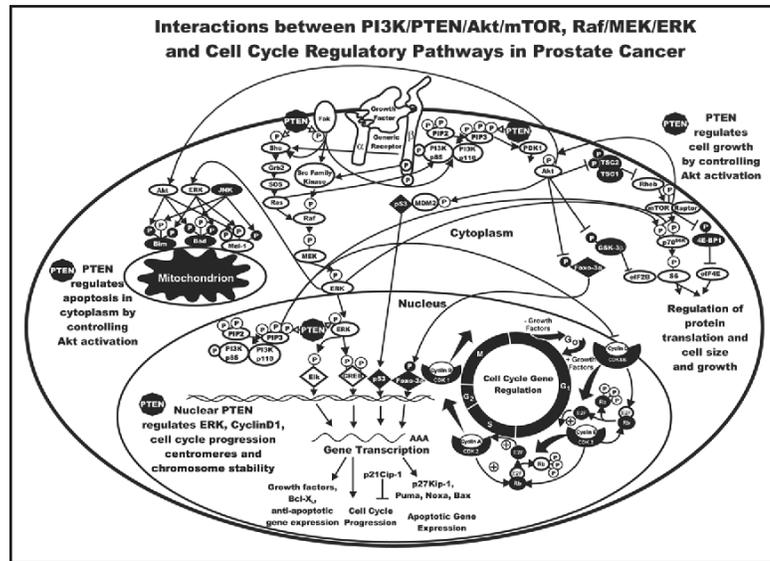
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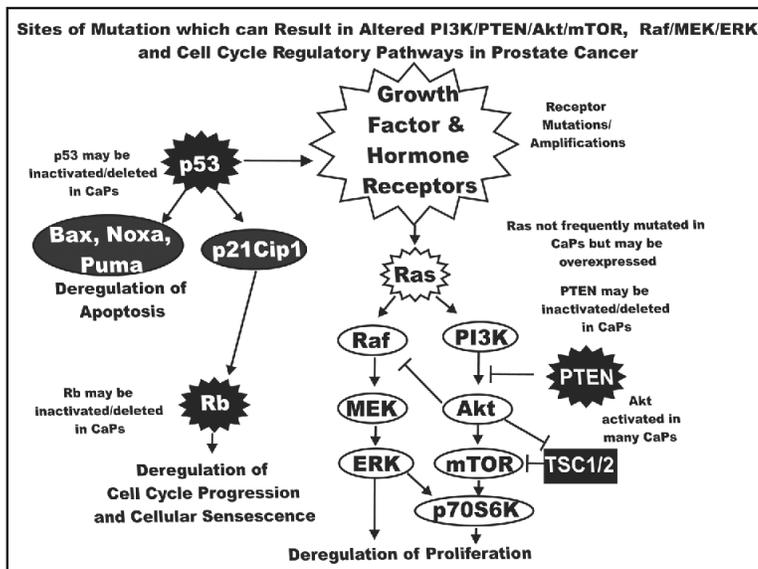
**Figure 1.** Overview of PI3K/PTEN/Akt/mTOR Pathway. The PI3K/PTEN/Akt/mTOR pathway is regulated by Ras as well as various kinases. The PI3K/PTEN/Akt/mTOR pathway is also activated after receptor ligation. The PTEN phosphatase (black octagon) inhibits activation of PI3K. Downstream of PI3K, Akt has many downstream targets that regulate cell growth and apoptosis. The transcription factors regulated by these pathways are indicated in diamond-shaped outlines. Dotted lines in front of AAA indicate that there is suppression of expression of some genes due to Akt phosphorylation of transcription factors such as Foxo3. Some of the interactions between the PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK pathways are also indicated.



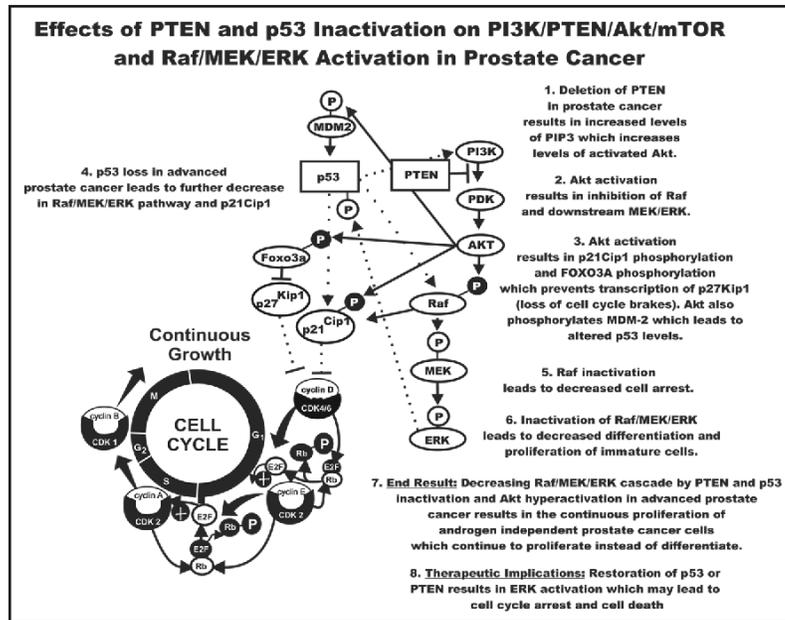
**Figure 2.** Overview of Raf/MEK/ERK Pathway. The Raf/MEK/ERK pathway is regulated by Ras as well as various kinases (PKC, PAK, PKA). Many kinases serve to phosphorylate S/T and Y residues on Raf. Some of these phosphorylation events serve to enhance Raf activity (black P in a white circle) whereas others serve to inhibit Raf activity (white P in a black circle). Moreover there are phosphatases such as PP2A, which remove phosphates on certain regulatory residues. The downstream transcription factors regulated by this pathway are indicated in diamond-shaped outlines. Raf can also exert effects which are independent of MEK/ERK and can interact with mitochondrial proteins to regulate apoptosis.



**Figure 3.** Interactions Between PI3K/PTEN/Akt/mTOR, Raf/MEK/ERK and Cell Cycle Pathways in Prostate Cancer. All of these pathways interact to regulate the induction of cell cycle progression and apoptosis. The Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways normally serve to suppress apoptosis while p53, which is induced by certain chemotherapeutic drugs and ionizing radiation, will result in increases in pro-apoptotic family members and in some cases, growth factors which may activate certain growth factor receptors. p53 can also regulate the transcription of many genes including p21<sup>Cip-1</sup>, Bax, Puma, Noxa and other genes which serve to regulate cell cycle progression and the induction of apoptosis. Furthermore, p53 activity can be altered by phosphorylation by ERK as well as MDM2 levels, whose activity is in turn previously regulated by Akt. Hence these pathways are interconnected and serve to regulate each other.



**Figure 4.** Sites of Mutation which can Result in Altered PI3K/PTEN/Akt/mTOR Raf/MEK/ERK and Cell Cycle Pathways in Prostate Cells. Mutations and deletions have been detected in p53, PTEN, Rb, AR and many other genes in prostate cancer. Many of these mutations and chromosomal trans-locations result in activation or inactivation of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR cascades as well as pathways which control cell cycle progression and apoptosis. The most frequently mutated genes are indicated by a starburst symbol.



**Figure 5.** Effects of PTEN and p53 Inactivation on PI3K/PTEN/Akt/mTOR and Raf/MEK/ERK Activation in Prostate Cancer. Some of the complex interactions between the PI3K/PTEN/Akt/mTOR, Raf/MEK/ERK, p53 and cell cycle pathways and how they influence cell cycle progression in prostate cancer are presented.