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REDOX-REGULATED RELB-AR AXIS MEDIATES PROSTATE SPECIFIC ANTIGEN EXPRESSION: INSIGHT IN PROSTATE CANCER RESPONSE TO RADIATION THERAPY

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REDOX-REGULATED RELB-AR AXIS MEDIATES PROSTATE SPECIFIC ANTIGEN EXPRESSION: INSIGHT IN PROSTATE CANCER RESPONSE TO RADIATION THERAPY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
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By

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ABSTRACT OF DISSERTATION

REDOX-REGULATED RELB-AR AXIS MEDIATES PROSTATE SPECIFIC ANTIGEN EXPRESSION: INSIGHT IN PROSTATE CANCER RESPONSE TO RADIATION THERAPY

Although the prostate specific antigen (PSA) test is widely used in clinical settings for prostate cancer (PCa) diagnosis and post-treatment follow-up monitoring, false positive PSA test results, which contribute to over-diagnosis of PCa, and false negative results, which miss some patients with aggressive PCa, remain problems of clinical importance.

Our study demonstrates that radiation therapy, which is widely used for treatment of localized PCa, generates TNF-α in tumor cells and stromal fibroblasts, redox dependently. Interestingly, TNF-α rapidly and transiently triggers the RelA-mediated NF-κB canonical pathway, but its effect on RelB expression is more robust and long lasting, which leads to sustainable suppression of PSA expression. TNF-α further amplifies endogenous reactive oxygen species (ROS) partially through NADPH oxidase activation and mediates redox-dependent downstream signaling pathways. Addition of the NADPH oxidase inhibitor or ROS scavengers such as superoxide dismutase (SOD) mimetic can abrogate TNF-α-mediated suppression of PSA expression by inhibiting the RelB-AR axis. Treatment with TNF-α suppresses PSA expression and it confers minor yet statistically significant protection to LNCap cells against irradiation, indicating that radiation-induced TNF-α may not only interfere with the PSA-based PCa diagnosis and post-treatment monitoring but may also diminish the efficacy of radiotherapy.

In addition, we uncover a role for RelB in suppressing PSA expression at the advanced stage of PCa, which could be a mechanism for the low PSA level in some patients bearing aggressive PCa. Experiments with both RelB overexpression and siRNA knockdown indicate that RelB negatively regulates androgen receptor (AR) and PSA levels in human prostate cancer, LNCap, cells. RelB directly interacts with AR to form a complex on the enhancer elements of the PSA promoter. Thus, the RelB-AR axis is an important contributor to PSA suppression at the advanced stage of PCa.

Overall, this study is the first to reveal a redox-mediated association among radiation-generated TNF-α, activation of the RelB-mediated alternative NF-kappaB pathway and PSA suppression. This mechanistic information provides new insights with practical and clinical implications for PSA-based PCa diagnosis and post-treatment monitoring as well as redox intervention in radiation therapy.

KEYWORDS: radiation, prostate cancer, RelB-AR axis, PSA, oxidative stress

Lu Miao
Student's Signature

July 20, 2013
Date

REDOX-REGULATED RELB-AR AXIS MEDIATES PROSTATE SPECIFIC ANTIGEN EXPRESSION: INSIGHT IN PROSTATE CANCER RESPONSE TO RADIATION THERAPY

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Chapter One

Introduction

Cancer is a major public health issue around the world and accounts for about 25% of all deaths in the United State. Prostate cancer accounts for close to 29% of newly diagnosed cancer cases and 9% of cancer death in men in 2012 [1]. The common forms of treatment for prostate cancer are surgery, ionizing radiation (IR) therapy, chemotherapy and hormone management [2]. Radiation therapy may be effectively applied if the tumor is localized to the original site in the body or as a part of curative therapy to prevent cancer recurrence after surgical removal of the primary tumor. Unfortunately, as many as 30-40% of prostate cancer patients treated with radiation would have disease recurred and progressed to the advanced stages [2]. The presence of radiation-resistant prostate cancer cells and cancer stem cells, the complexity of tumor microenvironment, such as hypoxia, inflammatory cytokine and growth factor secretion, as well as the elevated relevant receptors expression are contributing factors that influence the outcomes of radiation therapy.

The effects of radiation therapy should not be considered just in terms of isolated cells since the entire tissue has a role to play in determining the response of individual cells to any regulatory or damaging signals [3, 4]. The localized release of radiation energy generates free radicals, mainly via ionization of water, which constitutes about 80% of cells, and produces various reactive oxygen species (ROS). The ROS can then rapidly diffuse and react with other molecules to damage DNA, protein and lipid targets. This indirect effect of IR, such as X-Rays and γ–Rays, is expected to cause a majority of radiation-induced damage [3, 5, 6].

Different types of cells within tumor tissue are subject to complex regulatory mechanisms depending on their interactions with other cells and cellular products that comprise their microenvironment. Many proteins are sensitive to oxidative modification, making ROS important signaling molecules when an organism is exposed to IR. Aerobic organisms are continuously exposed to a modest level of oxidative stress, which provides the essential signaling functions for maintaining metabolic homeostasis in mammalian cells [7]. Because ROS plays crucial dual roles in inducing cancer development (initiation, promotion and progression) and maintaining metabolic homeostasis, both pro-

oxidant and antioxidant-based agents have been developed for cancer prevention and treatment [8-12].

One of the major obstacles in radiation therapy for prostate cancer is that tumor cells that are initially sensitive to radiation treatment gradually develop resistance to radiotherapy. Even though the development of radiation resistance in human tumors is not fully understood, extensive research indicates that antioxidant enzyme-mediated adaptive responses to IR are partially involved in this process [13-15]. IR-stimulated inflammatory factor production can not only alter redox status within tumor microenvironment directly but also activate a wide spectrum of genes, which regulate anti-apoptosis, invasion and angiogenesis pathways and confer radioresistance to tumor cells. In this introductory chapter, commonly elevated cytokines and growth factors, such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β), as major mediators of IR response found in the prostate cancer after radiation therapy will be reviewed and different redox signaling pathways and redox sensitive transcription factors controlled by these proteins will be discussed. Biological significances of such information can be particularly useful in understanding the development of cancer radioresistance to improve radiation therapeutic effects in humans.

Radiation in prostate cancer treatment

Ionizing radiation and radiation therapy

IR, which is both a carcinogen and a therapeutic agent, can be generated from radioactive materials, X-ray tubes and particle accelerators or present in the environment [3]. Cancer radiotherapy is the medical use of ionizing radiation to control or kill malignant cells. For prostate cancer treatment, radiation is most commonly given from an external source (external beam radiotherapy) but may also be administered by inserting small radioactive seeds directly into the tumor (brachytherapy) for some men with early prostate cancer [2]. External beam radiotherapy is much more widely used than brachytherapy. Delivery of a lethal dose of radiation to the tumor lesion while minimizing damage to normal surrounding tissues is one of the major challenges in radiotherapy. Progress has been made in imaging technology to facilitate the accuracy

and precision of radiotherapy, especially with the use of 3-dimensional conformal radiation therapy (3DCRT) and intensity modulated radiation therapy (IMRT) [16, 17].

External beam radiotherapy delivers one of two types of radiation, photons, such as X-rays and γ-rays, or charged particles, such as protons, to damage the DNA and other macromolecules of cancerous cells. X-rays are generated extranuclearly from X-ray machines whereas γ-rays are produced intranuclearly from radioactive materials. While they differ in the source of generation, X-rays and γ-rays share the same radiophysical properties [4, 5, 18] (Figure 1.1). Direct action of IR refers to direct interaction of radiation beams or particles with critical target molecules in cells, such as DNA, to cause various types of damages in DNA structure, leading to lethal chromosome aberrations [19, 20]. Indirect action of radiation are the multicellular effects by water radiolysis that produce free radicals, such as hydrated electrons (e-aq), ionizied water (H_2O^+), hydroperoxyl radical (HO_2•), hydrogen radical (H•), and hydroxyl radical (•OH), which can diffuse far enough to reach and damage the DNA, protein and lipid targets [3-5, 20]. Both direct action and indirect action of IR are closely linked, for example, direct damage to DNA by IR can induce ROS generation via histone H2AX-mediated mechanisms involving NADPH oxidase 1 (NOX1) and ras-related C3 botulinum toxin substrate 1 (Rac1) GTPase [21]. To a large extent, it is these free radicals that break chemical bonds, produce chemical changes, and initiate the chain of events that results in the final expression of biological damage. Oxygen is known to sensitize the tumor and oxygen molecules present in the tumor can react with free radicals (R•) to produce organic hydroperoxide (RO_2•), which is a nonrestorable form of the biological target that "fixes" the radiation lesion [4, 5]. This so-called oxygen effect and the associated reoxygenation strategy may enhance the radiation-induced cell killing.

Radioresistance: an important impediment in prostate cancer treatment

The development of resistance to radiation is one of the most important obstacles in prostate cancer radiotherapy. Thus, understanding of mechanisms of radioresistance is quite important for developing strategies that can either sensitize tumor cells to radiation treatment or protect normal tissue from radiation damage, which can be of significant benefit to patients. Some of the molecular entities associated with the development of radioresistance have been identified in prostate cancer although the underlying

mechanisms are still not fully understood [22]. For example, a novel tumor suppressor gene DAB2IP (DOC-2/DAB2 interactive protein) is a member of the RAS-GTPase-activating protein family and downregulation of DAB2IP in prostate cancer cells not only leads to epithelial mesenchymal transition (EMT) but also results in resistance to IR [23]. A proteomics study comparing individual parental cell lines with three ionizing radiation resistant prostate cancer cells lines revealed higher levels of androgen receptor (AR), epidermal growth factor receptors (EGFR) and activation of their downstream pathways, such as Ras- mitogen-activated protein kinase (MAPK), phosphatidyl inositol 3-kinase (PI3K)-AKT and janus kinase-STAT in the IRR cell lines [24]. Mutations in dominant tumor suppressor gene or constitutive activation of some prosurvival pathways, such as NF-κB pathway, Ras-MAPK and PI3K pathways, have also been correlated with resistance to ionizing radiation-induced cell killing [23-27].

The neuroendocrine differentiation (NED) of prostate cancer cells is closely correlated with radioresistance [28, 29]. In the prostate gland, neuroendocrine (NE) cells are less than 1% of total epithelial cells compared with the luminal and basal cells; however, the number of NE-like cells increases in advanced prostate cancer [30, 31]. Fractionated ionizing radiation can induce NED in the LNCaP prostate cancer cell line and in patients by activation of cAMP response element binding protein (CREB) and cytoplasmic sequestration of activating transcription factors 2 (ATF2) [28]. NE-like cells can de-differentiate back to a proliferating state, which may contribute to tumor recurrence [28, 32]. The neuroendocrine-like cells secrete a variety of factors, including parathyroid hormone–related peptides, serotonin, calcitonin, bombesin-related peptide, and neurotensin that enhance DNA synthesis, proliferation, and migration of prostate cancer cells *in vitro* [29]. The selective antagonist to the high-affinity neurotensin receptor 1 (NTR1) can sensitize prostate cancer cells to ionizing radiation by inhibiting downstream signaling events such as EGFR and *Src* activation [29]. Both of these two independent lines of studies suggest that the process of NED and/or the specific receptor to the protein secreted by NE-like cells may represent possible intervention opportunities to enhance the sensitivity of prostate cancer to radiotherapy. Interestingly, by utilizing LNCaP cell clones with stably overexpressed manganese superoxide dismutase (MnSOD) with lower superoxide levels and higher H_2O_2 levels, Quiros-Gonzalez. *et al* showed that

MnSOD upregulation was sufficient to drive NE differentiation, resulting in androgen independence and cell survival in prostate cancer cells [33]. It is believed that the balance between $O_2^{\bullet-}$ and H_2O_2 can determine pathways that drive the NED process [33]. Thus, it is conceivable that MnSOD might affect NED by modulating the level of H_2O_2 and balance between $O_2^{\bullet-}$ and H_2O_2. Further investigation on the roles of MnSOD in regulating prostate cancer cell NED and significance of NED in prostate cancer radioresistance and recurrence may lead to new discoveries that can be explored to overcome radioresistance.

Reactive oxygen species and prostate cancer

Reactive species, which include ROS and reactive nitrogen species (RNS), can be categorized into two groups: free radicals that contain one or more unpaired electrons, such as superoxide ($O_2^{\bullet-}$), •OH, nitric oxide (NO•), and non-radicals, such as H_2O_2. To maintain a delicate redox homeostasis, biological organisms are endowed with a complex intracellular "redox buffer" network including both enzymatic and non-enzymatic antioxidants. The major enzyme defense system against ROS includes SOD, catalase, glutathione peroxidase (GPx), peroxiredoxin (Prx) and glutathione S-transferase （GST） [34]. In addition to these antioxidant enzymes, small thiol-containing peptides, such as glutathione (GSH), glutaredoxin (Grx) and thioredoxin (Trx) systems also help to scavenge ROS and maintain appropriate redox homeostasis [12, 35].

The redox status (oxidizing/reducing conditions) of cells can regulate various transcription factors/activators such as AP-1, nuclear factor kappa B (NF-κB) and p53, thereby influencing target gene expression and modulating cellular signaling pathways. Appropriate levels of ROS and RNS are necessary for normal physiological function of the living organisms [36]. The increase in production of reactive species and/or the decrease in antioxidants can lead to oxidative stress, which can damage DNA, inhibit cellular enzyme activities and induce cell death through activation of kinases and caspase cascades [37-39]. Oxidative stress resulting from an imbalance between pro-oxidant and antioxidant in favor of the former is believed to play a critical role in prostate carcinogenesis and prostate cancer progression (reviewed in [40-42]).

Sources of reactive oxygen species

ROS derived from incomplete reduction of oxygen can be produced either endogenously (e.g., mitochondria respiration) or exogenously (e.g., ionizing radiation) [18, 43, 44] (Figure 1.2). The most important endogenous source of ROS is the mitochondrial electron transport chain [45]. ROS are produced as an inevitable byproduct of oxidative phosphorylation. The electrons leak from some components of the mitochondrial ETC, especially from complex I (NADH-dehydrogenase) and complex III (ubiquinone-cytochrome b), leads to the one electron reduction of O_2 and generation of $O_2^{\bullet -}$. Exogenous IR (i.e., 10 Gy of X-rays) can induce a time-dependent increase in the mitochondrial ROS level and raise mitochondrial membrane potential, mitochondrial respiration, and mitochondrial ATP production, which are indicative of upregulated mitochondrial ETC function [46]. Superoxide can be dismutated by SOD to yield hydrogen peroxide and O_2. In the presence of transition metal ions, especially iron ions, hydrogen peroxide is subsequently converted through Fenton and Haber-Weiss reactions to a hydroxyl radical, which is the most toxic form of ROS, leading to various types of lipid peroxidation, protein modification and particularly oxidative DNA damages, such as 8-hydroxy-deoxyguanosine (8OH-dG) [4]. IR-induced ROS/RNS generation, which occurs within minutes after irradiation, is inhibited by mitochondrial permeability transition inhibitor cyclosporine A and is absent in the mitochondria-deficient ρ^0 cells, indicating that mitochondria are the primary source of radiation-induced reactive species [47]. Mitochondrial dysfunction that causes persistent oxidative stress may contribute to radiation-induced genomic instability [48].

Somatic mutations in the mitochondrial genome are relatively frequent events in prostate cancer. Certain mutations are associated with elevated prostate specific antigen (PSA) levels [49] or increased degree of tumor malignancy in prostate cancer patients [50, 51]. The circular, multi-copy mitochondrial DNA (mtDNA) is a sensitive target for most exogenous carcinogens, including IR, and mtDNA damage is implicated as having a causal role in oncogenic transformation and metastasis [52]. Petros. *et al* provided convincing evidence that prostate cancer patients have a significantly increased frequency of functionally important cytochrome oxidase subunit I (COI) mutations and the introduction into prostate cancer cells of a mtDNA ATP6 T8993G mutation, which

inhibits oxidative phosphorylation and increases ROS production, increased *in vivo* growth of these cells as compared to the wild-type (T8993T) cybrids [53]. Compared to nuclear DNA, mtDNA is more susceptible to radiation-induced loss of integrity due in part to the lack of protective histones, an inefficient DNA repair system, and continuous exposure to the mutagenic effect of ROS [54], which is exacerbated by GSH depletion in mitochondria [52]. ROS-induced mtDNA damage can alter polypeptides encoded by mtDNA for respiratory complexes, resulting in further decreased electron transfer activity and increased ROS generation, thereby establishing a "vicious cycle" of oxidative stress [55] and decline in mitochondria energy production after initial oxidative damage of mtDNA [56]. Considering the genetic association between mtDNA mutations and prostate cancer, quantitative traits such as PSA levels and Gleason score as well as high-quality sequencing to detect differences in mtDNA may help clinicians to monitor prostate malignant transformation, tumor progression and metastasis.

ROS can also be generated by other enzymes, such as xanthine oxidase (XO), membrane-associated NOX and cytochrome P450 in endoplasmic reticulum and oxidases in peroxisomes [57]. The NADPH oxidase family, which catalyzes the NAD(P)H dependent reduction of molecular oxygen, is responsible for the generation of superoxide anion, which is then dismutated to form hydrogen peroxide. The association of NOX enzymes with prostate cancer growth and malignant phenotype has been extensively reviewed [42, 58]. Importantly, NADPH oxidase has significant therapeutic implications in prostate cancer radiotherapy. A useful strategy for prostate cancer treatment is to sensitize cancer cells to radiotherapy by specifically activating NADPH oxidase in prostate tumor cells, which already have high levels of oxidative stress, and pushing prostate cancer cells over to death while sparing normal cells that are capable of maintaining redox homeostasis through adaptive responses [59]. Compared with uncharged diffusible hydrogen peroxide, superoxide radicals exert signaling functions locally with a shorter lifespan. Low levels of hydrogen peroxide are utilized for many signaling pathways, such as the one for cell survival [60], and are responsible for integrating redox homeostasis and disulfide formation in the endoplasmic reticulum [61]. However, exogenous H_2O_2 may strongly enhance lysosome-dependent radiation-induced apoptosis in PC-3 human prostate cancer cells. A combined treatment with X-rays and

H_2O_2 can injure the mitochondrial cytoplasmic organelles and lead to production of ROS and apoptosis [62]. Depending on the prostate cancer cell type and the level of the intracellular ROS, IR-induced oxidative stress can lead to very different consequences, ranging from elevated proliferation, adaptive response, to cell injury, senescence and death.

Reactive oxygen species and prostate cancer progression

Increased oxidative stress plays a significant role in several physiological situations such as aging and aging-associated diseases. Prostate cancer cells generally have a higher level of oxidative stress as compared to normal prostate cells, and the level of oxidative stress is associated with prostate cancer occurrence, recurrence and progression [58, 63, 64]. It has been demonstrated that, at an early stage of cancer development, tumor cells are exposed to high oxidative stress in part due to the inhibition of various antioxidant enzymes activities (Figure 1.3). Consistently, lower antioxidant enzymes, such as MnSOD, Cu/ZnSOD, catalase [63, 64], and defects in several classes of GSTs [65] have been observed in prostate adenocarcinoma as compared with benign prostate cells and tissues. However, after cancer has progressed, ROS partially renders cancer cells more dependent on the function of antioxidant enzymes, such as SODs, to protect from damages caused by increased levels of superoxide radicals [66, 67]. Our laboratory has provided *in vivo* evidence and proposed the underlying molecular mechanism by which p53 differentially regulates the MnSOD expression between early and advanced stages of cancer [68]. A meta-analysis involving 8,962 subjects has been performed to derive a more precise estimate of the association between prostate cancer risk and MnSOD $Val^{16}Ala$ polymorphism that disrupts proper targeting of the enzyme from cytosol to mitochondrial matrix. The results suggest that the Ala allele of MnSOD gene is a low-penetrance susceptible gene in prostate cancer development, especially in Caucasians [69]. It will be interesting and potentially useful to investigate the association between this specific gene polymorphism and radiosensitivity in prostate cancer patients.

Although the protein levels of various antioxidant enzymes or signaling molecules are associated with cellular redox status, activation/inhibition of enzymatic activities and redox modification of those proteins during redox signaling or in response to cellular redox change in specific cellular compartments will play a more dominant role (Table

1.1). For example, it has been suggested that redox-sensitive molecule Trx1 functions as a protective cellular antioxidant and its upregulation protects cancer cells from oxidative stress [70]. However, despite the significant increase in its protein level, oxidation of nuclear Trx1 resulted in a loss of antioxidant activity, which clearly demonstrated the redox imbalance and an adaptation of prostate cancer cells to oxidative stress [71]. Therefore, characterization of redox-sensitive protein structure and cellular localization, identification of potential redox modifications based on structure information and modeling strategies and investigation of different functions before and after modification will provide insightful knowledge of cellular redox status at each stage of cancer development.

Based on the biomedical property of increased ROS and altered redox status in cancer cells, many avenues of research have been proposed to modulate the unique redox regulatory mechanisms of cancer cells for therapeutic benefits [9]. Mitochondrial ROS have been shown to promote production of proinflammatory cytokines [72] and fuel NLRP3 inflammasome activity [73]. NLRP3 inflammasome, a molecular platform triggering innate immune defense to cellular danger, senses mitochondrial dysfunction and links mitochondrial damage with inflammatory diseases [73-75]. Targeting prostate cancer cells by ROS-mediated mechanisms as a radical therapeutic approach has been proposed previously [9, 40]. Blocking androgen-induced ROS production by inhibiting polyamine oxidase could delay prostate cancer progression and death in animals developing spontaneous prostate cancer [76]. Compared with reducing ROS level at early stage of prostate cancer development, a highly oxidizing condition is strongly cytotoxic and is the primary mechanism for tumor cell killing by radiation therapy and some chemotherapeutics, such as taxol and adriamycin. Since tumor cells are under more oxidative stress and normal cells usually carry higher redox buffering capacity, specific mild prooxidants, such as parthenolide, have shown to be a redox-modulating reagent capable of selectively pushing the tumor cells beyond the tolerance to oxidative stress and sensitizing cancer cells to radiation induced cell killing [59].

ROS are not only involved in radioresistance but also implicated in prostate cancer progression and castration resistance. Growth and proliferation of castration-resistant prostate cancer is mediated by gain-of-function changes in the AR and AR reactivation.

MnSOD down-regulation is directly responsible for AR reactivation in prostate cancer and occurs through a ROS-mediated mechanism [77]. Masaki Shiota. *et al* have extensively reviewed both the effects of AR signaling on oxidative stress and the effects of oxidative stress on AR signaling in the context of prostate cancer, especially castration resistant prostate cancer [78]. Castration-induced oxidative stress may promote AR overexpression through transcription factor Twist1 overexpression, which may result in a gain of castration resistance [79]. Thus, modulating redox status to sensitize cells and overcome radioresistance may result in castration resistance, which diminishes therapeutic benefits of the redox modulation. Thus, it is necessary to determine the stage of prostate cancer development and AR signaling carefully before applying redox intervention strategies.

Role of reactive oxygen species in a reciprocal interaction between the stroma and the prostate cancer cells

It becomes increasingly clear that the microenvironment of prostate cancer cells are crucial to their survival, progression, metastasis (reviewed in [80-83]) and resistance to chemotherapy and/or radiotherapy. Redox status within such a microenvironment is complicated at different stages of prostate cancer development, due to the considerable heterogeneity with respect to cellular composition of the stroma and tumor. In addition to highly reactive free radicals generated from IR, it has been well documented that stromal components, such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and endothelial cells, enhance oxidative stress, which promotes tumor progression [84, 85].

As the most abundant cell type in the microenvironment of solid tumors, fibroblasts are particularly prominent in prostatic carcinoma (Figure 1.4). The origin of CAFs and their significance in determining the cancer aggressiveness was elegantly demonstrated previously [35, 85-87]. Cunha. *et al* have shown that CAFs contribute to prostate tumor growth and metastatic potential. Human prostatic CAFs grown with initiated human prostatic epithelial cells dramatically stimulated the growth and altered histological characteristics of the epithelial cell population. However, this effect was not observed when normal prostatic fibroblasts were grown with the initiated epithelial cells under the same experimental conditions [87].

Radiation-induced alterations in metabolic oxidation/reduction and signal transduction have been reviewed previously [6]. Ogawa. *et al* found that ROS formation increased immediately after irradiation and continued for several hours, resulting in the production of 8-oxoguanine (8-oxoG), which is a product of oxidative DNA damage [88]. Persistence of ROS-induced DNA damage could lead to deleterious mutations. Oxidative damage in DNA is repaired mainly via the base excision repair (BER) pathway [89-91]. To allow time for DNA repair, the cells activate cell cycle checkpoints, leading to cell cycle arrest and preventing the replication of defective DNA with unrepaired damages [91]. The BER pathway is initiated by removal of the base by DNA glycosylases, leaving an intact abasic site (AP site). Subsequently, AP endonuclease 1 (APE1/Ref1) nicks the damaged DNA strand upstream of the AP site, creating a 3'-hydroxyl terminus and a 5'-deoxyribose phosphate group flanking the gap [92, 93]. APE1/Ref-1 (APE1) possesses not only DNA repair functions but also transcriptional regulatory activities, controlling cellular response to oxidative stress [94]. It modulates the intracellular redox state by inhibiting ROS production. APE1 has been identified as a protein with nuclear redox activity, inducing the DNA binding activity of several transcription factors, such as AP-1 [95], NF-κB [96], hypoxia-inducible factors-1 (HIF-1α) [34], p53 [97, 98], Myb and ATF/CREB family [99] (reviewed in [94]). Thus, while IR-induced ROS lead to oxidative DNA damage, its repairing processes to remove those damages can also contribute to cellular redox status, at least in part, through APE1/Ref-1 functions.

Due to the diffusibility and abundance, multiple reactive oxygen species and inflammatory mediators associated with aging, infection or ionizing radiation exposure may provide a permissive environment for cancer development. Compelling experimental and clinical evidence indicates that ROS mediated stromal-epithelial interactions in both normal and malignant prostatic environments involve a number of soluble factors and their corresponding receptors [80]. Extracellular superoxide dismutase (ECSOD) plays predominant roles in scavenging superoxide in the extracellular space where redox state regulates intracellular signaling or tumor growth. ECSOD-derived H_2O_2 can promote vascular endothelial growth factor (VEGF) signaling in caveolin-enriched lipid rafts and stimulate endothelial cell migration and proliferation through oxidative inactivation of protein tyrosine phosphatases (PTPs), such as density-enhanced protein tyrosine

phosphatase-1 (DEP-1) and PTP1B [100]. VEGF is critical for not only angiogenesis but also prostate cancer-mediated osteoblastic activity [101]. Ionizing radiation modulates VEGF expression through multiple MAPK dependent pathways [102] and enhances glioma cell motility through vascular endothelial growth factor receptor 2 (VEGFR2) signaling pathways [103]. Since prostate cancer cells lack the expression of specific VEGF receptors, especially VEGFR2, IR-induced VEGF are more likely to promote prostate cancer progression indirectly through their functions in stromal cells, in particular, endothelial cell survival and as a chemotactic agent within the tumor microenvironment [101]. Besides endothelial cells, CAFs can also exert their cancer promoting roles through release of growth factors, such as TGF-β and epidermal growth factor (EGF) as well as chemokines [84]. Recent advances in studies on metabolic communications between tumor and stromal cells attract more and more research interest. Oxidative stress dependent mono-carboxylate transporter 4 (MCT4) expression in CAFs is closely involved in a stromal-epithelial lactate shuttling [104]. According to a recently proposed model, increased ROS in CAFs drives tumor-stromal co-evolution, DNA damage and aneuploidy in cancer cells. Specifically, loss of stromal fibroblast caveolin-1 induces ROS, leading to the removal of defective mitochondria from CAFs by mitophagy and autophagy. CAFs provide nutrients, such as lactate, to stimulate mitochondria biogenesis and oxidative metabolism in adjacent cancer cells (the "reverse Warburg effect") [86]. Pavlides. *et al* provided very detailed information on how CAFs accelerate tumor growth and metastasis via oxidative stress, mitophagy and aerobic glycolysis [105]. The multiple roles of ROS in these new metabolic coupling interactions and models suggest that certain redox modulation based therapeutic methods can be helpful when used in combination with traditional radiotherapy in prostate cancer treatment.

Radiation-induced bystander effect, mediated through gap junctions and inflammatory responses, is defined as the response of cells to their irradiated neighbors [106]. Many types of cancer-infiltrating immune cells, such as macrophages, dendritic cells and T cells, are important stromal components of prostate tumor as well as prominent bystander targets of radiotherapy. In this review, emphasis is placed on the implications of IR-generated soluble effectors on prostate cancer radiosensitivity and the underlying redox-mediated mechanisms. More information on the mechanisms by which

IR influences tumor-associated immune responses and various immune cells to secrete different inflammatory mediators have been reviewed recently [85, 107, 108]. In summary, activated immune cells are not limited to induction of anti-tumor immunity but also involved in creating an immunosuppressive and prooxidant network promoting tumor progression and facilitating immune evasion. Since tumor cells are often under a higher oxidative stress with deregulated and/or less adaptive redox buffering capacity than their normal counterparts, tumor cells are probably less able to cope with additional incremental increases in oxidative stress than normal cells, which can be explored to enhance anti-tumor immunity while minimizing the possibility of unintended tumor progression and evasion.

Radiation therapy induced inflammatory mediator secretion

When cells are exposed to IR, DNA damage generated from either direct or indirect effects of IR induce a multicellular program through a variety of signaling pathways to start DNA repair and prevent the proliferation of damaged cells. Such programs are usually mediated by soluble factors composed of cytokines, growth factors and chemokines, which function on both tumor and stroma to determine the fate of the affected cells [3]. IR exposure commonly induces stromal cells, especially CAFs, into a senescence-like phenotype in an altered tumor microenvironment. The so-called senescence-activated secretory pathways (SASPs) in senescent stromal fibroblasts generate an inflammatory environment through the secretion of proinflammatory cytokines and proteases [85, 109, 110]. These soluble factors can exert paracrine, or autocrine functions mediated by their respective receptors or interactive partners to promote prostate cancer progression and to create a continuous loop that pushes prostate cancer to a more aggressive state.

Chronic inflammatory mediator secretion associated with aging has been involved in the etiology and progression of prostate cancer. Chronic inflammatory microenvironment leads to an increased fraction of epithelial cells to proliferate in local atrophy lesions, an event called proliferative inflammatory atrophy [111]. Proliferative inflammatory atrophy [111] may progress to high-grade intraepithelial neoplasia and prostate cancer [112]. Thus, inflammation, as the seventh hallmark of cancer, and oxidative stress are important etiologic factors in prostate cancer. The relationship between chronic inflammatory

microenvironment and prostate cancer is gaining a wide acceptance [113, 114]. Genetic factors, environmental factors, such as infection and dietary carcinogens, and aging are potential sources of prostatic inflammation that contributes to the development of prostate cancer (reviewed in [112]). The evidence that the microenvironment is altered as a result of radiotherapy, especially the generation of various types of cytokine, has been elegantly reviewed [3, 106]. There are many types of small molecule weight mediators induced by ionizing radiation [3], including EGF [115], fibroblast growth factor (FGF) [3], interferon-γ (IFN-γ) [116], TGF-β [117], proinflamamtory cytokines IL-6 [118], TNF-α [119], the chemokine IL-8 [120] and others. [121]. A range of studies has shown clear differences in the level of circulating cytokines in prostate cancer patients as compared with normal or benign controls and changes in levels of circulating cytokines after radiation exposure and/or androgen deprivation therapy [122-124]. For the purpose of brevity, we will highlight the signaling pathways mediated by IL-6, IL-8, TNF-α and TGF-β induced by IR as well as their implications in prostate cancer malignancy and their potential significance in radiotherapy of prostate cancer.

Interleukin 6

IL-6 is a multifunctional cytokine that signals through a cell-surface type 1 cytokine receptor complex composed of the ligand-binding protein of IL-6Rα (also called CD126) and the signal-transducing component gp130 (CD130) [125]. Another type of receptor for IL-6 is a soluble IL-6 receptor (sIL-6R) that lacks a membrane-signaling domain but can bind with IL-6 and then with the membrane receptor β chain (gp130) to mediate the intracellular signaling pathways [126, 127]. IL-6 mainly activates JAK/STAT3 signaling pathway [128] but also participates in MAPK and PI3K/Akt pathways to influence a wide range of biologic activities in tumor cells [129]. IL-6 also acts as an autocrine and/or paracrine proliferative factor in prostate cancer cell lines [130]. IL-6 treatment not only stimulates the IL-6 autocrine loop but also activates insulin-like type I growth factor receptor (IGF-1R) signaling. This signal transducion and activation of transcription 3 (STAT3) mediated cooperation between IL-6 signaling and IGF-IR signaling in the prostate plays a critical role in facilitating prostate malignancy and EMT. STAT3 has been shown to promote oncogenesis in human cancer due to the requirement of STAT3 for cell transformation by the *Src* oncogene [131]. In additional to its classical role in the

nucleus, STAT3 modified by serine phosphorylation augmented oxidative phosphorylation in mitochondria and supported cellular transformation by oncogene *Ras* [132, 133]. Considering the differential cellular localization of STAT3 implicated in intracellular energy metabolism and a variety of redox sensitive genes, it will be interesting to investigate mitochondrial functions and cellular transformation under IR-induced IL-6 activation (Figure 1.5).

Different cell types, such as B and T cells, macrophages, monocytes, fibroblasts and certain tumor cells can synthesize IL-6 [134] that regulates various cellular functions including immune response, proliferation, apoptosis, angiogenesis and differentiation [135]. Several clinical studies reported that elevated serum levels of IL-6 and sIL-6R were associated with metastasis and castration-resistance, suggesting that IL-6 correlates with prostate cancer progression and patient morbidity [136-141]. Most clinical data support the biological role of the IL-6 pathway in prostate cancer, especially the significance of IL-6 pathways in advanced castration resistant prostate cancer patients mediated by a crosstalk between IL-6 and AR pathways [142]. Under androgen deprivation conditions, IL-6 is able to promote intracellular synthesis of androgens in prostate[143], resulting in AR activation and up-regulation of AR-targeted PSA expression, via STAT3 and MAPK signaling pathways [142] as well as an androgen enhancer region within human PSA promoter [144].

Even though increased IL-6 may indicate the presence of advanced prostate cancer tumor in patients or *in vivo* experiments, some *in vitro* results supporting the significance of IL-6 pathways in prostate cancer cells growth are still controversial. IL-6 can act as either a growth inducer [130, 145, 146] or inhibitor [147-149] in androgen-dependent LNCaP cells [142]. It is possible that IL-6-induced growth arrest may be associated with neuroendocrine differentiation [150]. The presence of neuroendocrine-like cells is correlated with a radioresistant phenotype and unfavorable prognosis [28, 29].

IL-6 signaling is tightly regulated by several negative feedback inhibitors including suppressors of cytokine signaling (SOCS), *Src*-homology 2 (SH2) containing protein tyrosine phosphatase (SHPs) and protein inhibitors of activated STATs (PIAS) [151, 152]. More detailed mechanisms about how these inhibitors regulate IL-6 intracellular signaling pathways have been reviewed previously [152]. There are different approaches

to target IL-6. For example, the monoclonal antibody siltuximab (CNTO328) have been used in experimental and a phase I clinical study of prostate cancer treatment in combination with other chemotherapy agents. CNTO328 has some effects on sensitizing castration-resistant prostate cancer patients [153, 154]. However, since IL-6 also plays an inhibitory role in prostate cancer cells depending on signaling crosstalk and the difference between the cancer and normal cells in redox status and adaptive response to oxidative stress may influence the signaling crosstalk, blocking IL-6 with antibody or signaling inhibitors may promote prostate cancer progression instead. Thus, it is necessary to identify the role of IL-6 signaling in specific situations before applying anti-IL-6-related therapy.

Interleukin-8

IL-8, also known as CXCL8, is a member of the chemoattractant chemokines. IL-8 is usually associated with inflammation that predisposes cells to produce different chemokines for malignant transformation or progression [155, 156]. IL-8 secretion is increased by oxidative stress from either intracellular or extracellular sources. IL-8 can stimulate the recruitment of inflammatory cells, which further elevates oxidant stress mediators, thereby making IL-8 a key parameter in localized inflammation [157]. Two cell-surface G protein-coupled receptors, CXCR1 and CXCR2 [158], are responsible for the binding of IL-8 and regulating target gene expression through downstream signaling pathways, such as activation of serine/threonine kinases, protein tyrosine kinases and Rho-GTPases [159]. Depletion of CXCR1 leads to inhibition of IL-8 mediated androgen independent tumor growth by increasing proapoptotic proteins and decreasing antiapoptotic proteins [160].

Studies using *in situ* hybridization have indicated that increased IL-8 expression is associated with both high Gleason score and tumor pathologic stage [161, 162]. Elevation of IL-8 expression has been linked to various markers of progression of prostate cancer to advanced stage, such as castration-resistance, metastasis and enhanced angiogenesis *in vitro* [163, 164], *in vivo* [164, 165] and in human patients [166]. Several studies suggest a link between IL-8 signaling and chemotherapeutic resistance. It has been shown that IL-8 signaling, which is endogenous and induced by TNF-related apoptosis inducing ligand (TRAIL), can modulate the extrinsic apoptosis pathway in

prostate cancer cells through direct transcriptional regulation of c-FLIP, an endogenous caspase-8 inhibitor, and reduce the propensity of prostate cancer cells to undergo apoptosis [167]. Therefore, inhibiting IL-8 signaling may be a promising strategy to sensitize advanced prostate cancer to chemotherapy. The reduction of intrinsic IL-8 potentiates ansamycin-based heat shock protein 90 (HSP90) cytotoxicity through several mechanisms, including inhibition of IL-8 induced NF-κB activity [168], cell cycle arrest at G1/S boundary, and increased spontaneous apoptosis as well as enhancing the efficacy of multiple chemotherapeutic drugs, such as docetalxel, Staurosporine and Rapamycin significantly [169].

There are currently seven known CXC chemokine receptors in mammals, named CXCR1 through CXCR7. A variety of crosstalk exists between different chemokine-mediated signaling pathways due to remarkable redundancy within chemokines with multiple chemokines binding to the similar or same receptor(s) and multiple receptors binding with the similar or same chemokine(s) [170]. For example, IL-8 and and CXCL6 can both bind CXCR1 in humans, while all other ELR-positive chemokines, such as CXCL1 to CXCL7, bind only to CXCR2 [171, 172]. It has been shown that IL-8 can upregulate CXCR7 expression and ligand-independent functions of CXCR7, which usually binds to the CXCL11 and CXCL12 ligands to promote the growth, proliferation and angiogenesis of prostate cancer cells through increasing EGFR and ERK1/2 phosphorylation [173]. Therefore, effects of IL-8/IL-8 receptors signaling pathways in prostate cancer progression and radiation sensitivity may be orchestrated by communications and/or interactions with many chemokines and their receptors, such as CXCR1-7s.

The reciprocal correlation between IL-8 signaling and AR signaling pathways has been reviewed previously [155, 159]. The proteomic data illustrated that the androgen-stimulated LNCaP cells had increased expression of IL-8 [174], which was dependent on AR since inhibition of AR expression by siRNA prevented IL-8 secretion in response to androgen stimulation. Additionally, IL-8 signaling also increased AR expression with altered the distribution and transcriptional activation of AR, leading to increased expression of AR-targeted gene [163]. Since the transition of prostate cancer to an androgen independent state is partially due to IL-8 signaling induced AR activation,

targeting IL-8 expression and signaling pathways may significantly enhance the efficacy of androgen ablation therapy.

Besides the importance of IL-8 in developing chemoresistance [175], our laboratory found that up-regulation of IL-8 enhanced radioresistance of prostate cancer cells [176]. RelB-mediated NF-κB alternative pathway plays a crucial role in IL-8 upregulation [176]. This result is consistent with the observation of RelB in promoting prostate cancer progression and radioresistance [165]. The relationships between NF-κB pathway, increased antioxidant capacity and resistance to radiation treatment in many tumor cell types have been well documented [15, 177, 178]. RelB regulating MnSOD gene and resistance to ionizing radiation of prostate cancer cells has also been demonstrated [179] and reviewed previously [26]. Selective inhibition of RelB-mediated NF-κB alternative pathway can remarkably sensitize prostate cancer cell to IR-induced killing [180, 181]. Thus, it will be interesting to investigate the radiosensitizing effects of IL-8 signaling blockage with either inhibitors to IL-8 receptors or monoclonal antibodies against IL-8. This strategy may synergistically facilitate the killing of castration-resistant and/or radiation-resistant prostate cancer cells.

Tumor necrosis factor-alpha

TNF-α is synthesized as a 26 kDa (233 amino acids) membrane-bound pro-peptide (pro-TNF) and released as a 17kDa soluble polypeptide (157 amino acids) after cleavage by the TNF-converting enzyme (TACE) [182, 183]. The action of TNF-α is mediated by two distinct receptors named TNF-receptor I (55 kDa, TNFRI) [184], which mediates the majority of TNF-α biological activities, and receptor II (75 kDa, TNFRII) [185] with both having a similar affinity for TNF-α in human tissues. An imbalance between pro-survival and apoptosis signals by TNF-α-initiated signaling pathways (Figure 1.6) has been implicated in malignancies of a variety of organs and tissues, such as colon [186], skin [187], ovarian [188], breast [111] and prostate [139].

TNF-α is one of the central factors involved in stress responses, including the response to radiation exposure. This inflammatory cytokine was named because of its ability to induce rapid hemorrhagic necrosis via selective destruction of tumor blood vessels and generation of specific T cell antitumor immunity [189]. Antagonists of TNF-α action have been developed for the treatment of rheumatoid arthritis and other

inflammatory diseases [190-193]. When present chronically in the tumor microenvironment, TNF-α is a major mediator of cancer-related inflammation. TNF-α not only maintains the homeostasis of the immune system, inflammation and host defense but also plays its paradoxical role in promotion and progression of cancer with pathways leading to activation of NF-κB and AP-1 transcription factor complexes (reviewed by Balkwill [193-195]). Circulating TNF-α is normally not detectable in healthy individuals but can be detected in some cancer patients. In a few prostate cancer clinical studies, elevation of serum TNF-α has been shown to correlate with clinicopathological features and patient survival [139, 141]. For example, TNF-α levels are significantly higher with metastatic disease (6.3 ± 3.6 pg/mL) compared to localized disease (1.1 ± 0.5 pg/mL, $P<0.001$) [139]. However, some independent studies indicated that TNF-α levels were not significantly different among normal control, benign prostate hyperplastic (BPH) and prostate cancer [140, 196]. Also, whether TNF-α elevation in prostate cancer patients is the cause or consequence of cancer development and progression requires more mechanistic investigations. Even though these conclusions are still controversial, there is a relatively consistent association between increased TNF-α and cachexia in patients with prostate carcinoma [197, 198]. Approximately 60-70% of patients with advanced stage of prostate cancer suffer from cachexia, which is one of the most devastating conditions at late stages of cancer. Some typical characteristics of cachexia syndrome including weight loss, anorexia, asthenia and anemia are invariably associated with the presence and growth of malignant tumors [199]. Roles of TNF-α in cancer cachexia include most of the changes concerning nitrogen metabolism associated with cachectic states [200], blockage of muscle differentiation associated with muscle tissue regeneration [201], activation of transcription factors NF-κB and AP-1 to increase proteolysis [202] (reviewed in [199]).

TNF-α can be produced when NF-κB is activated and TNF-α is also an important stimulus of NF-κB signaling and further cytokine production. The NF-κB signaling pathway is critical in cancer-related inflammation and malignant progression as well as maintaining the immunosuppressive phenotype of TAMs [203] and inducing chemoresistance and radioresistance. It has been demonstrated by immunohistochemical analysis that TNF-α protein was strongly expressed in epithelial cells of prostate cancer

but not in normal prostatic tissue. TNF-α may play a role in the initiation of an androgen-independent state in prostate cancer through its ability to inhibit AR sensitivity [204]. The interplay of NF-κB and B-myb contributes to the negative regulation of AR expression by TNF-α [205]. Contrary to the response observed in prostate cancer cells, TNF-α-stimulated NF-κB binding to the AR promoter induced AR promoter activity and increased endogenous AR expression in primary cultures of Sertoli cells, indicating the significance of TNF-α signaling in spermatogenesis. Immunohistochemistry results showed that nuclear localization of NF-κB family member p65 was associated with PSA relapse, the first sign of prostate cancer recurrence, while cytoplasmic expression did not [206]. Our laboratory demonstrated that RelB-mediated alternative NF-κB pathway is involved in prostate cancer aggressiveness and radiation resistance [26, 165, 179]. TNF-α functions as a potent inducer of NF-κB signaling pathway and particularly mediates the crosstalk between classical and alternative NF-κB signaling pathways, as well as interactions with AR (as shown in Chapter 3). Thus, it is important to investigate the production of TNF-α after chemo/radiotherapy and potential influences of TNF-α on the activation of RelB-mediated alternative NF-κB pathway and implications in prostate cancer.

The expression and activation of several genes and kinases, such as cyclooxygenase-2, Cyclin D1, Bcl-2 family, survivin, Akt, and EGFR, are regulated by NF-κB in NF-κB mediated chemoresistance and radioresistance in various tumor cells [207]. The therapeutic potential and benefit of targeting NF-κB in cancer and the possible complications and pitfalls associated with NF-κB modulation have been reviewed and explored [208]. Inhibition of NF-κB has been proposed as a means to treat cancer or to overcome chemoresistance and radioresistance in cancer therapy [178, 207, 209]. Inhibition of IR-induced activation of NF-κB, but not of Akt or MAPK kinase, sensitized Ki-Ras transformed prostate epithelial cells (267b1/K-Ras) to ionizing radiation [25]. A group of potential therapeutic agents, including 1α, 25-dihydroxyvitamin D3 (1α, 25-$(OH)_2D_3$) [181], STI571 [210] and a protein peptide SN52 [180], have been used to selectively inhibit RelB nuclear activation and downregulate the RelB-targeted gene MnSOD, leading to improve IR-induced killing of PC3 cells.

Transforming growth factor-beta

TGF-β is a ubiquitous cytokine that plays a critical role in numerous pathways regulating homeostasis and injury response as well as in the progression of human cancer. Prior to tumor initiation and during the early phases of tumor progression, TGF-β acts as a tumor suppressor. At later stages of cancer development, TGF-β promotes processes associated with tumor aggressiveness, such as cell invasion, dissemination and immune invasion [211, 212]. In mammals, there are three TGF-β isoforms, i.e., TGF-β1, TGF-β2 and TGF-β3. With the assistance of co-receptors endoglin and betaglycan (known as type III receptors or TGFβRIII), active TGF-β binds to cell surface type I (TGFβRI) and type II (TGFβRII) serine/threonine kinase receptors, which phosphorylate and activate the Smad family of signal transducers [211, 213] (Figure 1.7).

Once activated by TGF-β binding to the receptors, Smad2 and Smad3 associate with Smad4 and translocate to the nucleus where they regulate the transcription of genes involved in cell cycle arrest and apoptosis, which are essential for the tumor suppressor role of the TGF-βs in normal epithelial cells and at early stages of oncogenesis [214, 215]. TGF-β-induced growth arrest is mediated by the inhibition of cyclin-dependent kinases (CDKs) and the downregulation of MYC (reviewed in [211]). Mutational inactivation of TGF-β signal-transduction components, such as TGF-β type II receptor (TGFβRII) [216-218] or its mediators, Smad2 and Smad4, leads to the defective TGF-β signaling in some cancers [219, 220]. Pu. *et al* developed a TRAMP based prostate cancer transgenic mouse model harboring the dominant negative mutant TGF-β type II receptor (DNTGFBRII) in epithelial cells to characterize the *in vivo* consequences of inactivated TGF-β signaling on prostate tumor initiation and progression and found that disruption of TGF-β signaling *in vivo* accelerated the pathologic malignant changes in the prostate by altering the kinetics of prostate growth and inducing EMT [221]. These findings indicate that TGF-β performs tumor suppressive functions through inhibition of cell proliferation, induction of apoptosis and regulation of autophagy.

TGF-β is expressed at high levels in the later stages of tumor development [222-224], during which it is utilized as a potent promoter of cell motility, invasion, metastasis and tumor stem cell maintenance, as demonstrated in experimental prostate cancer models [225]. Local TGF-β1 elevation has been associated with tumor grade, pathologic stage

and lymph node metastasis in prostate cancer patients [226]. Though some investigators were not able to find a discriminative difference in serum concentration of TGF-β1 between BPH and prostate cancer [227], elevated levels of plasma TGF-β1 [228], TGF-β2 [229] and urinary TGF-β1 [229] were found to be higher in patients with prostate cancer.

TGF-β1 plays a critical role in tumor-stromal cell interactions and modulates the growth of prostate cancer, either positively or negatively, through the balance between the amounts of IGF-1 and IGF binding protein-3 [230]. Resistance to TGF-β-mediated growth arrest results in highly malignant phenotypes with increased EMT, tumor invasion, metastatic dissemination and evasion of the immune surveillance [212]. Interestingly, TGF-β1 activates IL-6, which has been implicated in the malignant progression of prostate cancers as described above via multiple signaling pathways including Smad2, NF-κB, JNK, and Ras [231]. Zhu et al have provided a detailed description of the cross-talk between AR and growth factors, including TGF-β-mediated signaling pathways, in prostate cancer cells [232]. Smad3, a downstream mediator of the TGF-β signaling pathway, can function as a coregulator to enhance AR-mediated transactivation and increase AR-targeted PSA gene expression [233]. Considering the correlation between increased circulating levels of TGF-β1 with invasion[228], metastasis [228, 234] and poor prognosis in patients with prostate cancer [223, 226], TGF-β1 could be an additional serum marker for prostate cancer [235, 236].

TGF-β acts an important mediator for the response to IR, and its signaling is tightly regulated by redox status within tumor cells and tumor microenvironment. IR has been shown to induce the release and activation of TGF-β in cells and tissues [3]. A mechanistic study in a cell-free system demonstrated that oxidation of TGF-β latent complex acted as a sensor of oxidative stress to mediate the release and activation of TGF-β1 and orchestrate cellular responses to damage [237]. More aspects of TGF-β biology, particularly its involvement in the microenvironmental response to IR, have been described elegantly [3]. Intracellular redox equilibrium is essential for constitutive AP-1-dependent TGF-β1 expression [238]. Nitric oxide downregulates TGF-β1 expression in prostate cancer cells at the transcriptional level by suppressing the *de novo* synthesis of TGF-β1 mRNA [239]. TGF-β1 induces stromal oxidant/antioxidant

imbalance as a result of an elevated NOX4-dependent ROS production and inhibits the expression of MnSOD and catalase [240] that may be critical in the acquisition of epithelial migratory properties [241, 242]. In addition, TGF-β1 decreases ETC complex IV activity by decreasing phosphorylation of the subunit 6b of glycogen synthase kinase 3 (GSK3), which contributes to senescence-associated mitochondrial ROS generation [243]. The significant roles of TGF-β in modulating tumor intracellular and extracellular redox status suggest that TGF-β signaling is involved in mediating cell autonomous, local and systemic responses, which together regulate the initiation, promotion, progression and prognosis of prostate cancer.

Radiotherapy-induced TGF-β activation may have undesirable side effects implicated in late tissue damage, such as fibrosis [244, 245]. Several studies support the use of TGF-β inhibitors to ameliorate IR toxicity to normal tissues [246-249]. Anticancer therapies, such as ionizing radiation or doxorubicin, may accelerate the steps of tumor progression, such as EMT and metastasis, due to the promoting effect of TGF-β within the tumor microenvironment [45, 250]. This effect can be abrogated by administration of a pan-TGF-β neutralizing antibody [215]. Current strategies to target TGF-β in radiotherapy mainly focus on general inhibition of TGF-β signaling. It has been shown that blockade of TGF-β signaling prior to irradiation attenuated DNA damage responses, increased clonogenic cell death, and promoted tumor growth delay and, thus, enhanced radiation response and prolonged survival in patients with breast cancer [251] and glioblastoma [252, 253] but rendered a lung cancer cell line more radioresistance [254]. Genetic differences and tumor specificity can be important factors in determining the radiosensitizing effect of TGF-β inhibition in radiotherapy. For an example, a hypofunctional genetic haplotype of the *TGFB1* gene encoding TGF-β1 is associated with lower TGF-β1 plasma concentrations and increased sensitivity to radiation induced chromosomal aberrations and apoptosis in lymphoid cells [255]. There are three major approaches to inhibit TGF-β signaling: targeting TGF-β synthesis using antisense molecules, ligand traps that sequester TGF-β and small molecule inhibitors that hinder the kinase activity of TGF-β receptors (reviewed in [212, 256]). Since IR-induced TGF-β may not only provide a survival benefit to cancer cells that are radioresistant but also accelerate tumor progression, targeted disruption of the TGF-β signaling pathway for

therapeutic intervention may be an effective adjuvant in cancer radiotherapy.

PSA test in prostate cancer detection

Prostate specific antigen, PSA also known as human kallikrein-related peptidase 3, has been widely used as a biological marker of prostate cancer since the 1980s. PSA is a glycoprotein secreted by the epithelial cells of the prostate gland and found exclusively in prostate. The physiological function of PSA to liquefy semen in seminal coagulum and allow sperm to swim freely [257]. The FDA has also approved the PSA test to monitor patients with a history of prostate cancer for cancer recurrence. As shown in Figure 1.8, with prostate cancer progression, PSA level increases at early stage in which tumors are initially benign or sensitive to treatment. After clinical interventions such as radiotherapy or hormone ablation therapy, PSA levels dramatically drop to a relatively low level. In advanced stages of prostate cancer, PSA levels of some patients elevate gradually, with tumors becoming metastatic and eventually resistant to treatment.

However, major clinical challenges in PSA testing arise due ambiguous results, leading to over- and under-diagnosis of such a bimodal disease, with both indolent and aggressive forms [258]. Although overall sensitivity of PSA test is close to 80%, it is much more sensitive than rectal examination and able to detect more than 90% of tumors with a diameter bigger than 1cm. A relatively high false positive rate compromises the specificity of PSA test due to many factors besides prostate cancer, such as benign prostatic hyperplasia, prostatitis, prostatic infarction, recent sexual activity [257, 258]. Compared to false positive test results, false negative results raise more serious concerns. Many results analyzed from the cancer prevention trial conflict with the consensus that a cutoff PSA level of 4.0 ng/mL is predictive of prostate cancer. There are a significant number of prostate cancer patients carrying high-grade prostate cancers but showing low PSA levels [259]. Similarly, a comparison of four commonly used human prostate carcinoma cells lines demonstrates that PSA expression is inhibited in some highly aggressive prostate cancer cells (Table 1.2).

In summary, while early detection and improved treatment options have dramatically increased the number of prostate cancer survivors, research suggests that more than half of prostate tumors may be nonaggressive and not require immediate intervention, such as surgery or radiation therapy. For clinicians, the frustrating question is, which half ? On

the other hand, when PSA levels decrease below a certain level after clinical intervention such as radiotherapy, is this an indication of patient safety? Therefore, the current state of PSA test dependent prostate cancer detection is unacceptable in that it leads to random discovery of harmless cancers and often misses potentially lethal cancers.

Research objectives

This study aims to demonstrate that the IR-generated proinflammatory mediator TNF-α, secreted as part of radiotherapy bystander effects, regulates redox-dependent RelB-mediated PSA suppression and to determine how the RelB-AR axis can downregulate PSA gene expression in advanced prostate cancer.

In Chapter two, we report our investigation of whether IR can induce the secretion of proinflammatory mediator TNF-α, to cause bystander effects by using different cell lines including prostate cancer cell lines, human stromal fibroblast and mouse embryonic fibroblasts. This study was focused on investigating the sequential activation of canonical and noncanonical NF-κB pathways after TNF-α treatment in LNCap cells. As we compared the nuclear RelA and RelB levels at different time points after TNF-α exposure, as well as PSA gene expression, we found that RelB induction, but not RelA activation, participated in PSA suppression. Despite the absence or presence of radiotherapy, tumors located within such an inflammatory tumor microenvironment are usually subjected to sustained oxidative stress, which led us to investigate whether and how TNF-α enhances endogenous ROS and influences downstream signaling activation and PSA suppression.

In Chapter three, we report novel molecular mechanisms by which the RelB-AR axis inhibits PSA gene suppression. In combination with the analysis of human Oncomine database, our *in vitro* studies focused on how RelB downregulates PSA gene expression and provided a mechanistic explanation to our previous *in vivo* unexpected findings that expression of RelB reduces the level of PSA and increases the tumorigenicity of prostate cancer.

The overall objective of this research is to investigate whether IR generates TNF-α, which can contribute to bystander effects, how TNF-α induces ROS, and how RelB regulates PSA gene expression at the molecular levels. The results demonstrate that radiation triggers TNF-α production, which serves as a sustained source of ROS to activate the noncanonical NF- κB pathway resulting in RelB-mediated PSA suppression.

The results also suggest that redox intervention, together with anti-proinflammatory mediators, can be useful to improve radiotherapy efficacy by radiosensitizing cancer cells.

Figure 1.1. Electromagnetic spectrum of radiation and medical use of ionizing radiation.

The direct and indirect actions of ionizing radiation on generating DNA damages are descripted. Direct action of IR leads to damages in DNA structure. Indirect action of radiation is the multicellular effects by water radiolysis that produce free radicals, which can diffuse far enough to reach and damage the DNA. Radiation therapy is frequently used to treat early stage and inoperable locally advanced prostate cancer.

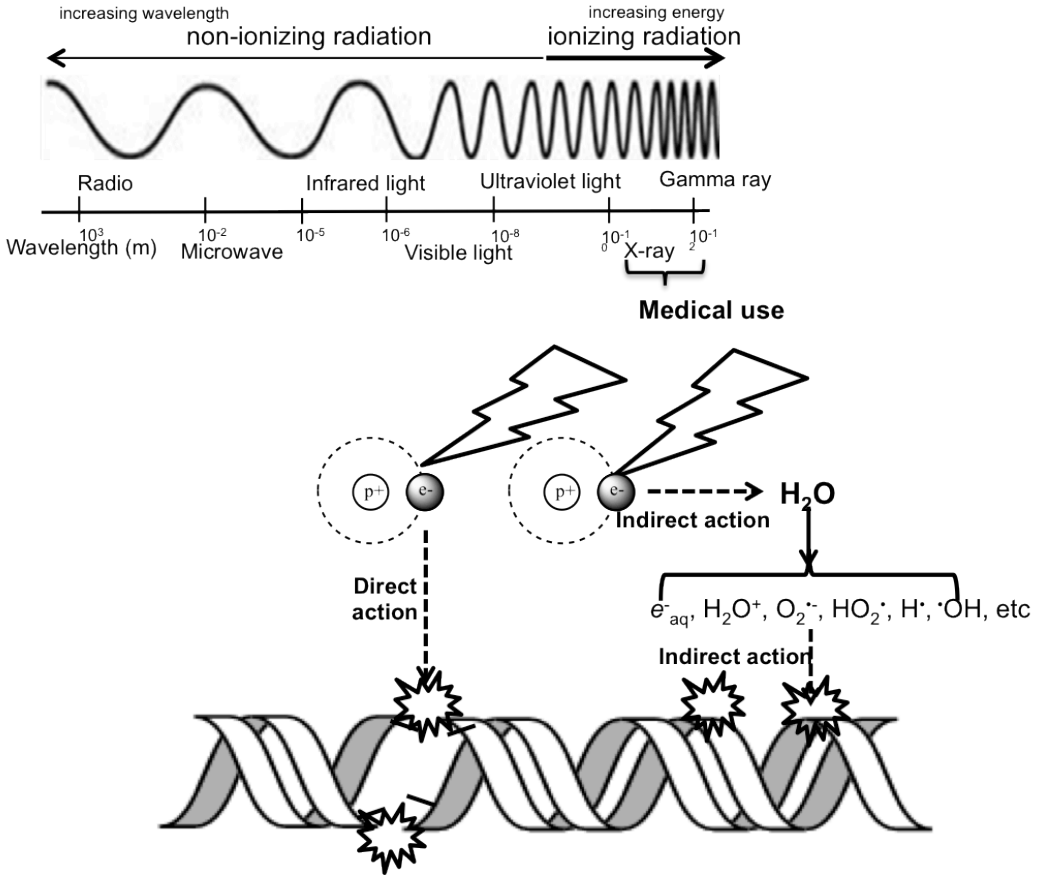


Figure 1.2. Scheme of cellular ROS generation and antioxidant system.

ROS that are generated from extracellular or intracellular sources can cause nuclear DNA and mtDNA damage. Various transcription factors such as NF-κB, p53, AP-1, STAT3, Nrf2, HIF-1α, Sp1, PPARγ and Ref1 are modulated by ROS. Extracellular sources of ROS: radiation, carcinogen, inflammation, hypoxia and etc. Intracellular sources of ROS: ETC, XO, xanthine oxidase; LPX, lipoxygenase; P450, cytochrome P450; COX. Antioxidant system: MnSOD, Cu/ZnSOD, ECSOD, GSH, GPx, Prx, GR, glutathione reductase; TrxR, thioredoxin reductase; TRXox, oxidized thioredoxin; TRXre, reduced thioredoxin.

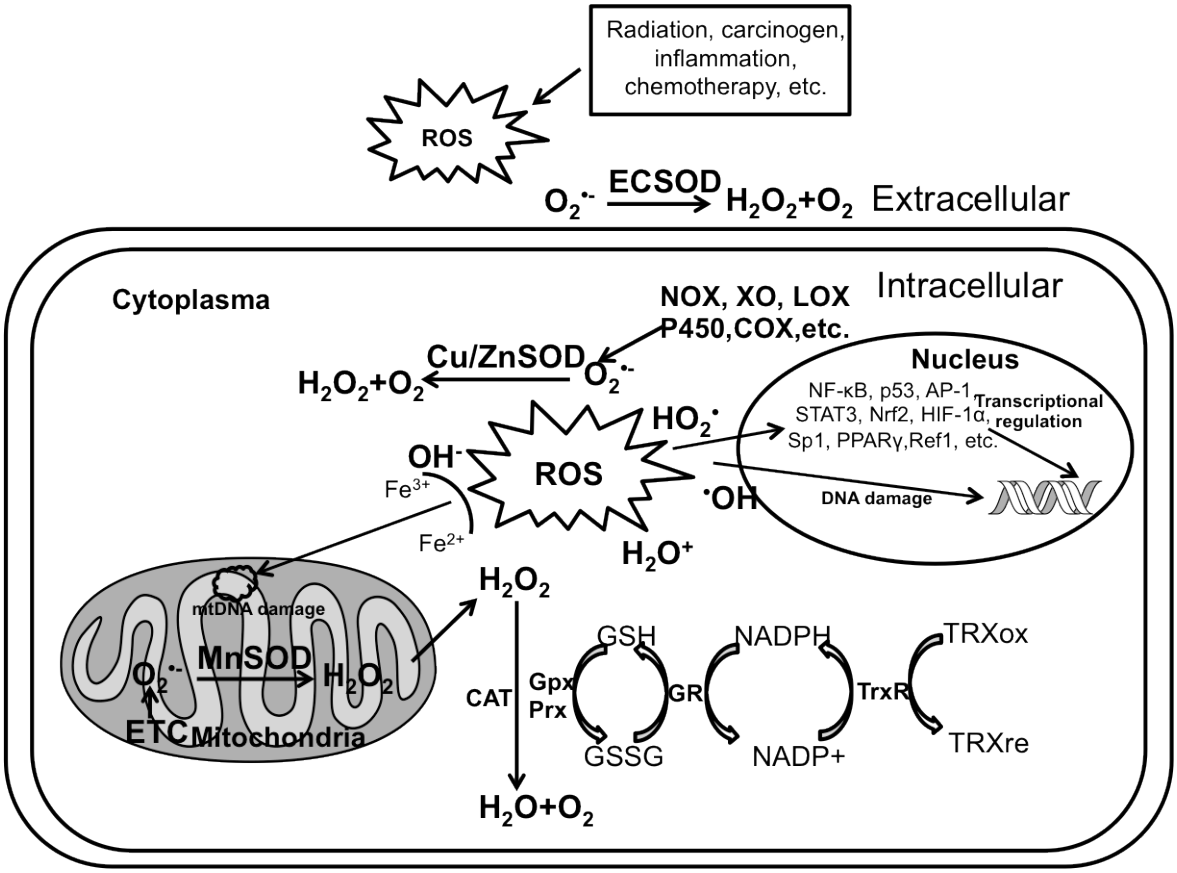


Table 1.1. Roles of MnSOD expression or activity and MnSOD-regulated cellular redox status in different stages of tumor development.

Tumor development	Normal cells	Early stage of cancer	Advance stage of cancer
MnSOD level or activity	high	Low	Very high
Cellular redox status	↓ prooxidant ↑ antioxidant Low oxidative stress	↑ prooxidant ↓ antioxidant Modest oxidative stress	↑ prooxidant ↑ antioxidant High oxidative stress
Sensitivity to ROS	++	+++	+
Implications in radiotherapy	Protect normal cells from oxidative insults such as radiation by inducing adaptive responses	Promote radiation-mediated cell killing due to low level of antioxidant capacity	Protect tumor cells from radiation and modulate neuroendocrine-like differentiation leading to radioresistance

Figure 1.3. Role of oxidative stress in cancer development and radioresistance. IR-induced genomic instability and oxidative stress are closely related to each other. Many human cancer cells harbor low levels of antioxidant at early stages of tumor, whereas those cancer cells may eventually develop tumor resistant and possess high levels of antioxidant at advanced stages of tumor. With cancer development, tumor cells are under increased oxidative stress and genomic instability continuously.

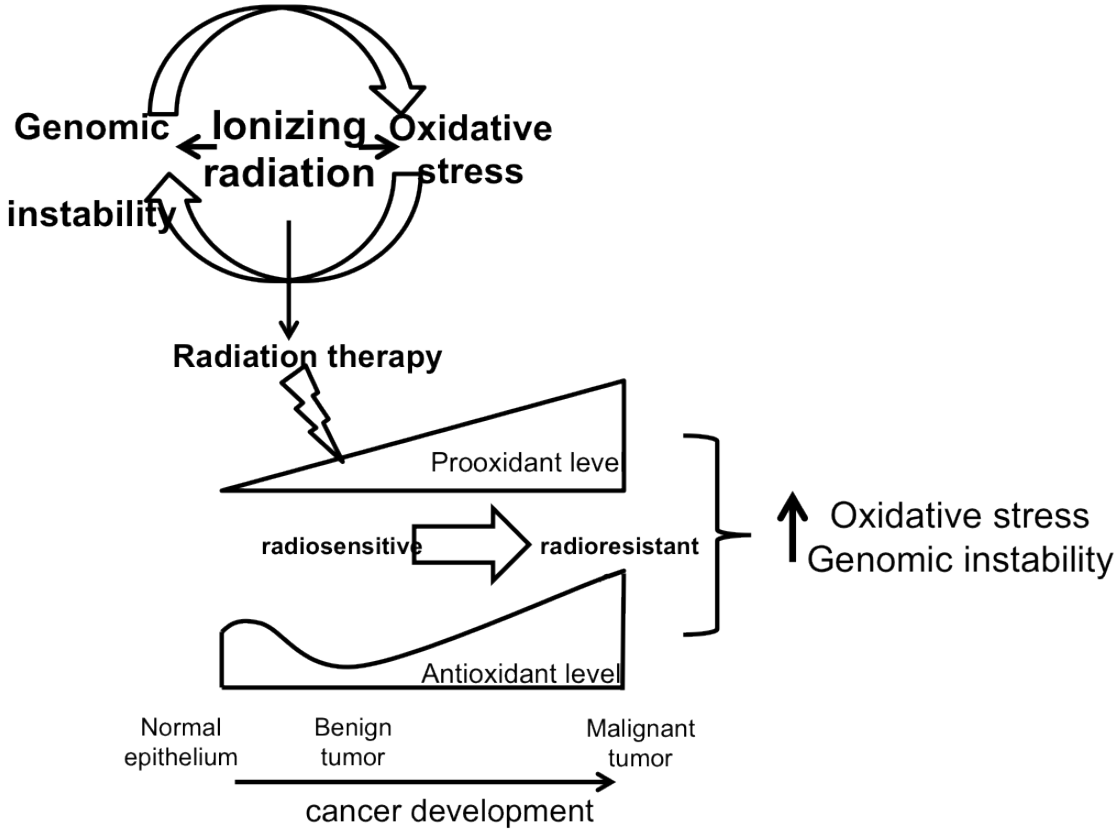


Figure 1.4. Radiation therapy induced cell killing and unintended effects on tumor stromal components leading to inflammatory mediator secretion.

Major cellular components in tumor microenvironment are described. There are many types of small molecule weight mediators induced by ionizing radiation, including EGF, FGF, IFN-γ, TGF-β, proinflamamtory cytokines IL-6, TNF-α, the chemokine IL-8 and others. Both radiation treatment and IR-induced inflammatory mediators result in increased ROS levels within the tumor microenvironment, and also contribute to the development of tumor radioresistance and tumor recurrence.

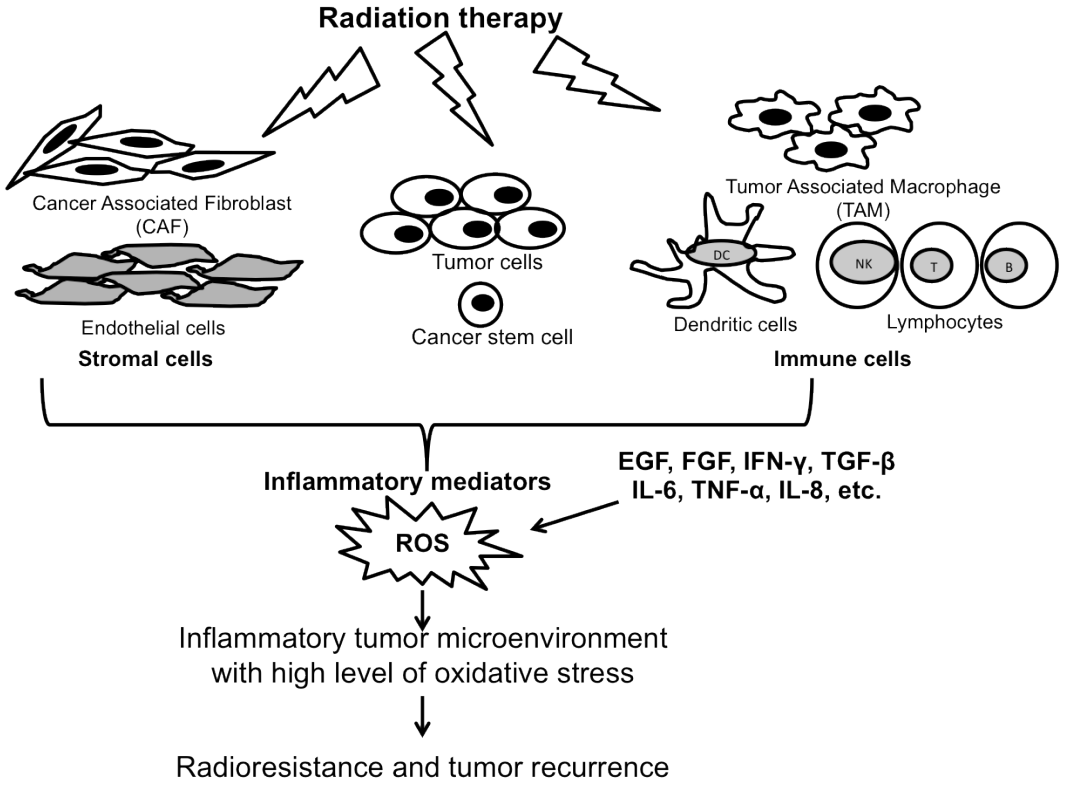


Figure 1.5. IL-6-mediated Jak-STAT3 signaling pathway.

IL-6 signals through a cell-surface type 1 cytokine receptor complex composed of the ligand-binding protein of IL-6Rα and the signal-transducing component gp130 (glycoprotein of 130kDa). IL-6 can also bind to sIL-6R, which lacks a membrane-signaling domain, and then with gp130 to mediate Jak phosphorylation and activation. Activated Jak family tyrosine kinases further phosphorylate STAT3, which in turn translocate to the nucleus and regulate target gene transcription. Many types of growth factors, such as EGF, VEGF, FGF, and IGF (insulin-like type I growth factor), can aggregate with respective receptors and activate STAT3 signaling pathways.

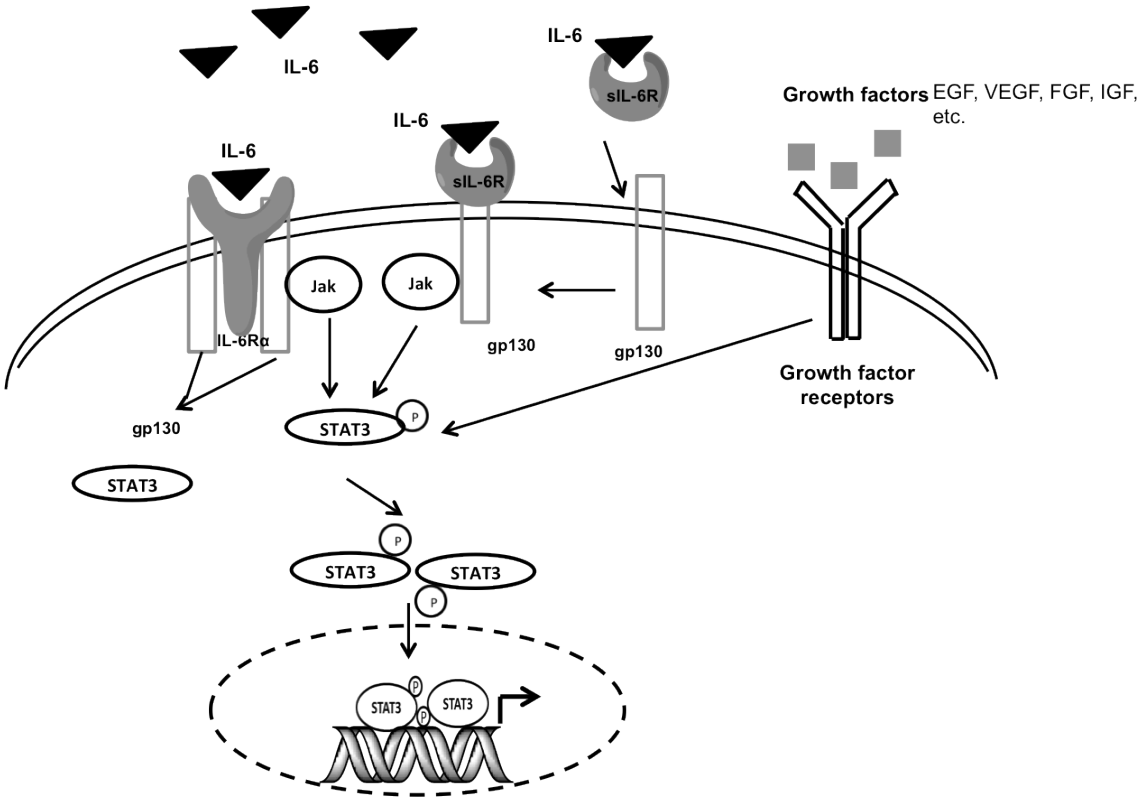


Figure 1.6. TNF-α-regulated major cellular signaling pathways.

Binding of TNF-α to TNFR1/2 leads to the rapid phosphorylation of the NF-κB, ERK, p38 and JNK pathways, and activates a group of transcription factors such as NF-κB, Elk1 and AP-1 in the nucleus. In addition to these pro-survival pathways, TNF-α can induce apoptosis through receptor-mediated caspase activation, and caspase-dependent and -independent components of the mitochondrial cell death pathway. A balance between these intracellular signaling pathways determines whether cells will die or survive after exposure to TNF-α. TNF-α-mediated ROS generation is mainly derived from mitochondria and membrane-associated NADPH oxidase, which contributes to signaling pathways.

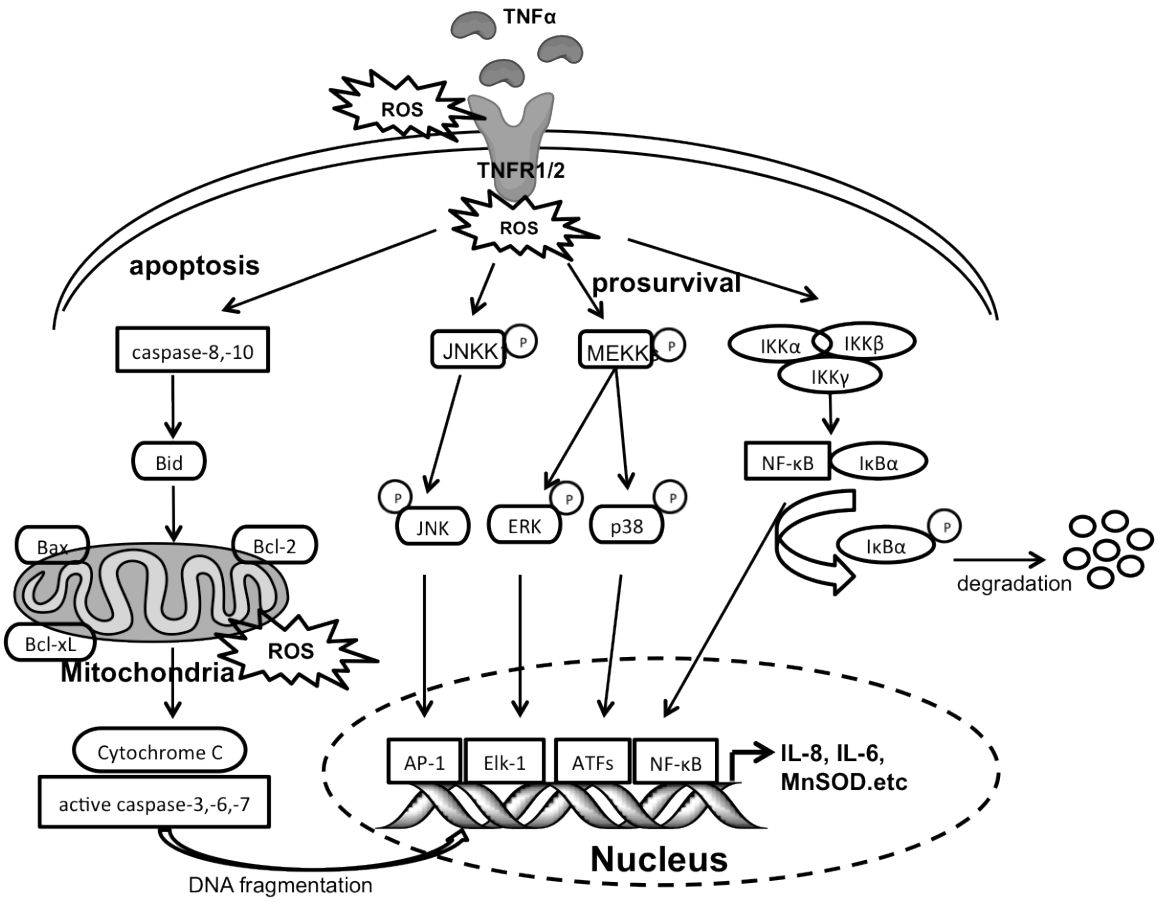


Figure 1.7. TGF-β-mediated classical Smads signaling pathway.

With the assistance of TGFβRIII, active TGF-β (three isoforms, i.e., TGF-β1, TGF-β2 and TGF-β3) binds to cell surface TGFβRI and TGFβRII, which phosphorylate and activate the Smad family of signal transducers.

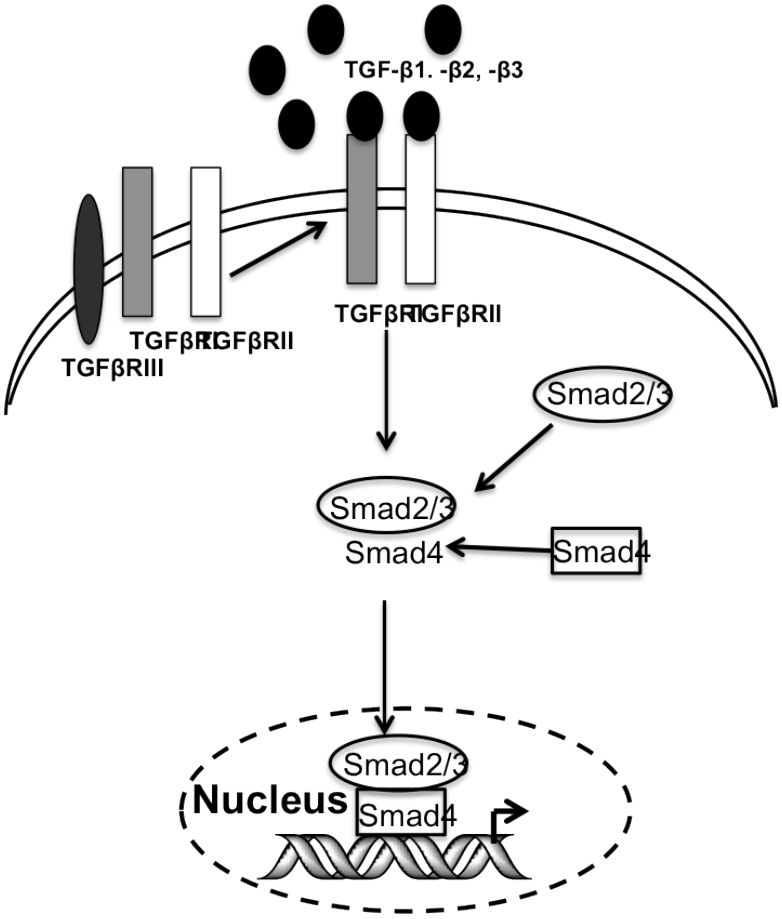


Figure 1.8. General alterations of PSA serum levels during prostate cancer progression.

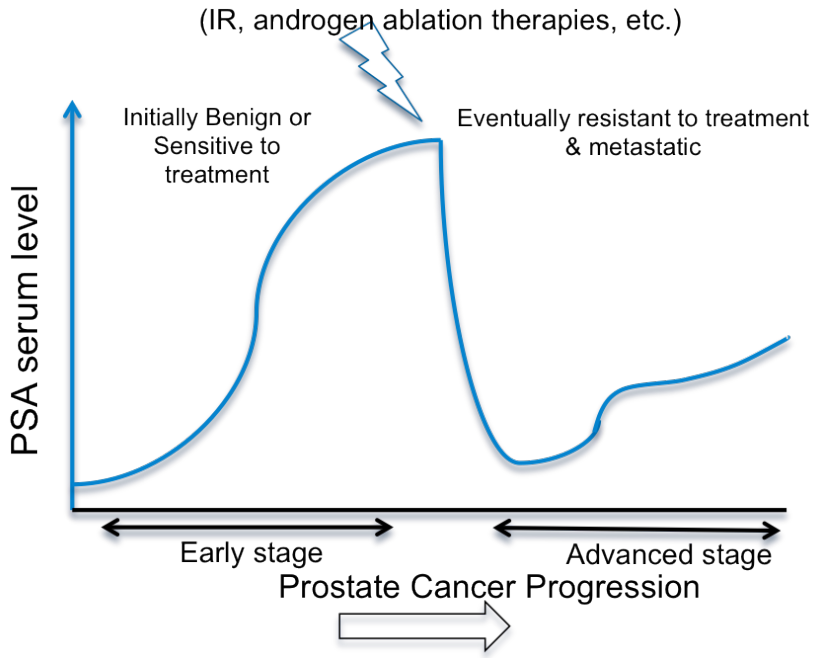


Table 1.2. Characteristics of four human prostate carcinoma cell lines

cell line	p53 status	AR status	**PSA**	doubling time	origin
22Rv1	wild type	+	+	40h	primary, xenograft CWR22r-2152
LNCap	wild type	+	++	34h	lymph node metastasis
DU145	mutated	-	-	25h	brain metastasis
PC-3	stop codon 169	-	-	24h	bone metastasis

Chapter Two

IR-generated TNF-α regulates the redox-dependent RelB-mediated PSA suppression

Introduction

Despite much recent progress, prostate cancer continues to be a major problem facing men, accounting for approximately 29% of newly diagnosed cancer cases and 9% of cancer deaths in men in 2012 to date [1]. The common forms of treatment for prostate cancer are surgery, ionizing radiation therapy, chemotherapy and hormone management. [2] Radiation can directly damage DNA and other biologically important molecules; however, most effects of radiation-mediated cell killing are derived from its indirect effects that generate free radicals that damage macromolecules and alter cellular signaling.

Radiation therapy is an integral part of prostate cancer treatment across all stages and risk groups [2]. IR can lead to rapid, global and persistent responses within the microenvironment [3, 260]. IR activates ROS-mediated cellular processes and alters intracellular oxidative stress, thereby linking external stimuli with internal signal-transduction pathways. ROS are crucial mediators within the microenvironment and are actively involved in the communication between primary tumor cells and various components of the surrounding microenvironment, such as TAM, CAF, tumor endothelial cells and cancer stem cells (CSC). In addition, evidence from clinical and experimental settings has shown a firm connection between radiation exposure and changes in cytokine, growth factor and chemokine levels [178, 261]. Cytokines, such as interleukin-1β (IL-1β), IL-6, and IL-8, TNF-α and TGF-β, convey information between cells through their secretion and interaction with receptors on neighboring or distant cells. Signaling by these molecules regulates cell proliferation, differentiation, motility, adhesion and apoptosis [121, 262]. IR-induced and macrophage-mediated immune response is a major contributor to the elevation of these inflammatory cytokines; however, CAFs and tumor cells exposed to IR can also directly regulate these cytokines [261, 263-265]. Altered inflammatory cytokine expression can affect many signaling pathways that converge onto a few important transcription factors, including NF-κB, AP-1 and STATs.

These transcription factors also up-regulate the expression of several cytokines, such as IL-1β and TNF-α [265]. Such positive feedback loops amplify the inflammatory response and increase chronic inflammation [266]. Accumulating evidence suggests that chronic inflammatory diseases are subject to tight redox control. The identity, source, regulation, and biological activity of redox molecules, as well as their key roles in multiple pathological conditions, including cancer, have been reviewed extensively [267].

IR-induced multiple proinflammatory mediators can also influence redox status in irradiated cells and the surrounding microenvironment, thereby affecting prostate cancer progression, radiotherapy efficiency, and prognosis. Depending on the type and stage of the prostate cancer cells, these proinflammatory mediators may play different roles ranging from apoptosis to development of radioresistance.

Extensive research has linked persistent inflammation and oxidative stress to the etiology and progression of PCa [112, 141, 268]. Reports indicate that some cytokine levels may be elevated due to multiple potential causes, such as infection, radiation therapy, aging and obesity [107, 112, 269]. For example, IL-6 and TNF-α levels correlate with clinicopathological features and patient survival in patients with PCa [139]. Despite the significant role of the IL-6 pathway in PCa, especially in advanced castration-resistant PCa (CRPC) patients [142], recent findings provide convincing evidence that IL-6 upregulates intracellular androgen synthesis [143], AR [270] and PSA expression [144, 270]. Thus, application of circulating IL-6 level in PCa diagnosis and/or disease monitoring is not a focus in the current study because it partially overlaps with PSA testing.

As one of the most well characterized cytokines, TNF-α is both a potent inducer of NF-κB and a target of activated NF-κB. The NF-κB family comprises five proteins RelA, p50, RelB, p52 and cRel. NF-κB is essential for the regulation of innate and adaptive immunity, and recent studies have provided evidence for a role of NF-κB in cancer initiation and progression [271, 272]. In contrast to IL-6, which enhances PSA and AR expression, the data presented here indicate sustained suppressive effects of TNF-α on PSA expression, which concomitantly depends on a time-dependent switch from immediate RelA-mediated NF-κB pathway to delayed RelB-mediated noncanonical NF-κB activation. While TNF-α can induce an acute and pro-death response in cells, chronic

elevation of TNF-α at a relatively low level can result in cytoprotection that is related to increased levels of antioxidant, antiapoptotic and other defense proteins, such as RelB and MnSOD. Both RelB and NF-κB-targeted MnSOD upregulation have been implicated in the adaptive response induced by low or fractionated doses of ionizing radiation, leading to radioresistance in prostate cancer [26, 273]. Furthermore, we present data indicating that TNF-α-triggered ROS generation, particularly NADPH oxidase-dependent superoxide radicals, serves as a secondary ROS source after ionizing radiation exposure to participate in the activation of the noncanonical NF-κB pathway and RelB-mediated PSA suppression. Addition of a NADPH oxidase inhibitor or ROS scavengers, such as a SOD mimetic, can abrogate TNF-α-mediated suppression of PSA expression. After radiation therapy, PSA test results can be an important indicator for estimating effective tumor killing and monitoring tumor recurrence. Our studies provide significant evidence that aggressive tumor may still be present in patients after treatment in spite of PSA reduction.

Materials and Methods

Cell culture and treatment. Human prostate cancer cell lines LNCap and PC3 were obtained from American Type Culture Collection (Manassas, VA). LNCap and PC3 cells were cultured in RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin mixture, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 1% NEAA mixture (Cambrex), 1% MEM vitamin mixture (Cellgro) and 2 mmol/L L-glutamine. Cells were grown in a 5% CO_2 atmosphere at 37°C. Recombinant human TNF-α was obtained from R&D Systems. NADPH oxidase inhibitor diphenylene iodonium (DPI, Sigma) was dissolved in DMSO. SOD mimetic (MnTE-2-PyP^{5+}) was kindly provided by Dr. Ines Batinic-Haberle. IR treatment was performed using a 250kV X-ray machine (Faxitron X-ray Corp.) with the peak energy of 130kV, 0.05mm Al filter, at a dose of 0 to 8Gy.

ELISA assay. Cultured media from radiated mouse embryonic fibroblast, LNCap cells or human immortalized foreskin fibroblast cell line BJ-5ta were changed to fresh medium containing only 1% FBS and were collected after 24hr at the end of experimental procedures. The levels of TNF-α secreted from cultured cells were quantified using either mouse or human TNF-α ELISA development kit (Promokine) according to the manufacturer's protocol. The relative TNF-α secretion was normalized by protein concentration.

MTT assay. Cells were plated at the density of 5,000 cells/well into 96-well plates and grew overnight. Then cells were pretreated with indicated concentrations of TNF-α for 24 hours, and exposed to radiation with indicated doses or were sham-irradiated. Five days after radiation, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 50 μg/well) was added and incubated at 37°C for 1 hour. After removal of medium, DMSO 200 μL was added to each well to dissolve the purple formazan crystal. The absorbance was measured at 540 nm. The cell survival was referenced to the control group.

Western blotting analysis. For each treatment group, a certain amount of the whole cell lysate was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After blocking in 5% milk for 1 hour, the membrane was incubated with the primary antibody and then the corresponding secondary antibody. The signals were detected by enhanced ECL system and quantified by Quantity One® (Bio-Rad). The

following antibodies were used in this study: anti-AR (N-20), anti-PSA (C-19), anti-RelB (C-19) and anti-RelA (C-20) (Santa Cruz Biotech); anti-MnSOD (Upstate); anti-Nox1 (abcam); β-actin (Sigma).

Preparation of whole cell extracts, cytoplasmic and nuclear fractions. Cell pellets were suspended in 100μL cell lysis buffer and incubated for 30 minutes on ice. The samples were then centrifuged at 13,000 rpm for 1 minute. The supernatant was collected as whole cell extract. Cytoplasmic and nuclear fractions were isolated using the Nuclear Extract Kit (Active Motif). Protein concentration was determined by Bradford assay (Bio-Rad).

RNA Isolation, cDNA synthesis and real-time PCR. Total RNA was isolated using RNasy kit (Qiagen). cDNA was generated from using 1μg total RNA using the SuperScript III first-strand synthesis system (Invitrogen) following the manufacturer's instructions. mRNA levels of RelA, RelB, AR or PSA were quantified using gene-specific primers in presence of Roche probes master by real-time PCR in a LightCycler 480 (Roche Applied Science) and normalized to internal control human 18s.

Reporter construct and luciferase assay. PSA (6.1 kb)-luciferase contains the promoter/enhancer regions of the PSA gene and was kindly provided by Dr. Alvaro Puga. The reporter constructs were cotransfected with and β-galactosidase (β-gal) construct into LNCap cells, treated with indicated concentrations of TNF-α for 24 hours to determine the effect of TNF-α on PSA promoter activities. Relative luciferase units were calculated as indicators of transcriptional activity.

Electron spin resonance [274] assay. All ESR measurements were performed using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly, as described previously [275, 276]. The intensity of the ESR signal is used to measure the amount of short-lived hydroxyl radical trapped and the hyperfine couplings of the spin adduct are generally characteristic of the original trapped radicals. 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), used as spin or radical trap, was charcoal purified and distilled to remove all ESR detectable impurities before use. The Acquisit program was used for data acquisitions and analyses (Bruker Instruments). LNCap cells were harvested, treated with TNF-α at various concentrations for 30min and mixed with DMPO

(100 mM). The samples were then transferred to a flat cell for ESR measurement. Experiments were performed at room temperature and under ambient air.

Quantitative ROS determination. Dihydroethidium (DHE) oxidation was used for quantifying $O_2^{\bullet-}$ production by intact cells. General ROS production was quantified by DCF assay using carboxy-H_2DCFDA (sensitive to oxidation, Invitrogen) and oxidized carboxy-DCFDA (insensitive to oxidation, Invitrogen) as optimized in [277]. The fluorescence in cells preloaded with carboxy-H_2DCFDA was normalized to that in cells preloaded with carboxy-DCFDA (ratio of H_2DCFDA/DCFDA) to control for the cell number, dye uptake, and ester cleavage differences between different treatment groups.

NADPH oxidase activity assay. This assay was measured by the lucigenin-enhanced chemiluminescence method as described previously [278]. Photoemission generated by the reaction of superoxide radical and lucigenin in terms of RLU was measured every minute for 15 minutes. NOX activity was calculated as relative chemiluminescence (light) units (RLU) per minute per microgram protein.

Trypan blue exclusion assay. Cell suspension (20 μL) was mixed with 20 μL 0.04% trypan blue solution and loaded on to a hemocytometer. Cells were counted under a light microscope. Dead cells retained the dye while the viable cells excluded trypan blue and appeared bright. Cell survival was calculated against the relative control group.

Statistical analysis. Statistical analysis was performed using either Student's t-test (for two-group comparison) or one-way ANOVA followed by Dunnett’s test (for multiple-group comparison). Data are reported as mean ± standard error (SE).

Results

Ionizing radiation induces TNF-alpha secretion in both human prostate cancer cells and fibroblasts.

While most cytokines are generated due to immune response, other factors such as aging, environmental factors, or therapeutic intervention, also contribute to the elevation of inflammatory mediators. Evidence that the microenvironment is altered as a result of radiotherapy, especially the generation of myriad cytokines, has been elegantly reviewed [3]. In PCa treatment, radiation therapy causes cancer cell death primarily by directly damaging DNA or producing ROS that can diffuse far enough to damage DNA indirectly. Due to the bystander effects of ionizing radiation, production of myriad proinflammatory mediators, including TNF-α, is triggered in various types of cells including leukocytes and tumor cells [279]. In the current study, we present data showing ionizing radiation significantly enhances TNF-α secretion both in LNCap cells and BJ-5ta human fibroblast cells in a dose-dependent manner (Figure 2.1 A). Radiation induced TNF-α secretion was independent of cell type or species since IR can also induce TNF-α secretion in mouse embryonic fibroblasts in a dose- and time-dependent manner (Figure 2.1 B). To investigate whether IR-induced TNF-α is correlated with intracellular redox status, we compared TNF-α elevation between wild-type and knockout *sod2* mouse embryonic fibroblast (MEF) cells after IR treatment. As shown in Figure 2.1 B, KO-*sod2* MEFs with high oxidative stress secrete more TNF-α than WT MEFs.

Compared with normal cells, redox status is often less adaptive in tumor cells than in their normal counterparts because tumor cells are usually under higher oxidative stress,. *sod2* gene encodes one of the most important antioxidant proteins, MnSOD, the loss of which would significantly increase intracellular oxidative stress levels. Our results provided direct evidence demonstrating that cancer and stromal cells may be able to secrete more proinflammatory mediators, such as TNF-α, after IR exposure, particularly when tumors are under high level of oxidative stress.

TNF-α suppresses PSA expression dose dependently and significantly.

IL-6 can be generated in the microenvironment of prostate cancer in response to radiotherapy, during which it appears to peak after 15 days of radiotherapy before returning to pre-radiotherapy levels [280]. Elevation of IL-6 or overexpression of IL-6-

induced STAT3 can cause radioresistance in solid tumors, such as glioma and non-small cell lung cancer [281-283]. Elevation of both IL-6 and TNF-α levels correlates with clinicopathological features and patient survival in patients with PCa [139]. IL-6 stimulates STAT3 phosphorylation to upregulate expression of target genes, and TNF-α-mediated activation of NF-κB in PCa cells has been documented in many publications [142, 205, 284].

In contrast to cytokines, such as IL-6, which upregulates PSA and AR expression, we demonstrate a suppressive effect of TNF-α-induced and RelB-mediated NF-κB noncanonical pathway on PSA expression (Figure 2.2 A). Given the evidence that the RelB-mediated, but not RelA-mediated, canonical NF-κB pathway plays a more important role in prostate cancer progression and radioresistance, we carefully evaluated the expression of RelA and RelB in addition to PSA levels. LNCap cells exposed to TNF-α for 24 h showed reduced PSA expression but increased RelB levels in a dose-dependent manner, as well as increased expression of MnSOD, which was used as the positive control of a NF-κB target gene in the study (Figure 2.2 B). TNF-α not only suppressed PSA protein levels, as shown by Western blotting, but also inhibited PSA promoter-driven luciferase reporter activity (Figure 2.2 C). Real-time PCR also showed a significant induction of RelB transcription, but a reduction of PSA transcription (Figure 2.2 D). There were no significant changes in RelA protein (Figure 2.2 B) and mRNA (Figure 2.2 C) levels with the TNF-α treatment. In addition, TNF-α significantly induced RelB expression by 24hr in multiple cell types, but the suppressive effect on PSA was detected in LNCap cells (Figure 2.2 D).

TNF-α sequentially activates the canonical and noncanonical NF-κB pathways, leading to PSA suppression.

As shown in Figure 2.2 D, RelB induction and PSA suppression in LNCap cells happened at late, but not initial time points. To delineate the sequence of events, we collected total cell lysate at different time points after TNF-α treatment. The suppressive effects of TNF-α on PSA expression were observed initially at 6 h after treatment, concurrent with RelB induction, while RelA in total cell lysate remained essentially unchanged (Figure 2.3 A).

Since translocation of either RelA or RelB into nucleus with specific partners is sufficient to regulate the targeted gene transcription, we further determined RelA and RelB levels in the nuclear fraction. The results show that RelA induction occurred immediately after treatment with TNF-α then diminished quickly. For RelB, there was a gradual and prolonged increase that lasted into late time points up to at least 24hrs after TNF-α treatment (Figure 2.3 B), suggesting a sequential activation of RelA-mediated canonical and RelB-mediated noncanonical NF-κB pathways, with the RelB-mediated noncanonical pathway contributing to sustained PSA suppression. In addition to this TNF-α-mediated switch from the RelA-mediated pathway to the RelB-mediated pathway, we also found that AR levels increased initially, but decreased gradually, in both total lysate and nuclear fractions (Figure 2.3), consistent with RelA binding directly to the 5' regulatory region of the AR gene and upregulating AR and its targeted PSA expression [285]. The alteration of AR levels during TNF-α exposure may result from decreasing RelA-mediated induction and increasing RelB-mediated suppression.

TNF-α amplifies endogenous ROS and induces superoxide radical generation.

Both increased production of ROS and decreased antioxidant levels can result in oxidative stress. TNF-α is often produced in response to oxidative stress and it also causes oxidative stress in its target cells. TNF-α-induced ROS, which can be inhibited by mitochondrial-specific MnSOD overexpression, may oxidize and inhibit JNK-inactivating phosphatases, leading to sustained JNK activation [286]. The relationship between TNF-α-induced ROS and TNF-α-mediated PSA suppression in PCa cells remains to be determined. ESR was used to detect free radical generation from TNF-α-stimulated LNCap cells. Figure 2.4 A shows the spectra recorded from mixtures containing cells treated with increasing doses of TNF-α for 30 min. Based on these splittings and 1:2:2:1 line shape, the spectra were assigned to the DMPO/•OH adduct [275], which is evidence of •OH radical generation. Addition of TNF-α resulted in a dose-dependent increase in the 1:2:2:1 quartet ESR signal. Pretreatment with SOD or catalase was able to diminish the TNF-α-induced DMPO/•OH peak to basal levels, indicating that ROS such as $O_2^{\cdot -}$ and H_2O_2 were generated in LNCap cells by TNF-α treatment (Figure 2.4 A). To further evaluate the ROS generation upon TNF-α treatment, we performed DHE assay and found a significant and dose-dependent increase in $O_2^{\cdot -}$ as

early as 5 min and as late as 24 h post-treatment (Figure 2.4 B). TNF-α also slightly increased normalized carboxy-H_2DCFDA fluorescence, a general indicator of cellular ROS level, in a dose dependent manner, but the increase was not as significant as DHE oxidation (Figure 2.4 B), suggesting that $O_2^{\cdot -}$ is likely to be the major type of reactive oxygen species generated by TNF-α in prostate cancer cells. The magnitude of the TNF-α induced increase in $O_2^{\cdot -}$ or general ROS diminished at 24 h as compared to 5 min post-treatment, which is most likely due to some adaptive response induced by TNF-α. To determine whether TNF-α-induced oxidative stress is involved in PSA and AR suppression, LNCap cells were treated with TNF-α in the presence/absence of the $O_2^{\cdot -}$ scavenging SOD mimetic, MnTE-2-PyP^{5+}. SOD mimetic pretreatment abrogated TNF-α-induced PSA and AR suppression, as well as RelB activation, significantly and dose dependently (Figure 2.4 C). These data support the notion that TNF-α treatment generates ROS, particularly $O_2^{\cdot -}$, which activates RelB-mediated noncanonical NF-κB pathway, resulting in PSA and AR suppression.

Activation of NADPH oxidase by TNF-α is an initial source of ROS that results in its downstream signaling and PSA suppression.

Many ROS generating systems, such as mitochondria, NADPH oxidase and nitric oxide synthase, may be influenced by TNF-α exposure. NADPH oxidase catalyzes the transfer of an electron to O_2 to generate $O_2^{\cdot -}$, which is one major source of ROS generation and is required for the aggressive phenotype in PCa cells [58, 287]. We measured NADPH oxidase activity to determine whether it is involved in TNF-α-induced $O_2^{\cdot -}$ in LNCap cells. Our results show that TNF-α enhanced NADPH oxidase activation rapidly which was sustained throughout the duration of TNF-α exposure (Figure 2.5 A). In LNCap cells, treatment with TNF-α increased NADPH oxidase activity in a dose-dependent manner (Figure 2.5 A).

There are seven members of the NOX family: NOX1 through NOX5 and Duox1/Duox2. We identified NOX1 as the major Nox isoform responsible for TNF-α-induced NADPH oxidase activation. Based on the findings that Nox1 expression is enhanced in a dose dependent manner (Figure 2.5 B) as early as 5min after TNF-α treatment. The levels of NOX4, another important NOX isoform in prostate cancer, increased slightly when LNCap cells were treated with a high dose of TNF-α. However,

NOX4 expression exhibited a declining trend time-dependently (Figure 2.5 B). Furthermore, RelB induction was inhibited dose-dependently by DPI, a NADPH oxidase inhibitor, indicating that NADPH oxidase activation is an upstream event of the NF-κB pathway (Figure 2.5 C). Knockdown of NOX1 by siRNA inhibited TNF-α-induced PSA and AR suppression as well as RelB induction (Figure 2.5 C), suggesting that NOX1 activation contributes to TNF-α-induced ROS generation, leading to PSA suppression.

Low dose TNF-α exposure has no cytotoxic effects and stimulates radiation resistence in LNCap cells

To evaluate the significance of low dose TNF-α exposure, LNCap or PC-3 cells were treated with increasing doses of TNF-α with or without IR. When exposed to TNF-α at concentrations that are within a physiologically relevant and/or IR-inducible range (<1.0ng/mL) [288], LNCap exhibited little change in cell survival and viability as determined by MTT assay (Figure 2.6 A) and Trypan blue exclusion assay (Figure 2.6 B), respectively. In addition to LNCap cells, low dose exposure of TNF-α prior to radiation in PC-3 cells had slightly cytoprotective or radioresistant effects (Figure 2.6 B). However, TNF-α treatment mitigated some of the effect of radiation on the cell survival as evidenced by the significantly higher survival of the cells treated 0.25 ng/mL TNF-α and exposed to 2 Gy radiation and the cells treated 0.5 ng/mL TNF-α and exposed to 2 or 4 Gy radiation as compared to radiation or TNF-α treatment alone (Figure 2.6 A). These results indicate that low dose TNF-α exposure at physiologically relevant doses or after IR may confer small yet statistically significant protection or radio-resistance to the cells without appreciable effects on cell survival.

Discussion

Our results demonstrate that IR directly induces the generation of proinflammatory mediators including TNF-α in both prostate cancer cells and stromal fibroblasts due to the bystander effect (Figure 2.1), an induction of some biological effect in cells that have not been directly traversed by radiation, but are in close proximity to a cell that has received radiation [289]. MnSOD, encoded by *sod2* gene, is the most critical enzyme in the mitochondria and a center for regulating cellular redox balance [66]. The difference in TNF-α secretion between wt-MEF and *sod2*-KO MEF after IR exposure provides the direct evidence that this bystander effect of IR is highly dependent on cellular redox status (Figure 2.1).

Various lines of evidence have suggested that chronic inflammation is causally linked to prostate carcinogenesis and PCa progression. While IL-6 elevation correlates with clinicopathological features and survival of patients with prostate cancer [139], the use of PSA to diagnose IL-6-based prostate cancer would be redundant because IL-6 positively regulates PSA expression and AR function [143, 144, 270]. Our study demonstrates that TNF-α, a major mediator of cancer-related inflammation when it is chronically present in the tumor microenvironment, can lead to significant RelB induction and PSA suppression significantly (Figure. 2.2).

Considering the cellular heterogeneity in tumors and dynamic communications between stromal and prostate cancer cells, TNF-α can be generated by many sources, such as chronic inflammation or radiation-induced bystander effect, and it is a prime factor involved in stress response [119, 189]. When we compared the effects of low dose TNF-α exposure on downstream signaling pathways and target gene expression among different cancer cell lines, a significant suppression of PSA gene expression raised our interest in the current study (Figure 2.2 D). Because the PSA gene harbors several AREs in its promoter and enhancer, PSA expression is tightly regulated by AR level and function [258, 290]. Among commonly used prostate cancer cell lines, LNCaP cells express functional AR, while both DU145 and PC3 cells are AR negative [291]. Consistent with our findings, TNF-α also has been implicated in initiation of an androgen-independent state in prostate cancer through its ability to inhibit AR sensitivity [204], negative regulation of AR expression through the tumor necrosis factor receptor–

associated death domain (TRADD) [284], and/or the interplay of NF-κB and B-myb [205]. Our results indicate that TNF-α immediately activates the RelA-mediated canonical NF-κB pathway, but the robust RelB-mediated noncanonical pathway contributes to sustained PSA suppression (Figure. 2.4). RelB upregulation by TNF-α or other factors may result in more aggressive characteristics in a tumor despite inhibition of PSA expression. Patients with the most devastating conditions of late stages of cancer [197, 198] can exhibit association between increased TNF-α and cachexia. The results of the current study are consistent with population-based national findings that obesity, which significantly induces TNF-α, is associated with an increased risk of prostate cancer mortality but negatively correlates with PSA [292].

Elevated oxidative stress plays a significant role in several physiological/pathological conditions such as aging and aging-associated diseases. Aging is one of the most important factors in prostate cancer, as tumors occur frequently in older men. Cancer cells tend to secrete more proinflammatory mediators and have a higher level of oxidative stress compared to normal cells, and the level of oxidative stress is associated with prostate cancer occurrence, recurrence and progression [58]. Generally, at an early stage of cancer development, tumor cells are under high oxidative stress, in part due to inhibition of various antioxidant enzyme activities. However, after cancer has progressed, cancer cells tend to be equipped to cope with incremental increases in oxidative stress by inducing more antioxidant enzymes, such as MnSOD, which may confer aggressive or radioresistant phenotype of prostate tumors [66].

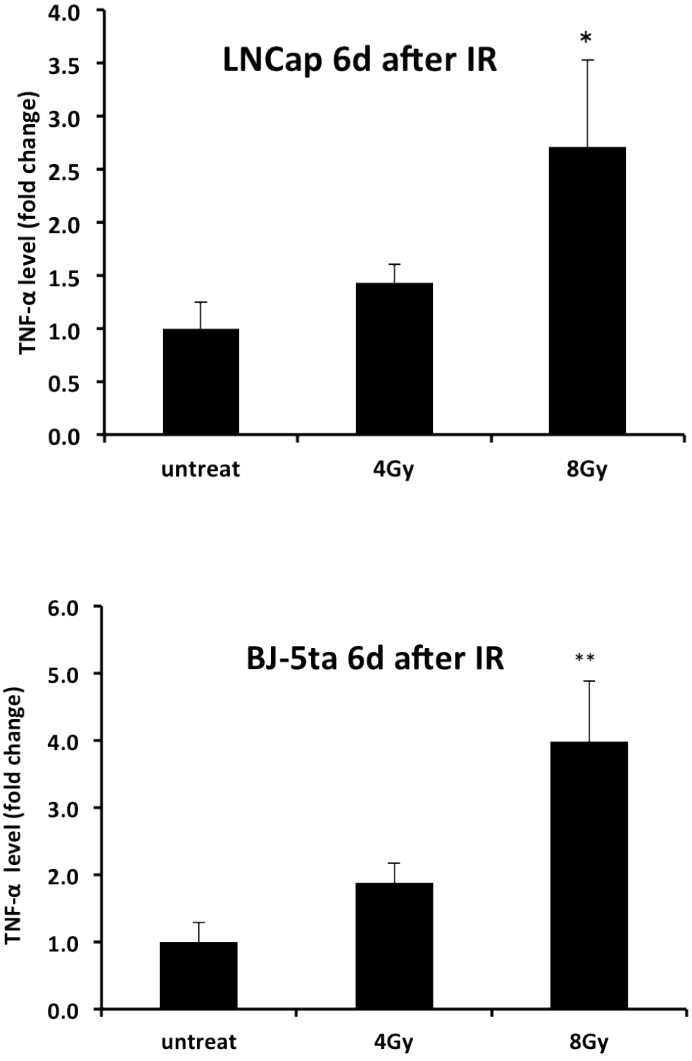
IR-generated proinflammatory mediators play pleiotropic roles in the complexity of tumor microenvironment at different stages of prostate cancer development and progression. TNF-α functions as an indirect and sustained source of ROS source due to activation of NADPH oxidases, which leads to activation of downstream signaling pathways (Figure. 2.4-2.5). Blocking ROS by adding SOD mimetic or knocking down NOX1 leads to suppression of RelB induction, as well as PSA and AR suppression (Figure. 2.5-2.6). Although a relatively high dose of TNF-α usually induces an acute and pro-cell death response, chronic elevation of TNF-α at a relatively low level can result in cytoprotection and tumor progression, which is related to increased levels of antioxidant, antiapoptotic, and other defense proteins, such as thioredoxins and MnSOD. Additionally,

TNF-α mediated activation of the NF-κB signaling pathway is critical in supporting cancer-related inflammation and malignant progression, as well as maintaining the immunosuppressive phenotype of tumor associated macrophages [203]. For example, in human and mouse ovarian cancer, TNF-α maintains TNFR1-dependent IL-17 production by CD4+ cells, which leads to myeloid cell recruitment into the tumor microenvironment and enhanced tumor growth [293]. It is of interest to note that while treatment with low dose of TNF-α had little effect on cell survival, it conferred a small, yet statistically significant, radioresistance to the irradiated cells (Figure. 2.6), implicating IR-induced proinflammatory mediators in cancer promotion and progression pathways.

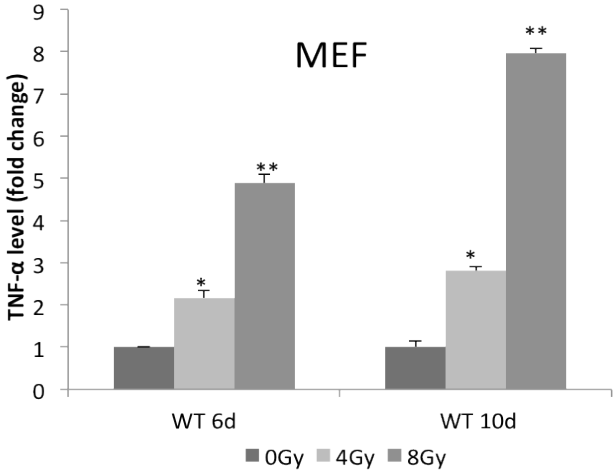
In summary, the present study demonstrates that radiation therapy generates TNF-α, leading to the formation of an inflammatory microenvironment with sustained oxidative stress, in part, through NADPH oxidase activation. TNF-α triggers RelA-mediated NF-κB canonical pathway rapidly and transiently but its effect on RelB expression is more robust and long-lasting, which led to sustainable suppression of PSA expression. Treatment with TNF-α within concentration ranges expected in the increased oxidative stress or after radiation exposure suppressed PSA expression and conferred a small, yet statistically significant, protection to LNCap cells against irradiation, which indicates that radiation-induced TNF-α may not only interfere with the PSA-based PCa diagnosis and post-treatment monitoring, but also diminish the efficacy of radiotherapy. Thus, redox intervention, together with anti-inflammatory mediators, can be useful to improve radiotherapy efficacy by radiosensitizing cancer cells while protecting normal cells and tissue from excessive radiation damage.

Figure 2.1. Ionizing radiation induces TNF-α secretion in both human prostate cancer cells and fibroblast.

A. Ionizing radiation induces TNF-α secretion in prostate cancer LNCap cells and human immortalized foreskin fibroblast cell line BJ-5ta. Cells were irradiated at the indicated doses and then cultured for 6 days. Cultured media were changed to fresh medium containing only 1% FBS and conditioned media were collected 24 h later for ELISA. The relative TNF-α secretion was normalized against protein concentration. The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.



B. Ionizing radiation induces TNF-α secretion in mouse embryonic fibroblasts time (upper panel) and redox dependently (lower panel). Cells were irradiated at the indicated doses and then cultured for 6 days. Cultured media were changed to fresh medium containing only 1% FBS and conditioned media were collected 24 h later for ELISA. The relative TNF-α secretion was normalized against protein concentration. The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.



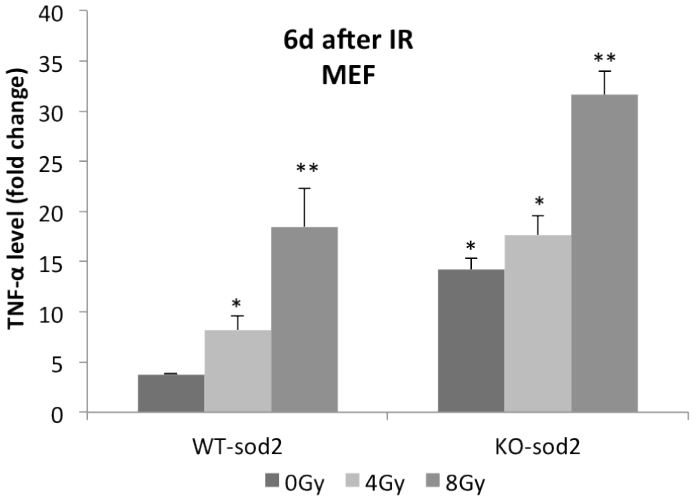
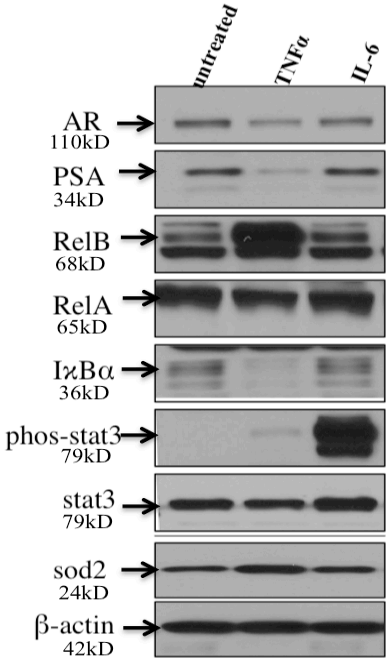
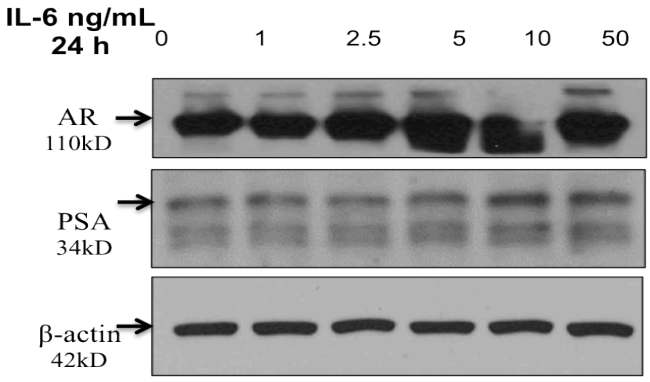


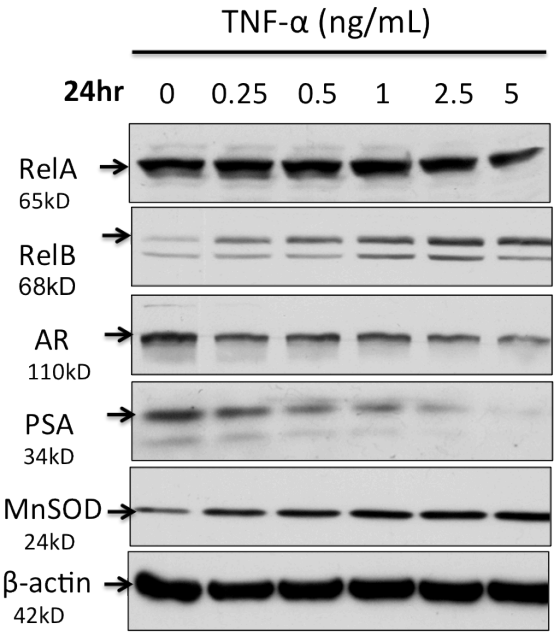
Figure 2.2. TNF-α dose dependently suppresses PSA expression.

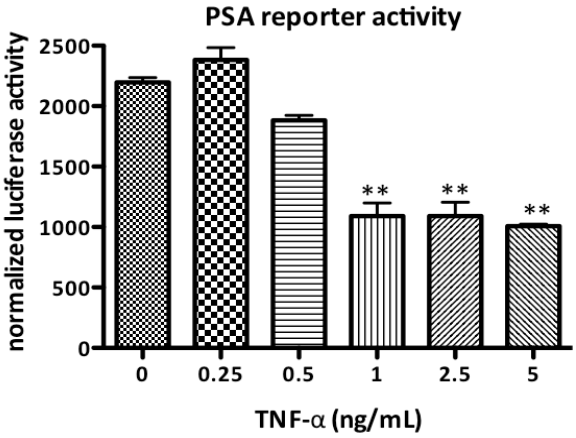
A. TNF-α activates NF-κB pathway while IL-6 induces STAT3 phosphorylation in LNCap cells leading to different effects on PSA and AR expression. LNCap cells were treated with TNF-α and IL-6 (1ng/mL) as indicated for 24 h (upper panel) or different concentration of IL-6 as indicated for 24 h. Total cell lysate was prepared and analyzed for the Western blotting assay.



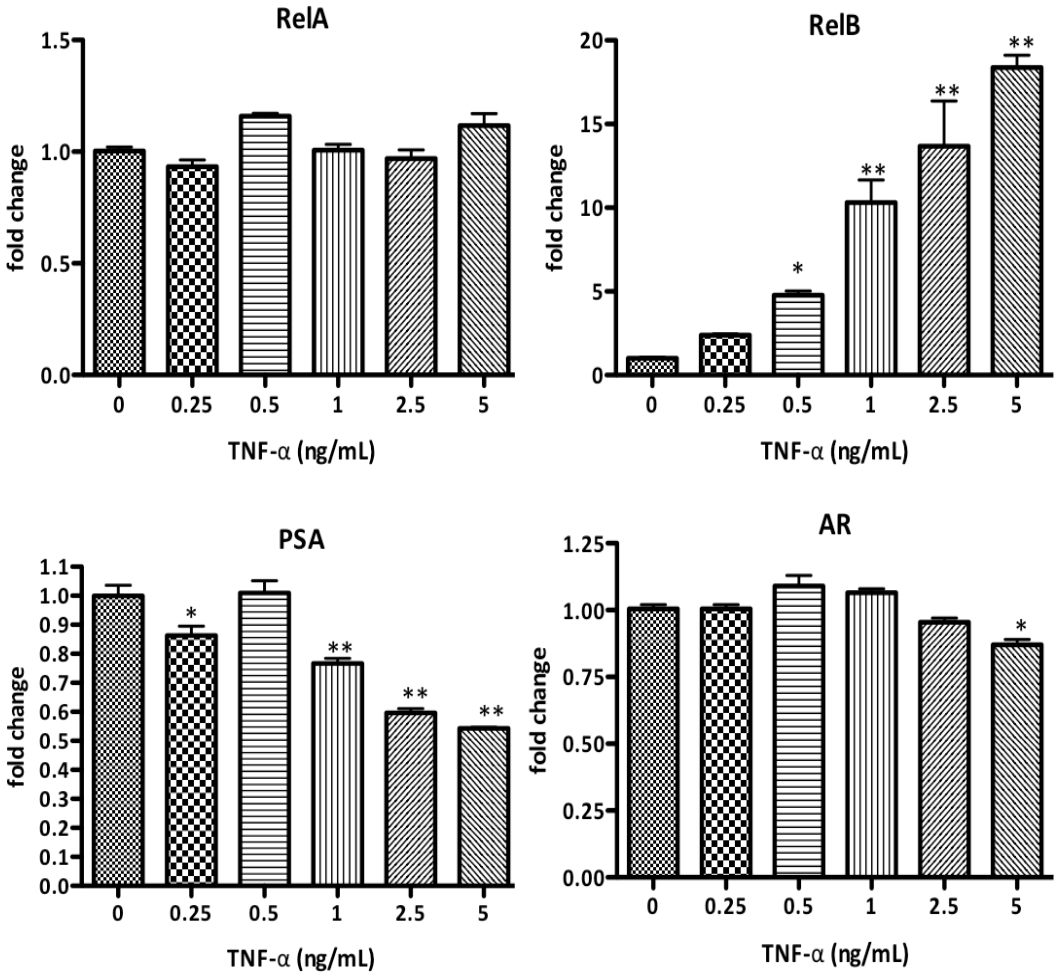


B: TNF-α induces RelB but suppresses PSA in dose dependent manners. LNCap cells were treated with different concentration of TNF-α as indicated for 24 h. Total cell lysate was prepared for the Western blotting assay (upper panel). PSA promoter activity assay. LNCap cells were transiently transfected with PSA promoter construct (~6.1kb) and treated with different concentrations of TNF-α for 24 h. Firefly luciferase activity Normalized against internal β-gal activity was used as a surrogate measurement of PSA gene transcription (bottom panel).





C: Real-time PCR. Total RNAs were isolated and converted to cDNA to determine mRNA levels of respective genes following normalization against human 18S rRNA levels, which served as the loading control.



D. TNF-α induces RelB robustly in multiple cell types but suppresses PSA in LNCap cells. Multiple cell types were treated with TNF-α (1ng/mL) for 24 h as indicated. LNCap cells were treated for 30 min and 24 h for comparison. Total cell lysate was prepared for the Western blotting assay.

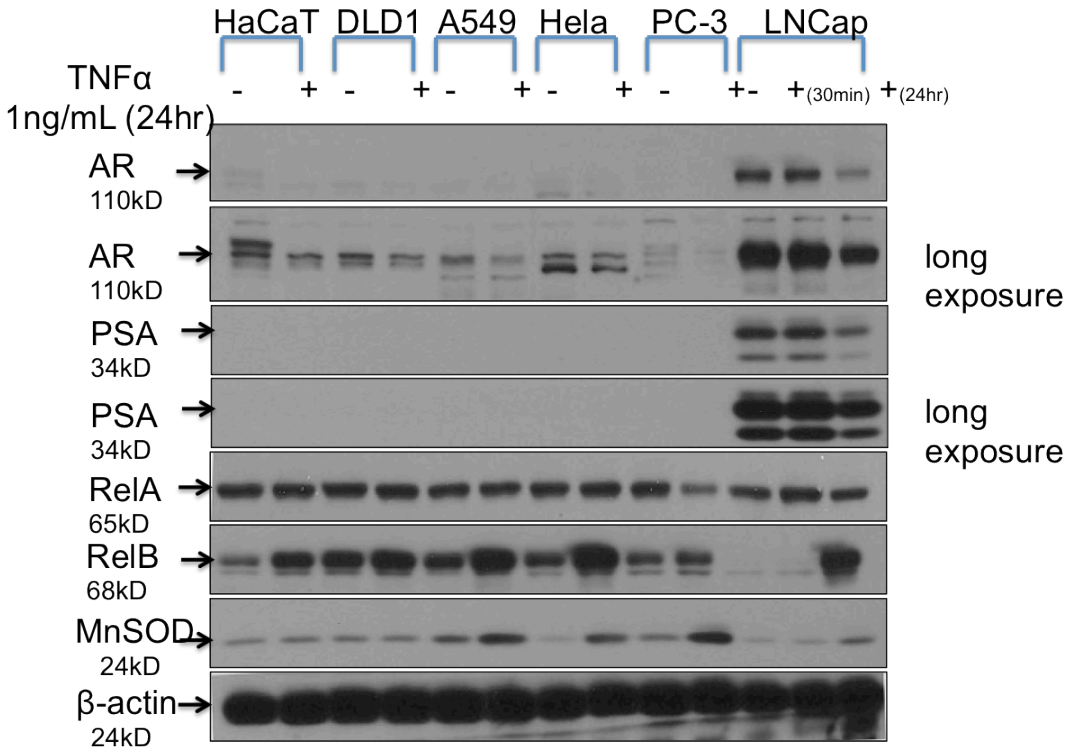
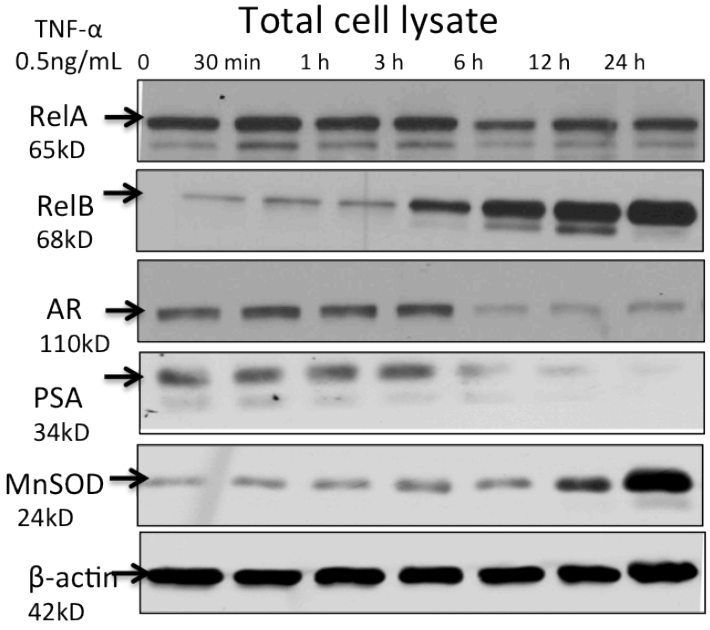
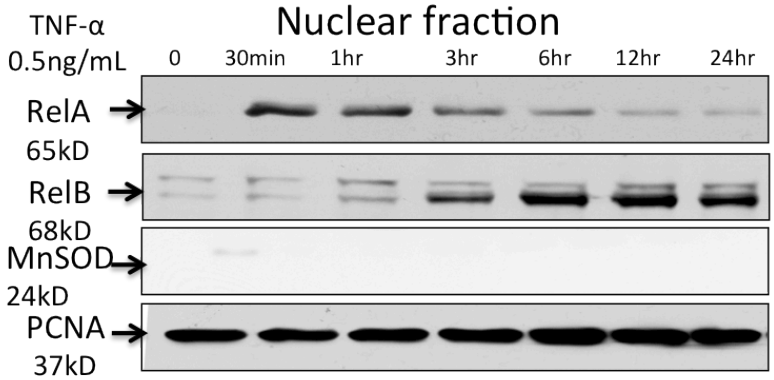


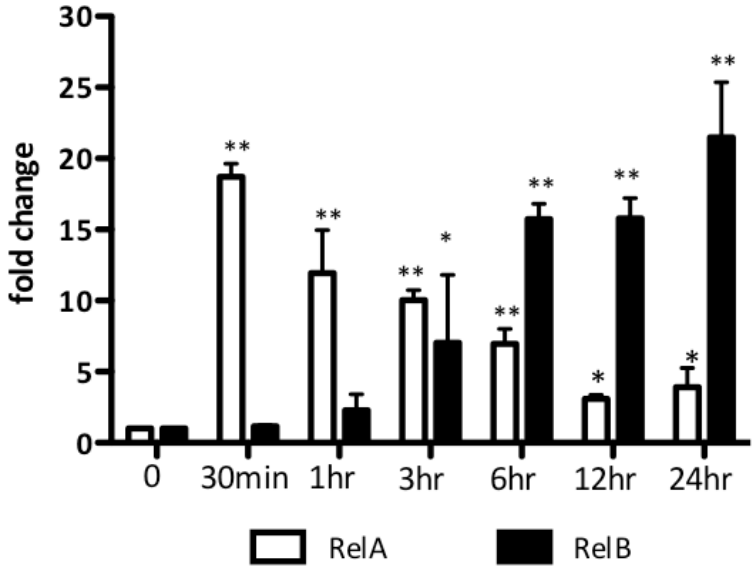
Figure 2.3. TNF-α sequentially activates the canonical and noncanonical NF-κB pathways, leading to PSA suppression.

A: Time course analysis. To determine the sequence of canonical and noncanonical NF-κB pathways after TNF-α treatment (0.5ng/mL), total cell lysate (upper panel) and nuclear fraction samples (bottom panel) were prepared at indicated time for Western blotting analysis. Staining signals obtained with anti-MnSOD and anti-PCNA antibodies were used as loading controls for cytosolic and nuclear fractions, respectively.





B. The nuclear RelA, RelB and AR protein bands were quantified and normalized against the band intensity of PCNA. Each data point is shown as the mean ± SE of triplicate experiments, and significant difference as compared to the untreated control is indicated by *p<0.05 and **p<0.01.



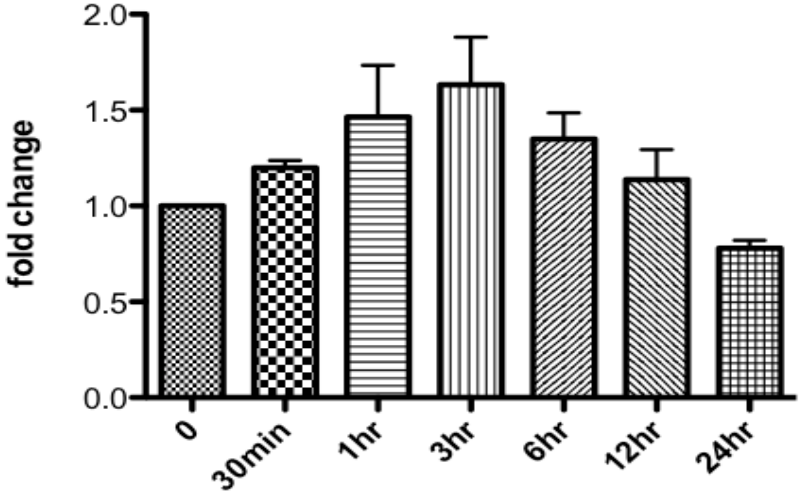
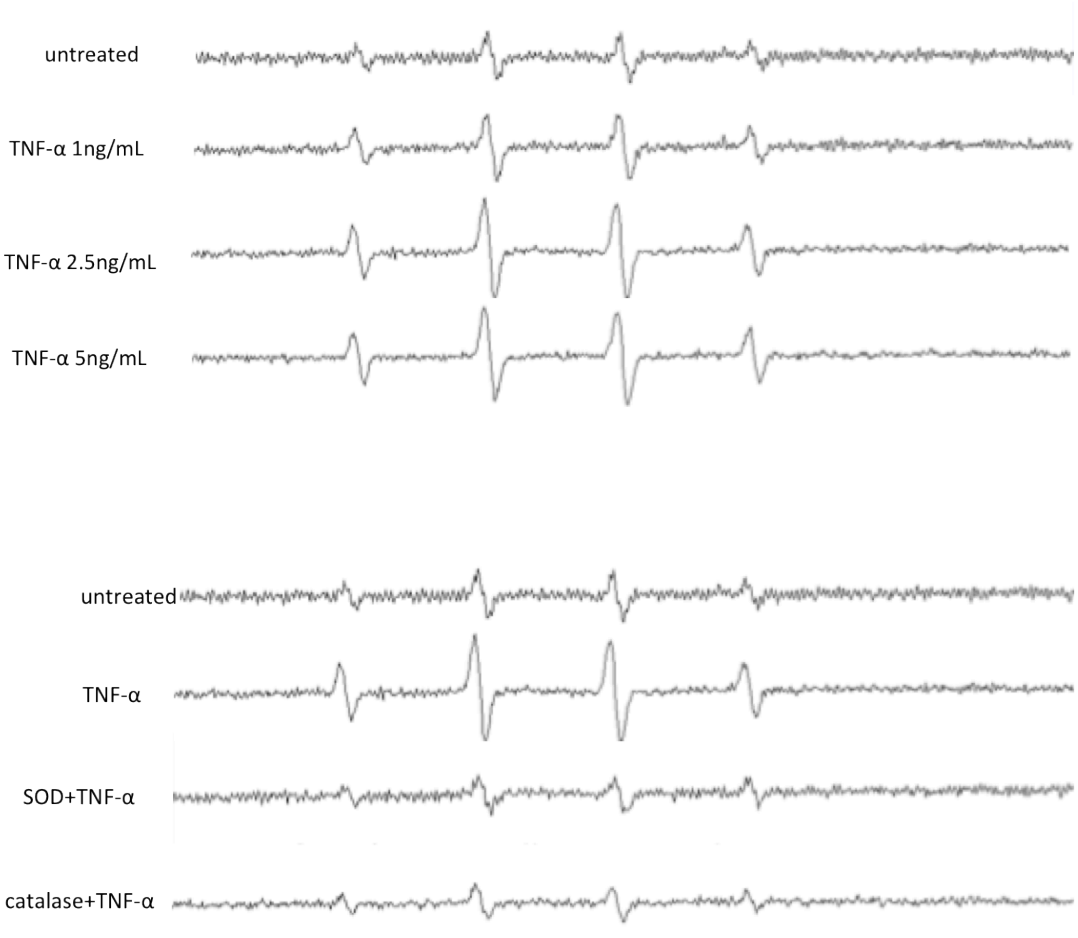
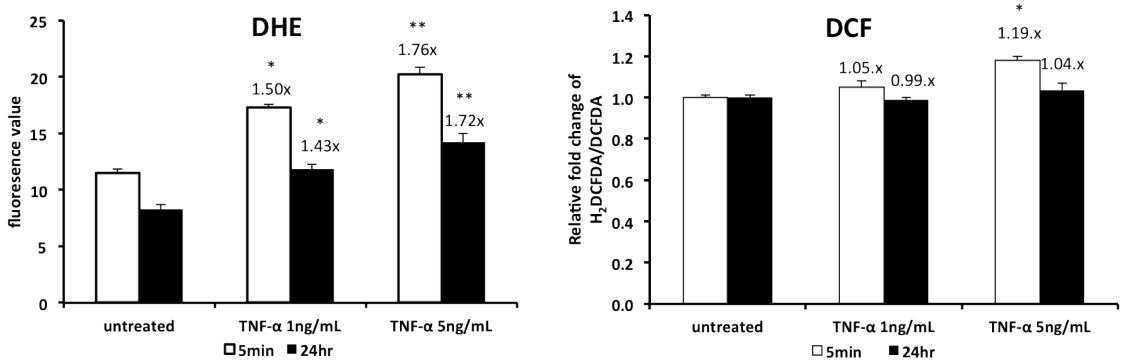


Figure 2.4. TNF-α amplifies endogenous ROS and induces superoxide radical generation.

A: ESR spectrum for LNCap cells treated with TNF-α. ESR spectrum was recorded 15 min after the addition of different concentrations of TNF-α for 30min as indicated and 100 mM DMPO to 5×10^5 cells (upper panel). ESR spectrum for LNCap cells pre-treated with SOD or catalase and exposed to TNF-α. Cells were pretreated with SOD (500U/mL) or catalase (500U/mL) for 5min and exposed to TNF-α (1ng/mL) for 30min. ESR spectrum was recorded as described above (bottom panel).



B: DHE assay. Cells were treated with different concentration of TNF-α as indicated for 5min or 24 h; the oxidation of DHE was measured to quantify $O_2^{\cdot -}$ generation. D: DCF assay. Cells were treated as described above; the ratio of carboxy-H_2DCFDA (oxidation sensitive) to carboxy-DCFDA (oxidation insensitive) was compared.



C: Abrogation of TNF-α-mediated PSA and AR suppression by SOD mimetic. Cells were pretreated with SOD mimetic (100pg/mL) for 30min then exposed TNF-α (0.5ng/mL) for 24 h (upper panel). RelB induction diminished after treatment with SOD mimetic. Addition of SOD mimetic diminishes TNF-α-mediated RelB induction and alleviated PSA and AR suppression in a dose dependent manner (bottom panel). The results are shown as the mean ± SD of triplicate experiments and significant difference as compared to control was indicated by * ($p<0.05$) and ** ($p<0.01$). Significant difference for groups with SOD mimetic addition as compared to the untreated control in aspect of TNF-α-induced AR and PSA suppression is indicated by # ($p<0.05$) and ## ($p<0.01$).

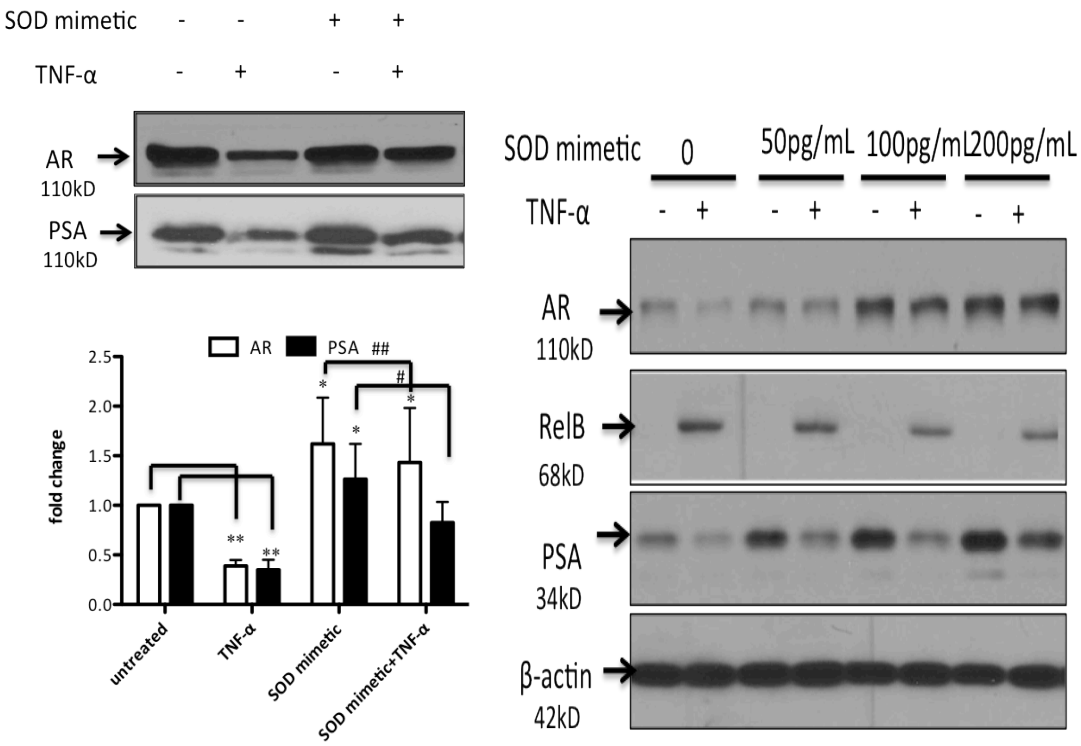
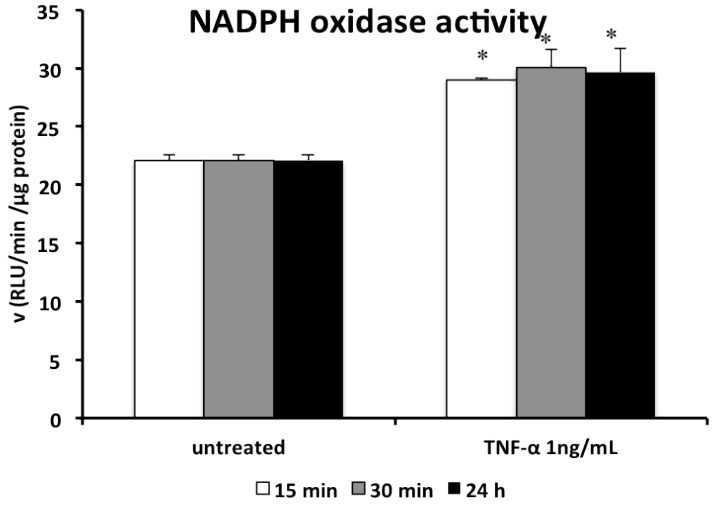
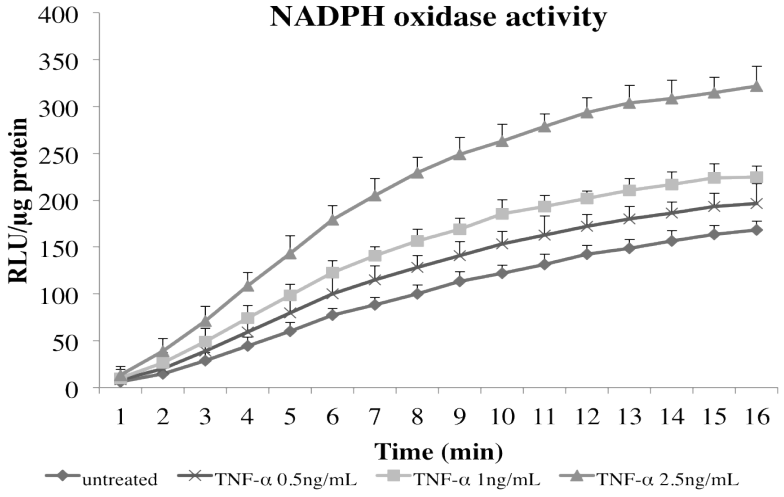


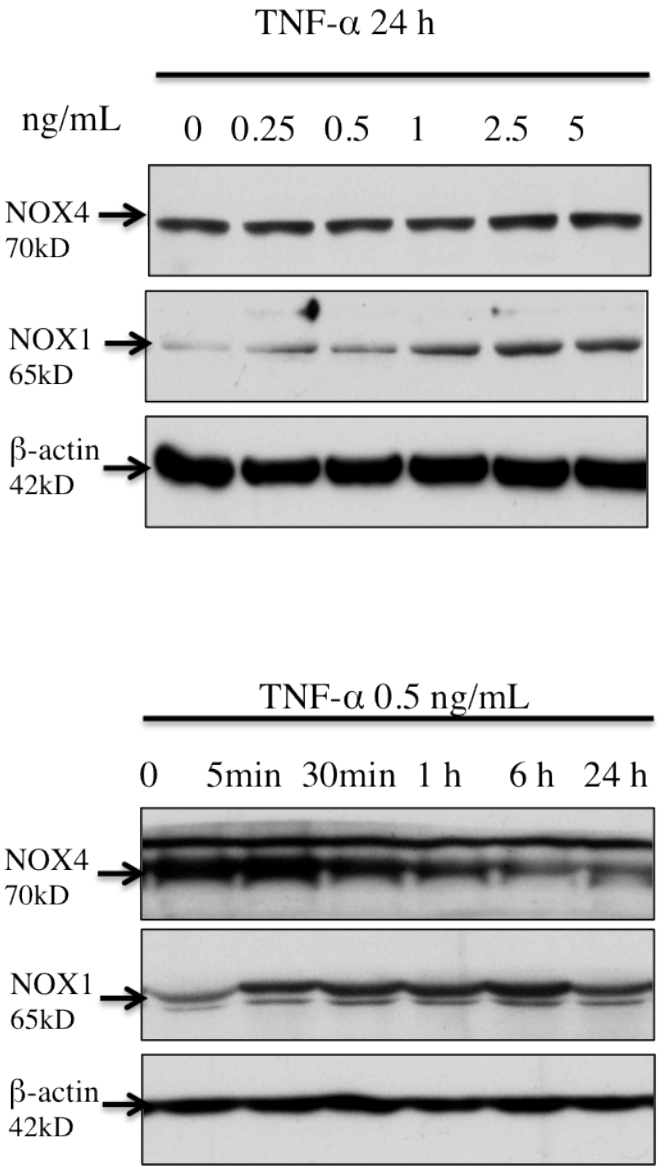
Figure 2.5. Activation of NADPH oxidase by TNF-α is an initial source of ROS leading to downstream signaling and PSA suppression.

A: NADPH oxidase activity assay of cells after treatment with 1 ng/mL TNF-α (upper panel). Cells were treated with TNF-α (1ng/mL) and harvested at different time for NADPH oxidase activity measurement. Reaction velocity (V) was calculated as the change of RLU per minute per μg protein. NADPH oxidase activity assay of cells treated with TNF-α at different concentrations (bottom panel). Cells were treated with different concentrations of TNF-α. Photoemission generated by the reaction of superoxide radical and lucigenin in terms of RLU was measured every minute for 15 minutes. Significant difference as compared to the untreated control is indicated by * ($p<0.05$).

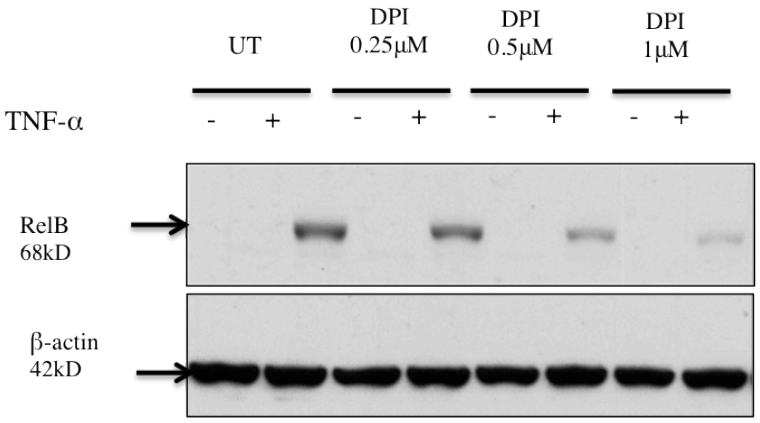




B: TNF-α induces NOX1 but not NOX4 expression in a dose dependent manner. LNCap cells were treated with different concentration of TNF-α as indicated for 24 h (upper panel). TNF-α induces NOX1 expression in a time dependent manner, as early as 5min. On the other hand, NOX4 expression level gradually decreased. LNCap cells were treated with TNF-α at a concentration of 0.5ng/mL. Total cell lysate were prepared at the indicated times and analyzed by Western blotting (bottom panel).



C: NADPH oxidase activation is the upstream event of TNF-α-mediated RelB induction. LNCap cells were pretreated with different concentration of DPI, a NADPH oxidase inhibitor, then exposed TNF-α (0.5ng/mL) for 24 h (upper panel). Knocking down NOX1 suppresses TNF-α-induced RelB activation, PSA and AR suppression. LNCap cells were transiently transfected with siRNA NOX1 or control siRNA for 12 h, then treated with or without TNF-α (0.5ng/mL) for 24 h. Total cell lysate was analyzed by Western blotting (bottom panel).



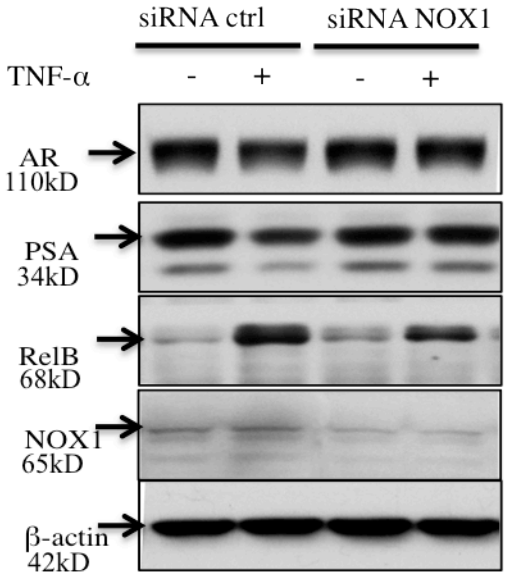
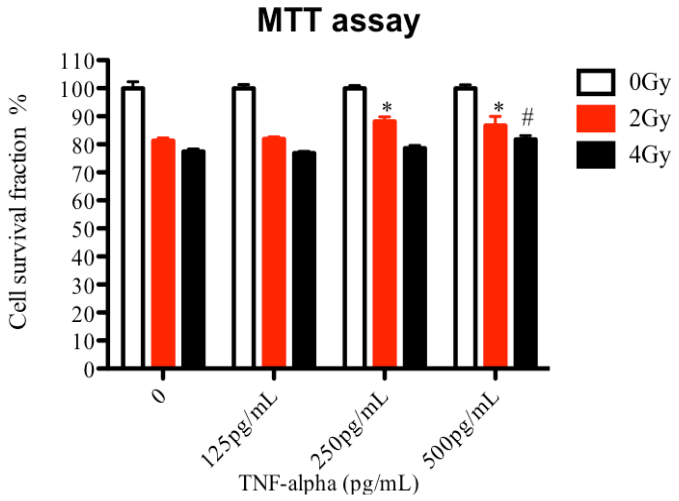
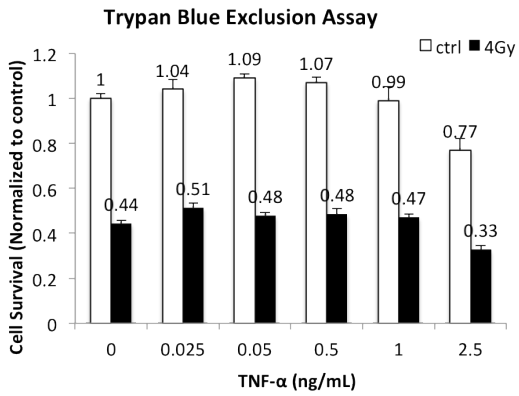


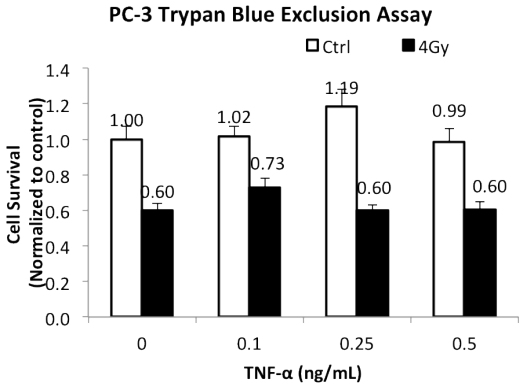
Figure 2.6. Low dose of TNF-α exposure exhibits little cytotoxicity but minor radioresistance in prostate cancer cells.

A. MTT assay for cell survival. Cells were treated with different concentrations of TNF-α for 24hr and then irradiated at indicated doses. Cells were then cultured for four doubling times (~5day) and cell mass, which was a surrogate measurement of cell survival, was measured using MTT assay. Each data point represents the mean ± SD of three independent experiments and significant difference as compared to the untreated control is indicated by * ($p<0.05$) and ** ($p<0.01$).



B. Typan blue exclusion assay for cell survival. LNCap or PC-3 cells were treated with different concentration of TNF-α for 24 h and then irradiated at indicated doses. Forty-eight hours after irradiation, cells viability was determined by Typan blue exclusion assay.





Chapter Three

RelB-AR axis mediates PSA suppression in advanced prostate cancer: significance in PSA dependent clinical applications

Introduction

According to the latest annual cancer statistics report from the American Cancer Society, 241,740 new cases of prostate cancer and 28,170 prostate cancer-specific deaths are projected for 2012 [1]. The advent of PSA testing has revolutionized both the initial diagnosis and the follow-up disease monitoring after treatment, such as hormone management and radiation therapy. However, PSA testing results can sometimes be ambiguous and its interpretation remains a major challenge to even very experienced clinicians, leading to over- and under-diagnosis of such a bimodal disease with indolent and aggressive forms [258]. Comparing to the overtreatment of patients who would otherwise require only conservative management, Thompson. *et al* reported that close to 15% of men whose PSA levels were below a conventional cutoff of 4.0ng/mL did harbor PCa, including high-grade carcinoma [259]. In human prostate cancers, cancer cells expressing little or no PSA population contain tumor propagating cancer stem cells that resist castration, exhibit high clonogenic potential and possess long-term tumor-propagating capacity [294]. Therefore, the current state of PCa detection is not optimal and can miss potentially lethal cancers, which highlights the critical need for additional molecular markers and/or a better understanding of mechanisms by which PSA expression is downregulated in advanced PCa.

The above mentioned concerns have raised interest in fundamentally expanding our understanding of PCa progression and developing alternative and/or additional biomarkers that distinguish indolent versus aggressive forms of PCa, especially in patients with low or undetectable PSA levels.

The PSA gene encodes a 33kDa glycoprotein, which consists of a single polypeptide chain of 240 amino acids. In the normal prostate, PSA is secreted into the glandular ducts where it functions to degrade high molecular weight proteins synthesized in the seminal vesicles to inhibit coagulation of the semen [257, 295]. However, PSA enters the serum through leakage into the extracellular fluid of the normal prostate. In most situations, with prostate tumor progression, serum PSA levels are elevated due to the loss of the

normal glandular architecture [295]. The expression of PSA is regulated by the AR. Upon binding to androgen, the AR translocates into the nucleus and binds to the ARE on the PSA promoter [257, 295]. Thus, androgen and AR signaling play fundamental roles in PSA expression at the transcription level. In addition, AR signaling through cross-talk with various signaling transduction pathway is a major contributor to the promotion of prostate cancer progression.

Recent studies have provided evidence for a role of NF-κB in cancer initiation and progression [271, 272]. NF-κB pathway and increased antioxidant capacity are involved in causing resistance to radiation treatment in many tumor cell types [15, 177, 178]. The NF-κB family includes five proteins, RelA, p50, RelB, p52 and cRel, and is essential for the regulation of innate and adaptive immunity. All five NF-κB family members have a Rel-homology domain that contains a nuclear localization sequence and is important for DNA binding, dimerization, and interaction with IκB proteins [26]. Generally, the NF-κB pathway can be divided into RelA-mediated canonical pathway and RelB-mediated noncanonical pathway. Immunohistochemistry results show that nuclear localization of RelA is associated with PSA relapse, which is the first sign of prostate cancer recurrence [206], while *in vitro* mechanistic studies suggest RelA binds directly to the 5' regulatory region of the AR gene and upregulates AR and its targeted PSA expression [285]. However, RelB-mediated noncanonical NF-κB pathway plays more significant roles in prostate cancer aggressiveness and radiation resistance [26, 165, 296]. RelB is the most frequently detected NF-κB subunit in prostate cancer tissue nuclei that significantly correlated with patient's Gleason scores [296]. RelB regulates MnSOD expression through constitutively binding to the promoter region of the *sod2* gene ([179] and reviewed in [26]), which is important for the resistance of prostate cancer cells to ionizing radiation. RelB nuclear localization is significantly higher in the aggressive PC-3 prostate cancer cell line compared to the less aggressive LNCaP cell line, and correlates with increased MnSOD protein levels, enzyme activity and radioresistance in PC-3 cells [179]. Selective inhibition of the RelB-mediated NF-κB alternative pathway by a group of potential therapeutic agents, including 1α, 25-dihydroxyvitamin D3 (1α, 25-$(OH)_2D_3$) [181], STI571 [210] and a protein peptide SN52 [180], can remarkably sensitize prostate

cancer cells to IR-induced killing through inhibiting RelB nuclear activation and downregulating the RelB-targeted gene, MnSOD.

Our previous animal study showed that stable overexpression of RelB in LNCap cells enhanced prostate cancer tumorigenicity while, intriguingly, tumors with increased RelB level produced less PSA [165]. Here, we uncover a role for RelB in suppressing PSA expression at an advanced stage of prostate cancer, which could be a mechanism for the low PSA level in some patients bearing aggressive prostate cancer. In this chapter, four microarray gene expression datasets publicly available at Oncomine.org also consistently displayed an inverse relationship between RelB and AR expression in human prostate cancers. Our current study investigated the mechanisms by which RelB suppressed PSA expression and showed that RelB inhibited PSA promoter activity, AR expression and function, and interacted with AR to form a complex on the enhancer elements of the PSA promoter.

According to data presented in the previous chapter, unlike IL-6, that enhances PSA and AR expression, TNF-α exerts sustained suppressive effects on PSA expression, which concomitantly depends on a delayed RelB-mediated noncanonical NF-κB activation. The relationship of TNF-α-induced PSA suppression with concurrent robust RelB induction is as follows. In this chapter, a number of molecular mechanisms indicating how PSA gene transcription is regulated by RelB-AR axis are discussed extensively. We demonstrated this novel RelB-AR axis as an important contributor to the downregulation of PSA expression in aggressive and radioresistant prostate tumors. This mechanistic study provides new insights into the clinical relevance of the PSA test and the improvement of redox intervention in prostate cancer treatments.

In summary, this is the first study that reveals a fundamental association between the RelB-mediated noncanonical NF-κB pathway, and AR and PSA suppression in advanced prostate cancer. This mechanistic study provides new insights into the clinical relevance of the PSA test and the improvement of redox intervention in prostate cancer treatments.

Materials and Methods

Cell culture and treatment. Human prostate cancer cell lines LNCaP were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin mixture, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 1% NEAA mixture (Cambrex), 1% MEM vitamin mixture (Cellgro) and 2 mmol/L L-glutamine. Cells were grown in a 5% CO_2 atmosphere at 37°C.

Western blotting analysis. For each treatment group, a certain amount of the whole cell lysate was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After blocking in 5% milk for 1 hour, the membrane was incubated with the primary antibody and then the corresponding secondary antibody. The signals were detected by enhanced ECL system and quantified by Quantity One® (Bio-Rad). The following antibodies were used in this study: anti-AR (N-20), anti-PSA (C-19), anti-RelB (C-19) and anti-RelA (C-20) (Santa Cruz Biotech); anti-MnSOD (Upstate); anti-Nox1 (abcam); β-actin (Sigma).

Preparation of whole cell extracts, cytoplasmic and nuclear fractions. Cell pellets were suspended in 100 μL cell lysis buffer and incubated for 30 minutes on ice. The samples were then centrifuged at 13,000 rpm for 1 minute. The supernatant was collected as whole cell extract. Cytoplasmic and nuclear fractions were isolated using the Nuclear Extract Kit (Active Motif). Protein concentration was determined by the Bradford assay (Bio-Rad).

RNA Isolation, cDNA synthesis and real-time PCR. Total RNA was isolated using RNasy kit (Qiagen). cDNA was generated from using 1μg total RNA using the SuperScript III first-strand synthesis system (Invitrogen) following the manufacturer's instructions. mRNA levels of RelA, RelB, AR or PSA were quantified using gene-specific primers in presence of Roche probes master by real-time PCR in a LightCycler 480 (Roche Applied Science) and normalized to internal control human 18s.

Cell transfection. Cells were transiently transfected with RelB expressing construct (kindly provided by Dr. Natoli Gioacchino) or pCDNA 3.1 vector control plasmid using LipofectamineTM 2000 (Invitrogen). Stable RelB and vector-transfected clones were selected based on neomycin resistance, and the tumorigenicity of the selected clones was

investigated in a mouse xenograft tumor model as previously reported [165]. To knockdown endogenous RelB in PCa cells, the specific RelB siRNA (Santa Cruz) and control siRNA were transfected using Transfectin (Santa Cruz) according to manufacturer's protocol. The levels of protein overexpression or knockdown were confirmed by Western blot analysis.

Reporter constructs and luciferase assay. An AR Reporter (luc) kit was purchased from SABiosciences. The AR-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the AR transcriptional response element. LNCaP cells were cotransfected with a mixture of an AR-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct (40:1) with RelB expressing plasmid and pcDNA control for 48hr or treated with TNF-α (1ng/mL) for 24hr.

PSA (6.1 kb)-luciferase contains the promoter/enhancer regions of the PSA gene and was provided by Dr. Alvaro Puga. The reporter constructs were co-transfected with the RelB expression vector or RelB siRNA and β-galactosidase (β-gal) construct into LNCap cells to determine the effect of RelB on PSA promoter activities. Relative luciferase units were calculated as indicators of transcriptional activity.

Electrophoretic mobility shift assay (EMSA). Nuclear protein was extracted using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Double-stranded AR gel shift oligonucleotides (Santa Cruz) containing consensus AR binding element (5'-CTA GAA GTC TGG TAC AGG GTG TTC TTT TTG CA-3') were labeled with [^{32}P] ATP. The assay was performed as previously described [297].

Co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP assay). Cells were collected and processed using ChIP-IT kit (Active Motif). Fixed protein/DNA complexes were sheared and precipitated using anti-RelB antibody. The PSA promoter fragment containing putative binding element was amplified. The sequences of primer set were: upper-strand primer (5'-GCC TTT GTC CCC TAG ATG AA-3') and lower-strand primer (5'-CAG GAC TCC GCC CCT GCC CT-3').

Statistical analysis. Statistical analysis was performed using either Student's *t*-test (for two-group comparison) or one-way ANOVA followed by Dunnett's test (for multiple-group comparison). Data are reported as mean ± standard error (SE). Pearson's

correlation coefficient, a measure of the correlation (linear dependence) between two variables X and Y, giving a value between +1 and −1 inclusive, is widely used in the sciences as a measure of the strength of linear dependence between two variables [298]. This coefficient was calculated and shown together with 95% confidence interval (CI) to quantify the correlation between expression of RelB and AR for four human prostate cancer microarray datasets.

Results

RelB suppresses PSA and AR expressions *in vitro.*

We have previously demonstrated that RelB overexpression increased tumorigenicity of LNCap cells in a mouse xenograft tumor model. Although PSA level increased with the tumor load, the RelB overexpressing group exhibited a lower PSA elevation than the control group [165]. Perplexed with this unexpected *in vivo* finding of PSA suppression with concomitantly robust RelB induction after long-term TNF-α exposure, we explored the potential regulatory role of RelB on PSA suppression in this study. AR expression and function were evaluated together with PSA, since AR tightly regulates PSA expression. Upon androgen binding and following subsequent signal cascade, AR translocates into nucleus and binds to multiple androgen response elements (AREs) in the proximal promoters and enhancers of the PSA gene [258, 290]. Transient transfection of RelB in LNCap cells resulted in a dose-dependent suppressive effects on PSA and AR expression (Figure 3.1 A). On the other hand, knocking down RelB expression by siRNA led to the recovery of PSA expression significantly and in a dose dependent manner (Figure 3.1 B). The levels of respective proteins are presented in this representative blot (top panels) and quantified by densitometric scanning (bottom panels). The suppressive effect of RelB on PSA expression was statistically significant. RelA expression had no significant effects on RelB levels in cells (Figure 3.1 A and B). To further verify the role of RelB on PSA and AR suppression, stably transfected RelB overexpressing and vector control LNCap cell lines were established. RelB overexpression resulted in suppression of PSA and AR expression at the protein and mRNA levels (Figure. 3.1 C and D, respectively).

RelB negatively correlates with AR expression in human prostate cancer patients.

To determine whether an inverse correlation similarly exists in human prostate tumors, four microarray gene expression datasets publicly available at Oncomine.org were analyzed. Scatter plots of data from each microarray dataset were depicted according to mRNA levels of RelB and AR. Pearson's correlation coefficient was calculated to quantify the correlation between expression of RelB and AR. Samples were classified as AR high expression or AR low expression based on the median AR expression level, which allowed each dataset to be more clearly displayed in a boxplot

format. Two-sample t-test was used to compare RelB expression in the two groups. In Taylor's dataset obtained from 218 prostate cancer tumors (181 primaries, 37 metastases) [299], RelB and AR negatively and significantly correlated (Pearson's correlation coefficient=-0.34 [95% CI -0.48 to -0.2], p=1.2e-05). The levels of RelB mRNA were significantly higher in low AR groups than in high AR groups (p=0.00039) (Figure 3.2 A). Seventy-nine prostate cancer patients (37 with recurrent and 42 with nonrecurrent disease) [300] also showed an inverse correlation between RelB and AR (Pearson's correlation coefficient=-0.45 [95% CI -0.61 to -0.25], p=3.5e-05) (Figure 3.2 B). Despite differences in tumor biology derived from different races or ethnicities, RelB and AR consistently and significantly displayed an inverse correlation in Wallace's dataset including primary prostate tumors resected from 33 African-American and 36 European-American patients [301] (Pearson's correlation coefficient=-0.29 [95% CI -0.49 to -0.06], p=0.015) (Figure 3.2 C). Despite the limited sample size in Bittner's dataset [302], RelB and AR expression still correlated negatively (Pearson's correlation coefficient=-0.32 [95% CI -0.56 to -0.04, p=0.026] (Figure 3.2 D). These results demonstrate the significant inverse correlation of RelB mRNA levels with AR mRNA levels in human prostate tumors.

RelB suppresses PSA expression through inhibiting AR functions.

The AR gene encodes a transcription factor that regulates the expression of androgen-responsive genes involved in prostate epithelial cell division and differentiation [295]. The AR gene is located on the long arm of the X chromosome (Xq11-12) and comprises eight exons that encode an mRNA of 11 kb. The mRNA has a 2.8 kb open reading frame, a 1.1 kb 5' untranslated region and a 6 kb 3' UTR [295]. AR protein has four domains: an N-terminal domain (TAD) involved principally in transcriptional activation, a DNA-binding domain (DBD) that is required for interaction with specific gene sequences, a so-called hinge region, and a C-terminal ligand-binding domain (LBD) that binds androgens [295]. AR regulated expression of PSA is mediated through multiple AREs in the proximal promoter (-600 to +12) and the 5' upstream enhancer (-3875 to -4325) of the PSA gene (as depicted in Figure 3.3 A). To examine AR functions in RelB overexpressing LNCap cells, AR DNA binding activity was evaluated by EMSA assay and AR reporter activity. As shown in Figure 3.3 B, AR DNA binding activity was lower

in RelB overexpressing cells than in the control. Following transient cotransfection of the AR reporter together with RelB expressing plasmid or vector control, AR reporter activity measured with a simple dual-luciferase assay was reduced by approximately 55% (Figure 3.3 C). The AR reporter is a mixture of a constitutively expressing renilla construct and an androgen-responsive luciferase construct, which encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter, and tandem repeats of the AR transcriptional response element. The AR reporter activity control experiments should have been performed in presence or absence of androgen. These results demonstrate that RelB significantly inhibits AR DNA binding activity and transcriptional activity.

RelB inhibits PSA promoter activity leading to suppress PSA expression.

The NF-κB family consists of five Rel-related proteins including RelA, p50, RelB, P52 and c-Rel. Structural analysis indicates that RelA and RelB contain both a N-terminal DNA-binding domain and a C-terminal transactivation domain to directly regulate expression of a specific set of target genes [272, 303]. Wang et al have demonstrated that RelB mediated transcription of target genes, such as Bcl2 in breast cancer cells, which contributes to radioresistance [304]. RelB is the most frequently detected NF-κB subunit in the nuclei of prostate cancer tissues, and its level correlates with PCa aggressiveness [296]. To investigate the role of RelB in PSA gene transcription, LNCap cells were transiently transfected with different concentration of RelB expression vector and PSA promoters. Overexpression of RelB with the ~6.1kb PSA promoter construct led to a dose-dependent inhibition of PSA gene transcription (Figure 3.4 A), whereas siRNA knockdown enhanced PSA gene transcription as measured by luciferase reporter gene activity (Figure 3.4 B). To further identify the functional binding region of RelB within PSA promoter, we subcloned the proximal promoter (-600 to +12) into a reporter vector. Overexpression of RelB with this PSA proximal promoter construct also inhibited PSA transcription (Figure 3.4 C) and RelB knockdown increased the reporter activity at high level of siRNA transfection (Figure 3.4 D). Our results suggest that the short fragment from PSA gene proximal promoter may be sufficient for RelB-mediated suppression of PSA transcription.

RelB physically binds to the responsive sites of PSA promoter and forms a complex with AR.

RelB, together with Aryl hydrocarbon receptor (AhR), binds to a NF-κB-like binding site (5′-GGGTGCAT-3′) located within IL-8 promoter [305]. Interestingly, this exact putative binding element can be found within the proximal promoter of PSA gene (Figure 3.5 A). To determine whether RelB physically binds to this element in PSA promoter, we first performed ChIP assay using RelB or non-immune antibody with the cell extracts prepared from cells with or without RelB overexpression. As shown in Figure 3.5 B, ChIP with RelB antibody and subsequent PCR amplification detected a 176bp PSA promoter fragment (-313 to -114). RelB overexpression resulted in a higher level of PCR product as compared to empty vector control in the quantitative real-time PCR performed to amplify the precipitated DNA fragment (Figure 3.5 C). As shown in Figure 3.5 D, a co-immunoprecipation assay suggested the formation of a specific AR and RelB complex. RelB inhibited AR function (Figure 3.3) and there was an increase in the interaction between RelB and AR in RelB overexpressing LNCap cells (Figure 3.5 D). These results suggest one mechanistic model based on the RelB-AR axis depicted in Figure 3.6. When RelB levels are low, AR can bind to ARE and transactivate PSA gene expression. However, in presence of high RelB expression (induced by TNF-α or other factors), RelB may directly bind to a responsive element within PSA promoter, interact with AR and displace AR binding from AREs, thereby resulting in the reduced AR functions and PSA expression.

Discussion

The PSA test has been used in clinics to detect and monitor prostate cancer for more than thirty years. Unfortunately, clinical results indicate that the PSA test often cannot distinguish between the most indolent prostate cancers, which require little or no treatment, and the small fraction of prostate cancers that progress rapidly, which require immediate treatment [306]. Many other conditions, such as benign prostatic diseases, prostatic infarction, recent sexual activity or over-the-counter drugs like ibuprofen, can stimulate PSA elevation in healthy men [258]. More critically, some negative PSA-based screening results may overlook aggressive tumors that do not overexpress PSA. Prostate cancer may progress in some patients with low or undetectable serum PSA levels, and close to 15% of men with PSA levels below the conventional cutoff of 4.0 ng/mL harbor prostate cancer, including high-grade carcinoma [259, 307]. Why some patients with advanced prostate cancer have a low PSA level remains unclear.

Because the PSA gene harbors several ARE in its promoters and enhancers, PSA expression is tightly regulated by AR level and function [258, 290]. An increase in PSA implies transcription of genes regulated by AREs and possibly suggests activation of the AR or other transcriptional signaling pathways [308]. AR transactivation is required for the maintenance and growth of the prostate, which forms the rationale for androgen ablation therapies for prostate cancer [309]. Various pathophysiologically relevant protein-protein interaction networks involving AR have been identified [310]. For example, the protein Fused/Translocated in LipoSarcoma (FUS/TLS), displaying a strong intrinsic transactivation capacity, functions as a novel co-activator of AR in prostate cancer cells [309]. Despite significant roles of AR in prostate cancer progression, highly invasive and metastatic prostate cancer and cell lines such as PC-3 and DU145 frequently have low or non-existent levels of AR. Niu. *et al.* demonstrated that the prostate AR might function as both a suppressor and promoter of prostate cancer metastasis. Through AR gain- and loss-of-function in epithelial–stromal cell coculture and coimplantation experiments, they provide convincing evidence that AR could function in epithelial basal intermediate cells as a tumor suppressor to suppress prostate cancer metastasis, in epithelial luminal cells as a surviving factor, and in stromal cells as a proliferator to stimulate cancer progression [311]. AR may directly or indirectly modulate expression of

metastasis-related genes and suppression of TGF-β1 signals, resulting in the partial inhibition of AR-mediated metastasis [311].

The NF-κB pathway contributes to the development of many cancers and confers radioresistance in prostate cancer [285]. Each NF-κB family member exerts distinctive effects on PSA expression. RelA-mediated canonical NF-κB pathway can enhance AR and PSA expression [285], and nuclear localized RelA is associated with PSA relapse in humans [206]. p52, another NF-κB subunit involved in the noncanonical pathway, induces androgen-independent growth of LNCap cells *in vivo*, and increases AR activation and transactivation of the response of AR to PSA gene expression [312].

A higher percentage of nuclear localized RelB than RelA has been observed in prostate cancer specimens [296]. Our laboratory has done extensive investigations to establish a better understanding of the mechanisms by which the alternative NF-κB pathway increases tumor growth, progression and radiation resistance in prostate cancer [26, 165, 179]. RelB nuclear localization is significantly higher in the aggressive and radioresistant PC-3 prostate cancer cell line compared to the less aggressive and radiosensitive LNCaP cell line [26, 179]. Consistently, inhibition of RelB activity in PC-3 cells by overexpression of a dominant-negative p100 mutant or by knock-down of RelB expression by specific siRNA resulted in a decrease in tumor incidence and growth rate. Stable over-expression of RelB in LNCaP cells results in increased colony formation in soft agar and *in vivo* tumorigenicity compared to vector control [26, 165].

In our previous animal study, we found that overexpression of RelB suppressed PSA serum level in androgen-responsive LNCaP cells compared to vector control group, which suggests a negative effect of RelB on PSA expression [165]. Here, we provide convincing evidence that RelB directly suppresses PSA and AR expression as well as AR function. The novel RelB-AR axis contributes to suppress the PSA gene in advanced stages of prostate cancer. The analyses from human prostate cancer microarray datasets confirm an inverse correlation between RelB and AR expression, and strengthen the existence and significance of this RelB-AR axis (Figure 3.1-3.3). RelB is shown to physically interact with a putative binding element within the PSA proximal promoter and form a complex with AR to mediate the suppressive effect on PSA transcription

(Figure 3.5). This is the first study to provide a molecular mechanism by which PSA expression is downregulated in prostate cancer.

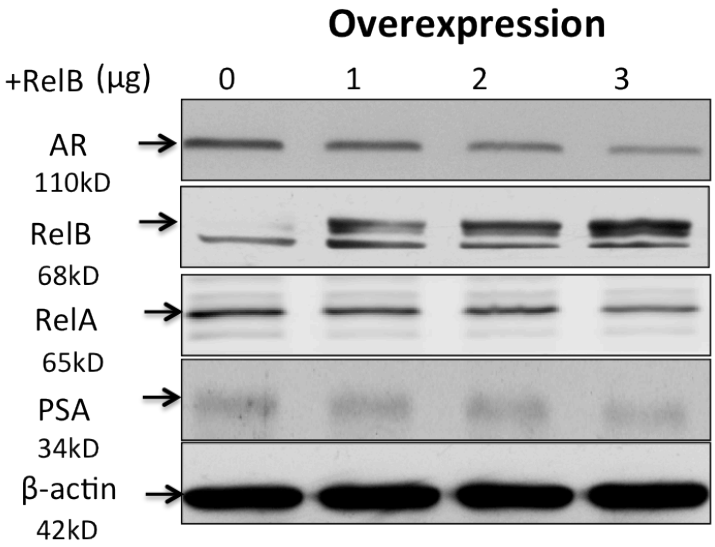
Although aberrant constitutive expression of c-Rel, p65 and p50 NF-κB subunits has been reported in over 90% of breast cancers [313, 314], similar to our findings in prostate cancer, an inverse correlation between RelB and ERα gene expression has been identified by Wang. *et al* in human breast cancer tissues and cell lines. Highly invasive, metastatic and hormone therapy resistant breast cancers are frequently ERα-negative [315]. Upregulation of de novo RelB synthesis targeting Bcl-2 can result in the more invasive phenotype of ERα-negative cancer cells. They further suggest that inhibition of de novo RelB synthesis represents a new mechanism whereby ERα controls EMT [304]. The suppressive effects of RelB on ERα or AR expression imply significant similarities between breast cancer and prostate cancer, especially at the advanced stage. Thus, the RelB-mediated noncanonical NF-κB pathway may be of paramount importance in inhibiting the expression and/or physiological functions of specific hormone nuclear receptors and promoting the aggressive characteristics of various hormone-related cancers, such as breast, ovarian and prostate cancer. Future investigations to explore the role of RelB as a biomarker or therapeutic approaches for treatment of metastatic prostate cancer or hormone-related diseases are warranted.

This study was aimed at understanding how PSA expression is regulated. PSA level is a widely used indicator for prostate cancer prognosis and post-treatment monitoring. False positive PSA test results, which contribute to over-diagnosis of prostate cancer, and false negative results, which miss some cases of aggressive prostate cancer, remain problems of clinical importance. This is the first study to reveal a significant inverse relationship between the RelB-mediated NF-κB noncanonical pathway and AR expression both *in vitro* and in human prostate tumors. This novel RelB-AR axis is of paramount importance in understanding the mechanisms by which the PSA gene is suppressed in advanced stages of prostate cancer with low PSA levels. PSA suppression by the RelB-AR axis will provide new insights with practical and clinical implications in PSA-based post-treatment monitoring and interventions.

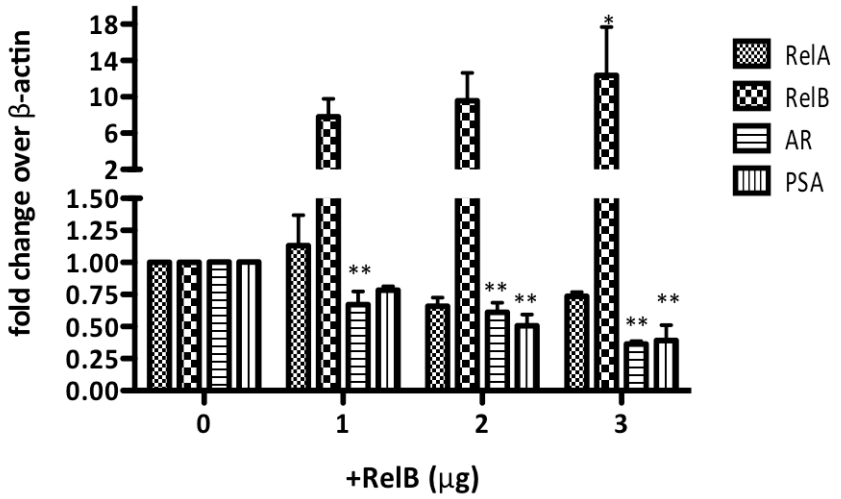
Figure 3.1. RelB suppresses PSA and AR expressions *in vitro.*

A. Overexpressing RelB resulted in dose-dependent suppression of PSA and AR expression. The levels of respective proteins were presented in this representative blot (top panel) and quantified by densitometric scanning (bottom panel). The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.

Representative Blot

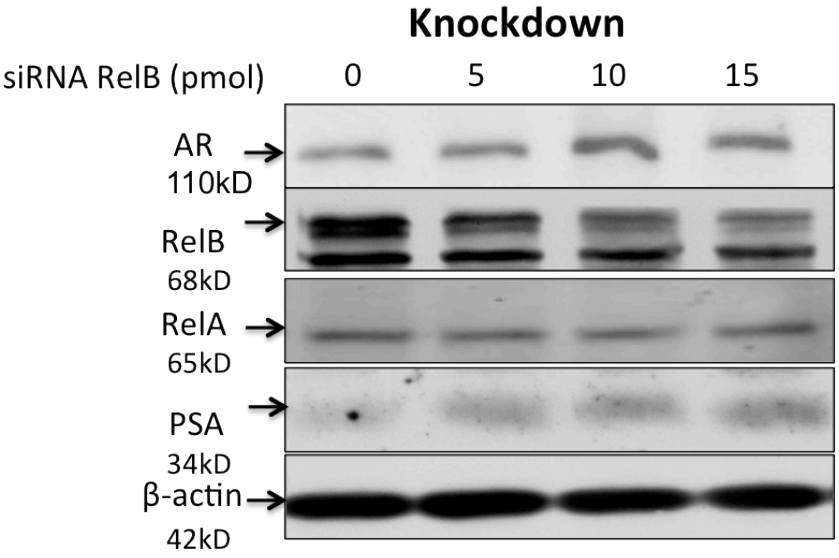


Quantification

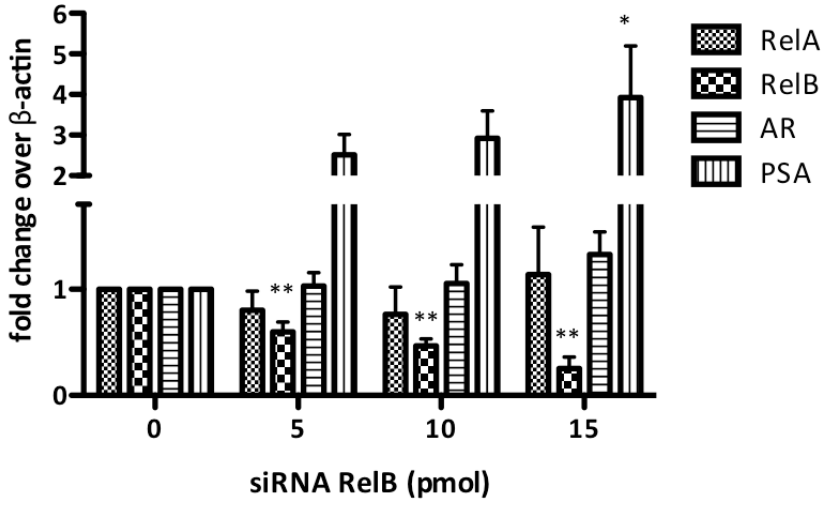


B: Knocking down RelB by siRNA increased PSA and AR expression in a dose dependent manner as shown in this representative blot (top panel) and the quantitative results by densitometric scanning (bottom panel). The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.

Representative Blot

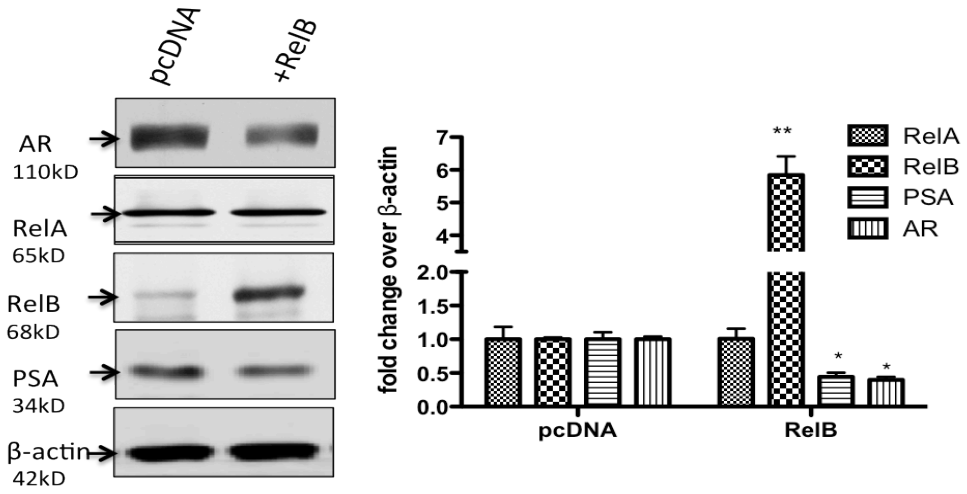


Quantification



C: Chacteristics of stablely transfected RelB in LNCap cells. RelB expression construct and empty vector were stably transfected into LNCaP cells and selected by neomycin resistance. Total proteins were extracted from the selected clones and respective proteins were separated by Western blotting (left panel) and quantified by densitometric scanning (right panel). The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.

Representative Blot **Quantification**



D: Real-time PCR. Total RNAs were isolated from stably transfected cells and converted to cDNA to determine mRNA levels of respective genes following normalization against human 18S rRNA levels, which served as loading control. The results are shown as the mean ± SD of triplicate experiments and significant difference compared to control is indicated by *p<0.05 and **p<0.01.

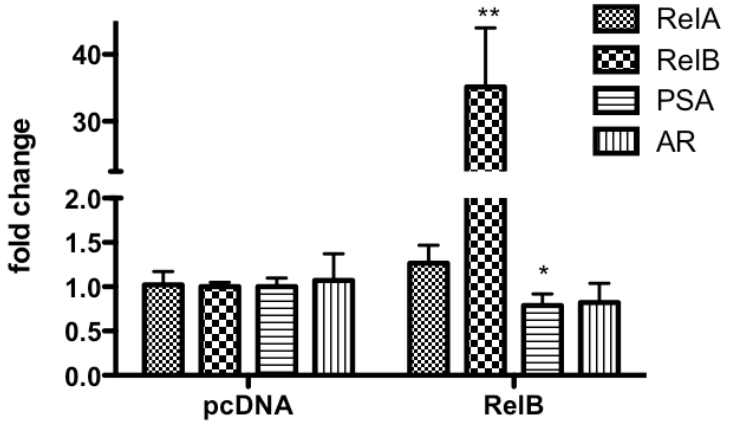
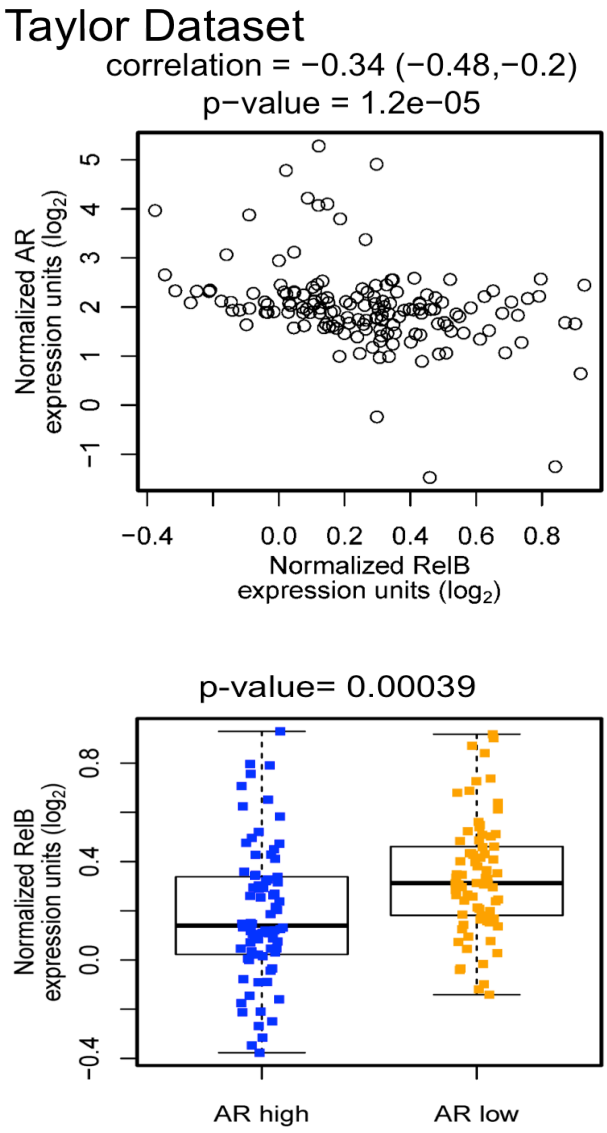
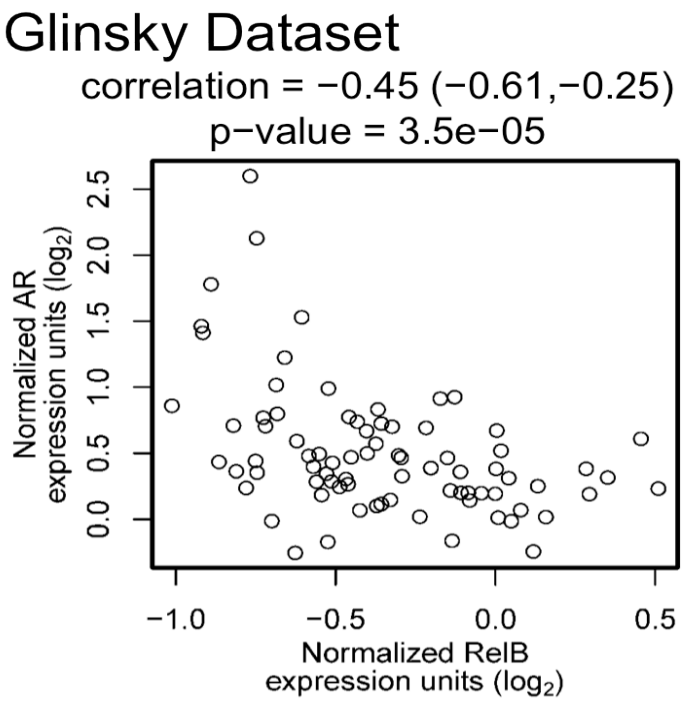


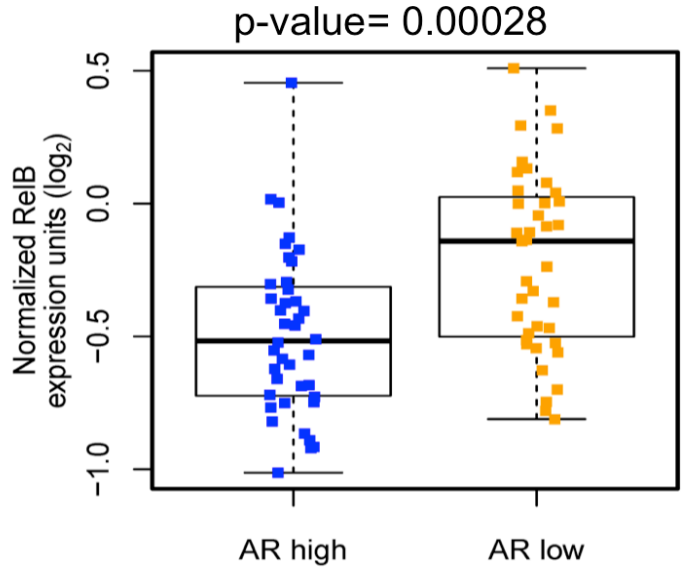
Figure 3.2. RelB negatively correlates with AR expression in prostate cancer patients.

Microarray and patient clinical data from four prostate cancer studies were accessed on the Oncomine Cancer Profiling Database (www.oncomine.org). Pearson's correlation coefficient was calculated to quantify the correlation between expression of RelB and AR. For each data set, samples were further classified into AR high expression and AR low expression groups based on the median AR expression level. Two-sample t-test was used to give a p-value for the correlation between RelB and AR in scatterplots and to compare RelB expression in AR high and low expression groups as shown in boxplots. A. Taylor dataset [299]; B. Glinksy dataset [300]; C. Wallace dataset [301]; D. Bittner dataset [302].

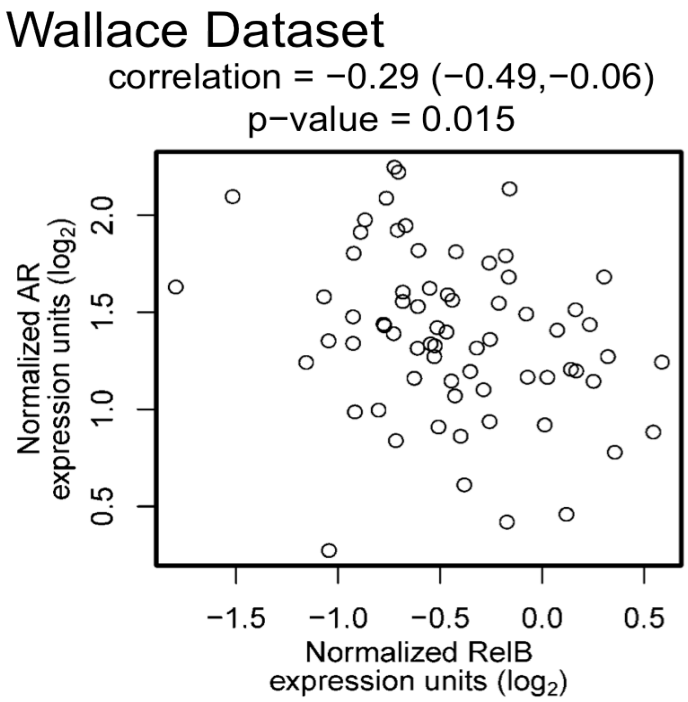


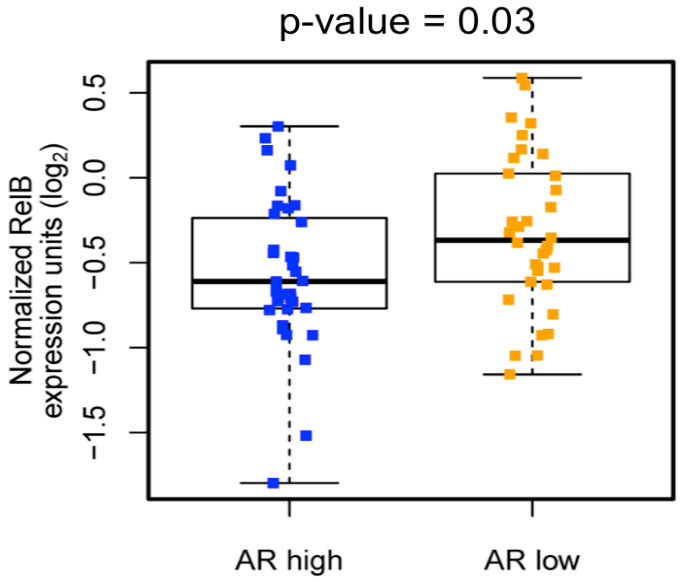
B:


Glinsky Dataset
correlation = −0.45 (−0.61,−0.25)
p−value = 3.5e−05
Normalized AR expression units (log2)
0.0
0.5
1.0
1.5
2.0
2.5
−1.0
−0.5
0.0
0.5
Normalized RelB expression units (log2)

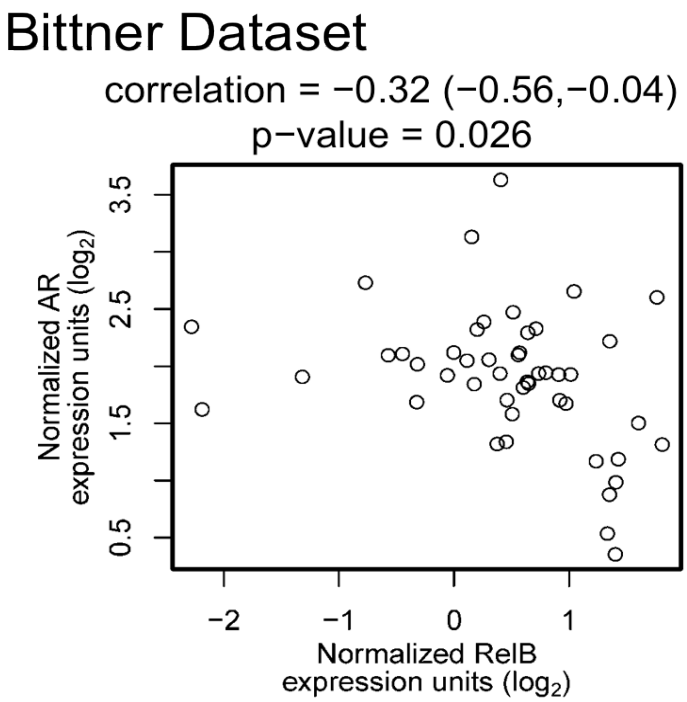

p-value= 0.00028
Normalized RelB expression units (log2)
0.5
0.0
−0.5
−1.0
AR high
AR low

C.


Wallace Dataset
correlation = −0.29 (−0.49,−0.06)
p−value = 0.015
Normalized AR expression units (log2)
0.5
1.0
1.5
2.0
−1.5
−1.0
−0.5
0.0
0.5
Normalized RelB expression units (log2)


p-value = 0.03
Normalized RelB expression units (log2)
0.5
0.0
−0.5
−1.0
−1.5
AR high
AR low

D:


Bittner Dataset
correlation = −0.32 (−0.56,−0.04)
p−value = 0.026
Normalized AR expression units (log2)
0.5
1.5
2.5
3.5
−2
−1
0
1
Normalized RelB expression units (log2)

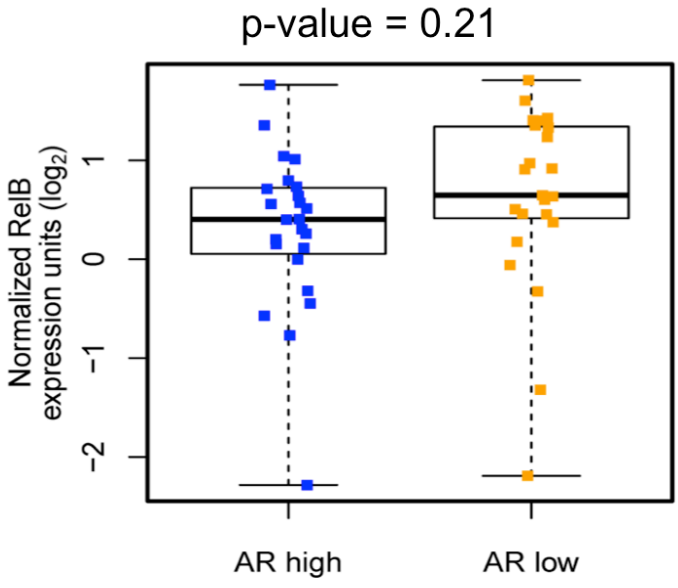
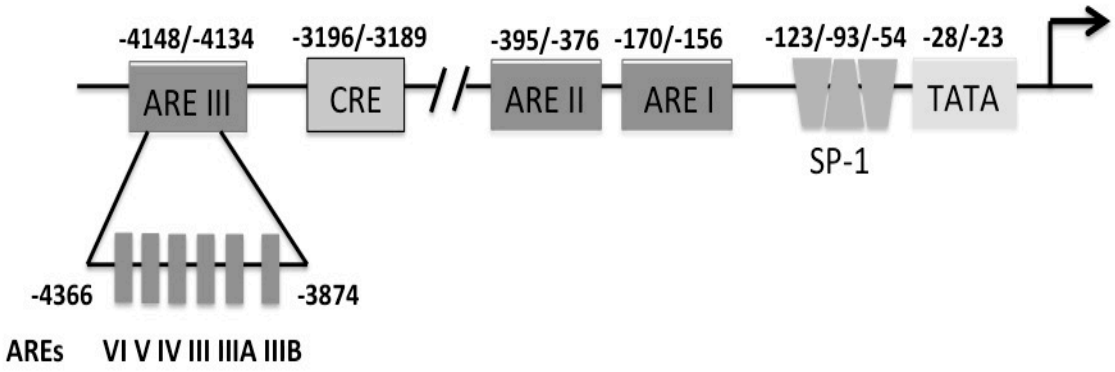
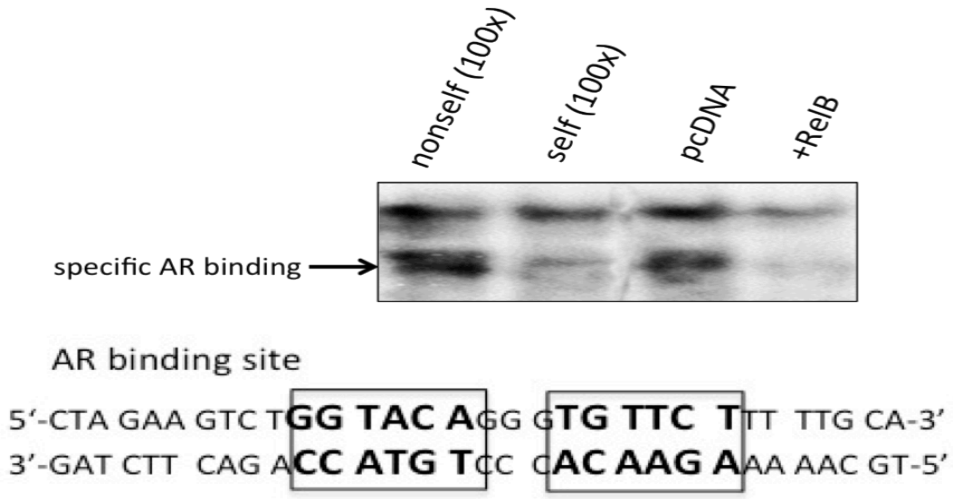

p-value = 0.21
Normalized RelB expression units (log2)
−2
−1
0
1
AR high
AR low

Figure 3.3. RelB suppresses PSA expression through inhibiting AR functions.

A. The schematic diagram of 5' regulatory regions in PSA promoter region that are required for high androgen-stimulated expression (adapted from [295]). Multiple AREs exist in both proximal promoter and upstream enhancer regions.



B: EMSA assay. Double-stranded AR gel shift oligonucleotides (Santa Cruz) containing consensus AR binding element (5'-CTA GAA GTC TGG TAC AGG GTG TTC TTT TTG CA-3') were labeled with [^{32}P] ATP. Nuclear extract was isolated from stably transfected cells for EMSA with radiolabelled AR probes.



C: AR reporter activity assay. To determine effects of RelB overexpression on AR activity, LNCap cells were cotransfected with the mixture of AR-responsive firefly luciferase construct, and constitutive expression renilla luciferase construct, as well as RelB expression construct or empty vector. Relative luciferase units were calculated as indicators of AR activity. Each data point represents mean ± SD of three independent experiments and significant difference as compared to the untreated control is indicated by *p<0.05 and **p<0.01.

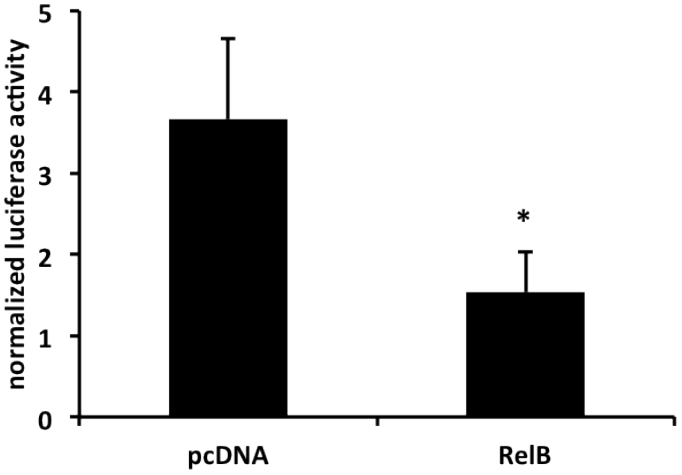
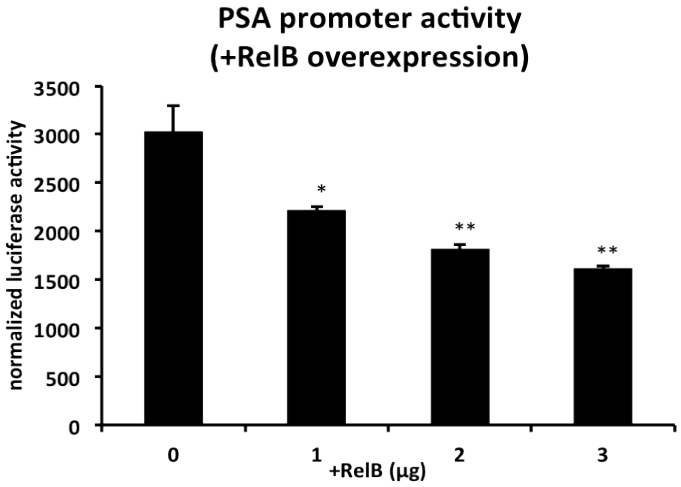
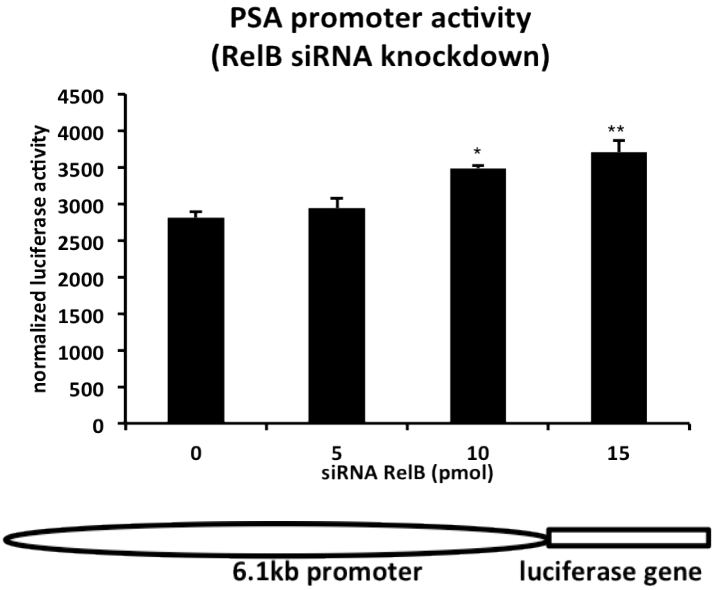


Figure 3.4. RelB inhibits PSA promoter activity leading to suppress PSA expression.

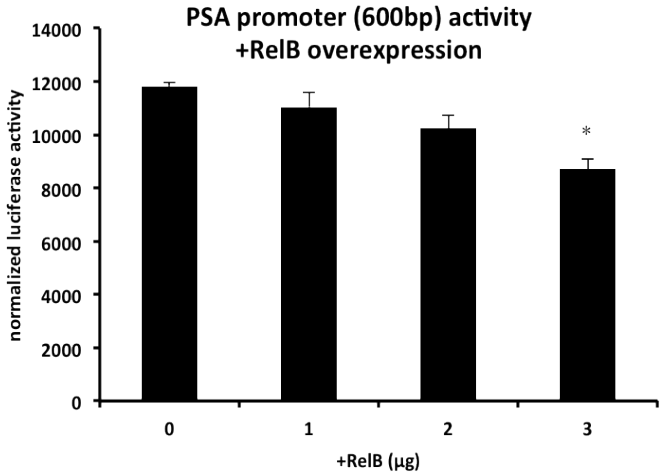
A: PSA promoter activity assay. To evaluate the effects of RelB on PSA promoter activity, the PSA promoter-driven luciferase reporter (~6.1kb) and RelB expression vector at different concentrations as well as internal control β-gal were cotransfected into LNCap cells. Firefly luciferase activity normalized against internal β-gal activity was calculated for comparing PSA promoter activity.



B: Cotransfection of RelB siRNA with PSA promoter construct (~6.1kb) as well as internal control β-gal. The cotransfection was performed and promoter activity was measure as described above.



C: PSA proximal promoter activity assay. The proximal promoter (-600 to +12) of PSA gene was PCR subcloned into reporter vector. To evaluate the effects of RelB on PSA promoter activity, the PSA proximal promoter-driven luciferase reporter and RelB expression vector at different concentrations as well as internal control β-gal were cotransfected into LNCap cells. The cotransfection was performed and promoter activity was measure as described above.



B: Cotransfection of RelB siRNA with PSA proximal promoter construct as well as internal control β-gal. The cotransfection was performed and promoter activity was measure as described above.

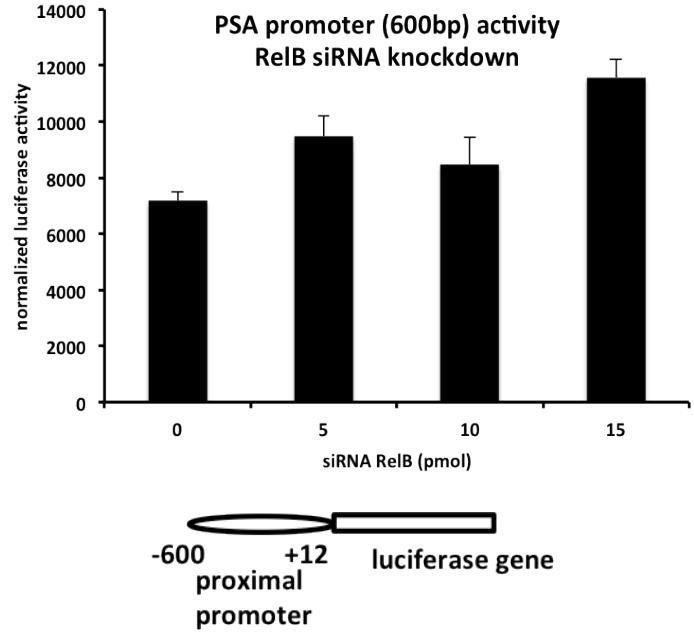
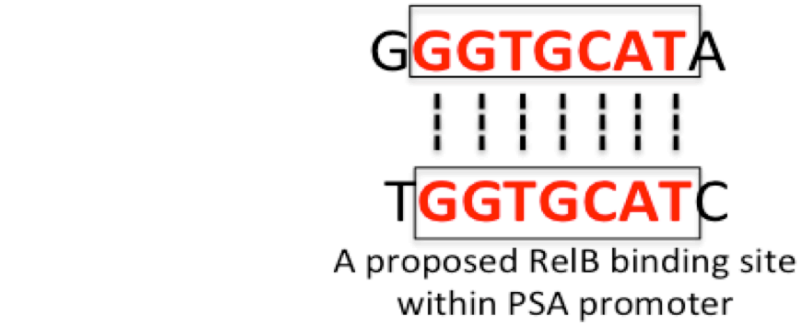


Figure 3.5. RelB physically binds to the responsive sites of PSA promoter and forms a complex with AR.

A. Alignment of RelB binding element as identified in IL-8 promoter together with the putative element in PSA proximal promoter. The putative RelB binding element was located and highlighted within PSA proximal promoter region.



PSA -313/-114

-313 **gcctttgtcc cctagatgaa** gtctccatga gctacagggc ctggtgcatc cagggtgatc

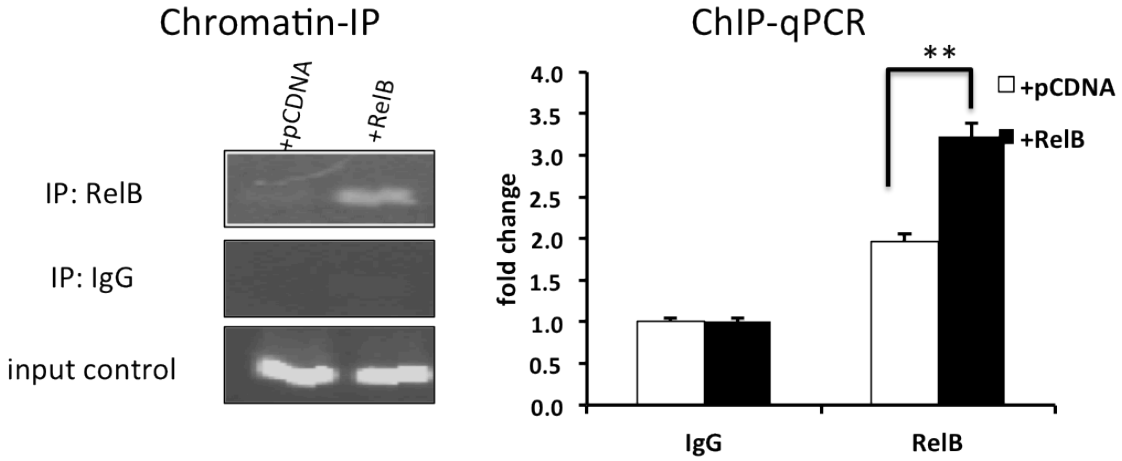
-253 tagtaattgc agaacagcaa gtgctagctc tccctcccct tccacagctc tgggtgtggg

-193 agggggttgt ccagcctcca gcagcatggg gagggccttg gtcagcctct gggtgccagc

-133 **agggcagggg cggagtcctg** gggaatgaag gttttatagg gctcctgggg gaggctcccc

-313 → -114

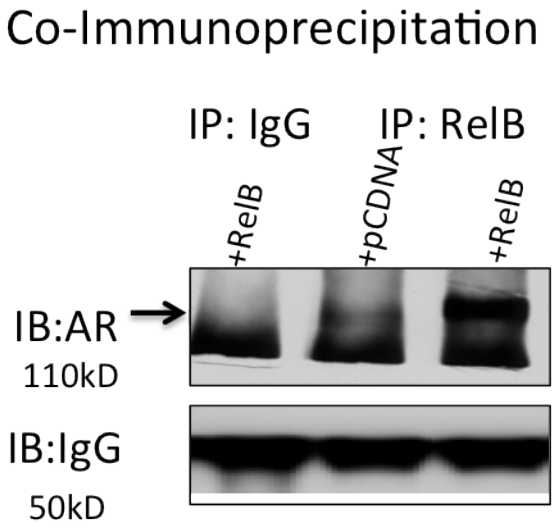
B: ChIP assay. The putative RelB binding element was highlighted within PSA proximal promoter region. ChIP assay was performed using stably transfected LNCap cells. Input controls were prepared before adding antibody. Chromatins were immunoprecipitated by RelB antibody or IgG as control and immunoprecipitated DNA was amplified by PCR using primers as indicated in Materials and Methods.



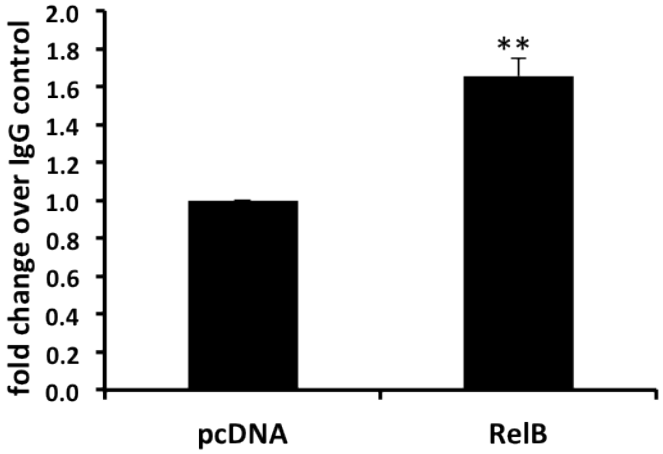
C: ChIP-qPCR assay (right panel). ChIP assay was performed as described above. Quantitative real-time PCR was also used to amplify the precipitated DNA fragment. Each data point represents mean ± SD of three independent experiments and significant difference as compared to the untreated control is indicated by **$p<0.01$.

D: Co-immunoprecipitaion. Total cell lysate from stably transfected cells were immunoprecipitated with RelB control IgG. Coimmunoprecipitated AR was quantified by immunoblotting with specific antibodies. The RelB-AR interaction were presented in this representative blot (top panel) and quantified by densitometric scanning (bottom panel). Each data point represents mean ± SD of three independent experiments and significant difference as compared to the untreated control is indicated by **p<0.01.

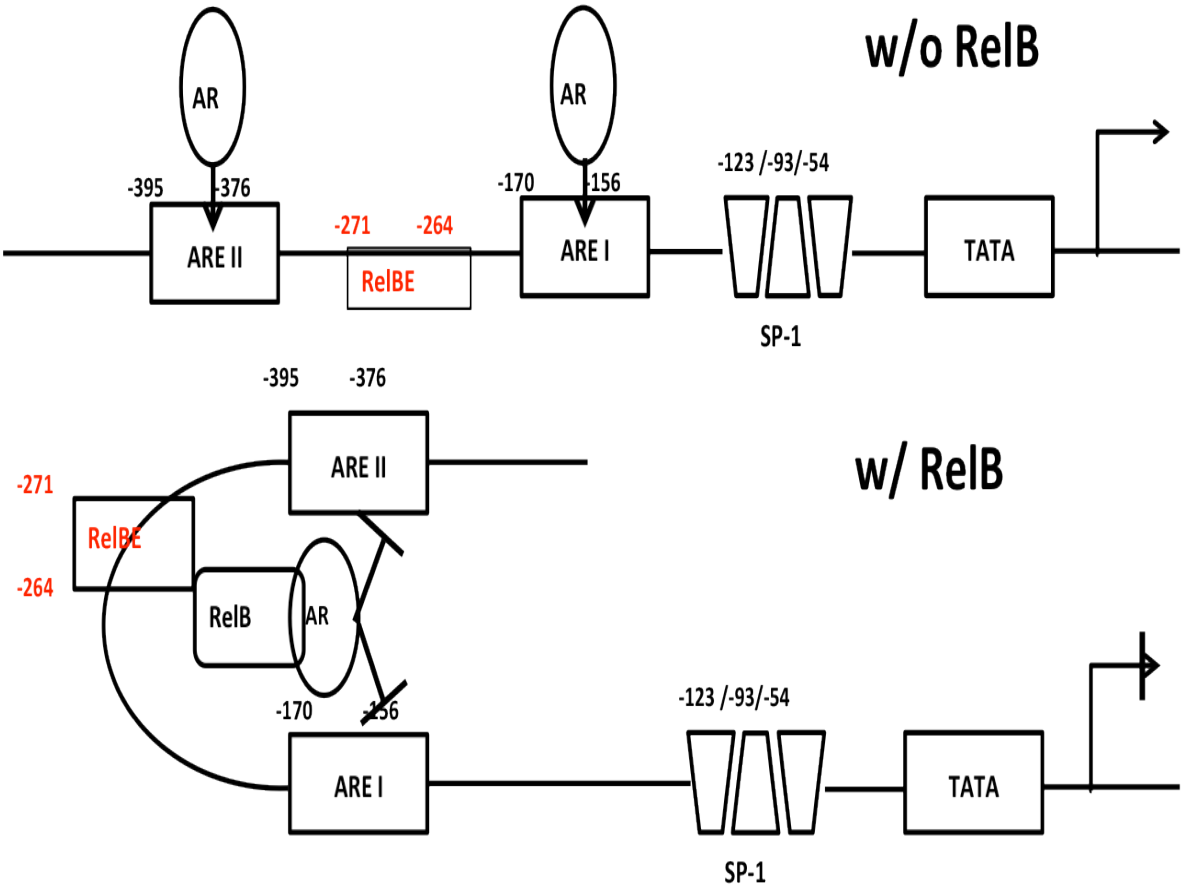
Representative Blot



Quantification



E: Schematic illustration of RelB suppressive effects on PSA proximal promoter.



Chapter Four

Discussion and Summary

Versatile partnership between radiation therapy and ROS elevation: insight in prostate cancer radioresistance

It has been reported that IR increases intracellular ROS levels quickly after exposure and the elevated levels of ROS are sustained for several hours after initial IR exposure [47, 316, 317]. IR-induced mitochondrial dysfunction, especially decreased electron transport chain complex I activity, contributes to the persistent oxidative stress after irradiation [48]. Tohru Yamamori. *et al* demonstrated that this process is accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content [46]. NADPH oxidase is responsible for a late increase in intracellular superoxide generation after exposure to IR [316-318]. IR-induced mitochondrial dysfunction, especially decreased electron transport chain complex I activity, produces a feed forward loop that contributes to persistent oxidative stress after irradiation [48]. Since the mitochondrion is the most important energy generating organelle, mitochondrial dysfunction due to direct effects of IR or indirect effects mediated by ROS may result in alterations or adaptive responses of metabolic pathways, such as glycolysis, involved in cancer development. Free radicals may amplify and prolong the deleterious effects of radiation, leading to chronic oxidative stress, alteration of multiple metabolic pathways, normal tissue injury, cell death and other bystander effects (reviewed in [106]).

DNA damage and ROS generated by IR exposure can stimulate many inside-out and outside-in cell signaling pathways involved in the control of cell survival and death, leading to either adaptive repair or apoptotic cell death [319]. However, the predominant mechanism for radiation killing of mammalian cells is the so-called mitotic cell death pathway in which damaged DNA with double-stranded breaks or dysfunctional chromosomal structure result in the loss of replicative potential after several mitotic cycles. Apoptosis is a minor form of cell death in irradiated solid tumors, including prostate tumors [320].

Since radiotherapy mainly induces cell death by generating oxidative stress, cellular antioxidant status also affects normal tissue injury and tumor sensitivity to radiation treatment (reviewed in [26, 321]). Inhibiting prooxidant enzyme expression, like COX-2

[322], and overexpression or upregulation of antioxidant enzymes, such as extracellular ECSOD [323] and heme oxygenase-1 (HO-1) [324], protected against radiation-induced thoracic, lung and skin injury [325]. Radiation-resistant mice had higher levels of SOD and catalase activities compared to radiation-sensitive mice [14].

Manganese superoxide dismutase (MnSOD) upregulation has been implicated in adaptive response induced by low or fractionated doses of ionizing radiation, leading to radioresistance [13, 15, 273]. MnSOD is one of the most important antioxidant enzymes and is located exclusively in mitochondria, the main source of ROS [66, 68]. The levels and activities of MnSOD modulate cellular redox status and influence the effects of chemotherapy or radiotherapy; therefore, MnSOD may confer radioresistence through its antioxidant enzyme activity. Our previous studies have demonstrated that selective inhibition of RelB-induced MnSOD after irradiation can sensitize prostate cancer cells to radiation treatment [26, 179], confirming the importance of MnSOD in radioresistance.

Tolerance for oxidative stress may influence different cancer cell types to exhibit opposite responses to ROS elevation. For example, the anticancer drug 2-methoxyestradiol (2-ME) is associated with upregulation of MnSOD as an adaptive response that protects pancreatic cancer cells from increased ROS [326]. In contrast, 2-ME can sensitize radioresistant MCF-7/FIR breast cancer cells by activating apoptosis, arresting the cell cycle and further enhancing radiation-induced ROS [327]. Therefore, applying redox modulating reagents such as ascorbate [328], arsenic trioxide [329], selenite [330] or a metalloporphyrin antioxidant mimetic (MnTE-2-PyP^{5+}) [331] in combination with ionizing radiation can either increase the cell killing effect of IR or protect against the radiation-induced oxidative stress. MnTE-2-PyP^{5+} treatment alone slowed prostate tumor progression and up-regulated immune parameters such as spleen mass relative to body mass, the numbers of splenic white blood cells (WBC) and lymphocytes (B and T) and circulating WBC, granulocytes, and platelets, but caused only minimal mitigation of the effects of 2 Gy total-body irradiation [331]. In response to ROS generation following radiotherapy, tumor reoxygenation leads to nuclear accumulation of HIF-1 and enhances translation of HIF-1-regulated transcripts, such as a prototypical angiogenic cytokine, VEGF, which inhibits endothelial cell apoptosis and limits treatment response by minimizing vessel damage [332, 333]. Inhibiting HIF-1 activation

after IR by blocking tumor reoxygenation processes may significantly increase tumor radiosensitivity as a result of enhanced vascular destruction, which has been implicated in tumor hypoxia condition [332, 333], growth factor expression [22] and tumor angiogenesis [333, 334]. In addition, the role of MnSOD activity in increased radioresistance through regulating cell cycle G2-checkpoint pathways suggests that mitochondria-derived superoxide and hydrogen peroxide signaling could regulate cellular responses to IR [335]. Additional understanding of the mechanisms of radioresistance under different redox status conditions in tumor cells will help to improve the outcome of radiation therapy.

IR-induced TNF-α functions as a sustained source of ROS, activates RelB-mediated noncanonical NF-κB pathways and has implication in PCa radioresistance.

Our data show that radiation therapy directly and dose dependently induces TNF-α, one of the most important proinflammatory mediators, in both prostate cancer LNCap cells and BJ-5ta human fibroblast cells. Because tumor cells are usually under higher oxidative stress, TNF-α secretion found in *sod2*-KO MEF after IR exposure is significantly higher than wt-MEF, suggesting that this bystander effect of IR is highly dependent on cellular redox status.

TNF-α is often produced in response to oxidative stress and acts, at least in part, by causing oxidative stress in its target cells. Whether they are promoting cell survival or death, reactive oxygen species produced by TNF-α have the important function of determining the fate of the impacted cells. The mitochondrion is the main site of ROS generation contributing to TNF-α-initiated signaling pathway [336-338]. Changes in mitochondria membrane permeability and electron transport chain activity, especially the impaired respiratory complex I activity, lead to TNF-α mediated ROS generation, resulting in mitochondrial damage [338]. For example, Kim. *et al* have shown that in response to TNF-α binding, TNF complex II, which is composed of receptor interacting protein 1, TNF receptor-associated protein with death domain (TRADD), TNF receptor-associated factor 2, Fas-associated death domain protein and pro-caspase-8, binds to the C-terminus of Romo1 (ROS modulator 1), which in turn recruits Bcl-xL to reduce mitochondrial membrane potential, resulting in ROS production and apoptotic cell death [339]. TNF-α-induced ROS, which can be inhibited by mitochondrial-specific MnSOD

overexpression, may also cause oxidation and inhibition of JNK-inactivating phosphatases, and sustained JNK activation is required for cytochrome c release and caspase 3 cleavage as well as necrotic cell death [286].

Chapter 2 demonstrates that TNF-α amplifies endogenous ROS by ESR and DCF assays. Pretreatment with SOD or catalase diminishes the TNF-α-induced DMPO/•OH peak to basal levels, indicating that ROS such as $O_2^{\bullet-}$ and H_2O_2 are generated in LNCap cells by TNF-α treatment. DHE oxidation, an indicator of superoxide radicals induction, changes immediately and significantly, suggesting that $O_2^{\bullet-}$ is likely the major type of ROS generated by TNF-α in prostate cancer cells. NADPH oxidase activation is involved in TNF-α induced ROS production, depending on the cell types and the extent of TNF-α exposure [340, 341]. Acute TNF-α exposure induced rapid (within 5 minutes) p47phox phosphorylation and increased p47phox-TNF-α receptor-associated factor 4 (TNAF4) association and membrane translocation, which further mediated p47phox-p22phox complex formation, leading to NADPH dependent $O_2^{\bullet-}$ production [340]. The binding of TNF-α to TNFR1 can activate NOX1 or NOX2 to generate ROS in the early endosome [342].

NADPH oxidase activity was measured to determine its role in TNF-α-induced $O_2^{\bullet-}$ in LNCap cells. The results show that TNF-α dose-dependently enhances NADPH oxidase activation and sustained NOX activity throughout the duration of TNF-α exposure. NOX1 is a major NOX member contributing to TNF-α-induced ROS generation and downstream signaling activation. Comparison of nuclear RelA and RelB levels, the data show that RelA induction occurred immediately after treatment with TNF-α then diminished quickly. For RelB, a gradual and prolonged increase last into late time points, up to as least 24 h after the TNF-α treatment, suggesting sequential activation of RelA-mediated canonical and RelB-mediated noncanonical NF-κB pathways. In addition to NF-κB signaling activation, a few important kinase signaling pathways, such as PI3K-Akt phosphorylation, ERK and p38 but not JNK pathways activated for a period of time, but were not sustained until very late time points (Figure 4.1 A). Addition of respective kinase inhibitors abrogated RelB induction to a certain extent, indicating theses kinase pathways are the upstream events contributing to activation of the RelB-mediated noncanonical NF-κB pathway (Figure 4.1 B).

Many studies have shown that a relatively high dose of TNF-α generally induces an acute and pro-cell death response. However, chronic TNF-α elevation at a relatively low level can result in cytoprotection, which is related to increased levels of antioxidant, antiapoptotic, and other defense proteins, such as thioredoxins and MnSOD. Increased mitochondrial ROS production induced by TNF-α leads to activation of nuclear genes, especially NF-κB. In human and mouse ovarian cancer, TNF-α maintains TNFR1-dependent IL-17 production by CD4+ cells, which leads to myeloid cell recruitment into the tumor microenvironment and enhanced tumor growth [293]. The survival data demonstrates that low dose of TNF-α exposure has no cytotoxic effects and enhances radiation resistance in prostate cancer cells. TNF-α-induced RelB and MnSOD expression may lead to more aggressive characteristics, as these two proteins are significant in prostate cancer progression.

Fortunately, a variety of novel strategies have been proposed to target TNF-α mediated signaling for treatment of human prostate cancer. For example, Gambogic acid can inhibit TNF-α-induced invasion of human prostate cancer PC3 cells *in vitro* by inhibiting PI3K/Akt and NF-κB pathways [343]. TNF-α induces MnSOD expression, which mediates delayed radioprotection [344] through an NF-κB binding site located within the second intron of the *sod2* gene [66]. The natural compound curcumin acts as a potent radiosensitizer in PC3 cells by inhibiting TNF-α-mediated NF-κB activity, resulting in bcl-2 protein downregulation [345]. There is a caveat to targeting TNF-α in prostate cancer therapy. TNF-α synergizes with γ-irradiation to induce apoptosis in LNCaP cells through a mechanism that may involve increased production of ceramide at 48-72 hours after exposure [346]. Anti-TNF-α treatment may mitigate the effect of γ-irradiation. Depending on TNF-α dose and prostate cancer cell type, different isoforms of C/EBPβ may regulate cell growth and confer TNF-α resistance to prostate cancer cells [347]. Although TNF-α is clearly linked with prostate cancer progression and radioresistance, it may also contribute to tumor immune surveillance and apoptosis-mediated antitumor pathways. Thus, there currently appears to be no reason to reject TNF-α or NF-κB as a drug target. The results provide direct evidence of IR-mediated TNF-α secretion, the consequences in downstream signaling activation and significance

in prostate cancer radiation responses. More work need to be done to increase the therapeutic efficiency of chemotherapy and radiotherapy in prostate cancer treatment.

Debate about PSA test in PCa clinical practice.

PSA level and rate have been widely used as biomarkers for prostate cancer because their risings are related to the extent and biological potential for the disease [348]. The percentage of men with a higher volume tumor, extraprostatic disease, higher grade disease, and biochemical failure after treatment usually increase as the PSA level increases [349]. Although the burden of suffering from prostate cancer in the United States is significant, recent epidemiologic studies have shown that the lifetime risk of prostate cancer diagnosis is about 16%, but the lifetime risk of dying from this disease is only 3.4% [350]. PSA testing may reduce the risk of death through early detection, but it is associated with a variety of harms. Multiple studies suggest that PSA screening and treatment for prostate cancer may have a marginal benefit on the lifespan of men, and may incur a considerable cost [351]. Given that the risk of a serious infection from a prostate biopsy in the United States has been estimated at between 2% and 4%, the risk of a potentially lethal variant of prostate cancer may be less than the risk of biopsy complications [352].

Approximately 25% of men with a PSA level >4.0 ng/mL had prostate cancer [353]. Much controversy exists about the potential harm and benefit of screening. A relatively high false positive rate compromises the specificity of the PSA test, leading to significant issues of overdiagnosis, overtreatment, adverse events and diminished quality of life. Thus, several population-based groups cited inconclusive evidence to recommend for, or against, PSA screening for men younger than age 75 years [354, 355]. As for PSA test dependent prostate cancer screening, the American Society of Clinical Oncology addressed provisional clinical opinions as follows, 1) In men with a life expectancy of less than 10 years, it is recommended that general screening for prostate cancer with total PSA be discouraged, because the harm seems to outweigh potential benefits; 2) In men with a life expectancy of more than 10 years, it is recommended that physicians discuss with their patients whether PSA testing for prostate cancer screening is appropriate for them. PSA testing may save lives but it is associated with harm, including complications from unnecessary biopsy, surgery, or radiation treatment [350]. Thus, it is of paramount

importance to reduce the proportion of overdetection of indolent disease and to improve the ability to identify lethal tumors early.

In addition to the false positive issues of PSA test, false negative results also raise tremendous concerns. There is no "safe" PSA value below which a man may be reassured that he does not have biopsy-detected prostate cancer [356]. Biopsy-detected prostate cancer, including high-grade cancers, is not rare among men with PSA levels of 4.0 ng/mL or less - levels generally thought to be in the normal range. Thompson. *et al* observed an extraordinarily high prevalence of prostate cancer among 2950 healthy men (age range, 62 to 91 years), participating in a prostate cancer chemoprevention study. All of these men had PSA levels below 3.0 ng/mL at the start of the study, and all of the men studied had PSA levels that remained below 4.0 ng/mL during the seven years of followup. Prostate cancer was diagnosed in 449 (15.2%); 67 of these 449 cancers (14.9%) had a Gleason score of 7 or higher. The prevalence of prostate cancer was 6.6% among men with a PSA level of up to 0.5 ng/mL, 10.1% among those with values of 0.6 to 1.0 ng/mL, 17.0% among those with values of 1.1 to 2.0 ng/mL, 23.9% among those with values of 2.1 to 3.0 ng/mL, and 26.9% among those with values of 3.1 to 4.0 ng/mL [259].

Chapter 3 discusses a role for RelB in suppressing PSA expression at the advanced stage of prostate cancer, which could be a mechanism for the low PSA level in some patients bearing aggressive prostate tumors. Analysis of Oncomine datasets obtained from prostate cancer patients demonstrates an inverse correlation between RelB expression and AR expression in four human prostate cancer microarray datasets. RelB not only suppresses AR expression but also directly interacts with AR to form a complex on the enhancer elements of the PSA promoter. This RelB-AR axis is identified as an important contributor to PSA suppression in an advanced stage of prostate cancer. Furthermore, TNF-α induces RelB and MnSOD but suppresses PSA expression in spite of the status of the androgen receptor. TNF-α-induced RelB substantially suppresses PSA expression in the presence of androgen DHT or the anti-androgen compound casodex. Overexpressing RelB significantly suppresses DHT-induced PSA elevation (Figure 4.2). Thus, compared to the roles of AR on PSA activation, RelB exerts a dominant role in PSA suppression.

Applications of biomarker such as PSA may detect prostate cancers or predict the relapse earlier, but better approaches are needed, such as risk stratification for screening and assessing individualized risk for prostate cancer initiation or recurrence. Since most prostate tumors remain silent and cause a patient no morbidity, the use of additional risk assessment tools may help to determine whether the specific tumor is clinically significant or need to be subjected to further biopsy or more careful monitoring. In addition to PSA elevation, a few important risk factors, including age, race/ethnicity, family history, DRE findings and alterations of many other proinflammatory mediators, should be given serious attention during prostate cancer diagnosis and treatment follow-ups.

Summary

Radiation therapy is generally used to treat early stage and inoperable locally advanced prostate cancer. Radiation kills prostate cancer cells and extends long-term patient survival by direct and indirect actions leading to macromolecule damages and altered redox signaling. However, IR is also responsible for the induction of neoplastic transformation and tumor progression, as well as normal tissue injuries. The development of radioresistance is a significant impediment to prostate cancer treatment. The side effects and late complications resulting from IR exposure limit the full potential of radiotherapy efficacy. Considering the heterogeneity of tumors, dynamic communications between stromal and prostate cancer cells, as well as the complicated redox-regulated mechanisms within the tumor microenvironment, simply applying generalized anti-inflammatory strategies might result in unintended adverse effects. Thus, it is important to develop individualized treatment regimes that will be the most effective and will not disrupt antitumor immunity. Additionally, redox-dependent proinflammatory mediator production from the directly exposed cells and their neighboring non-irradiated cells (bystander effect of radiotherapy) may play a critical role in the response of cells and tissues to IR. The key roles of IR-induced cytokines and growth factors and their interference with prostate cancer radiotherapy have been extensively discussed with an emphasis on TNF-α.

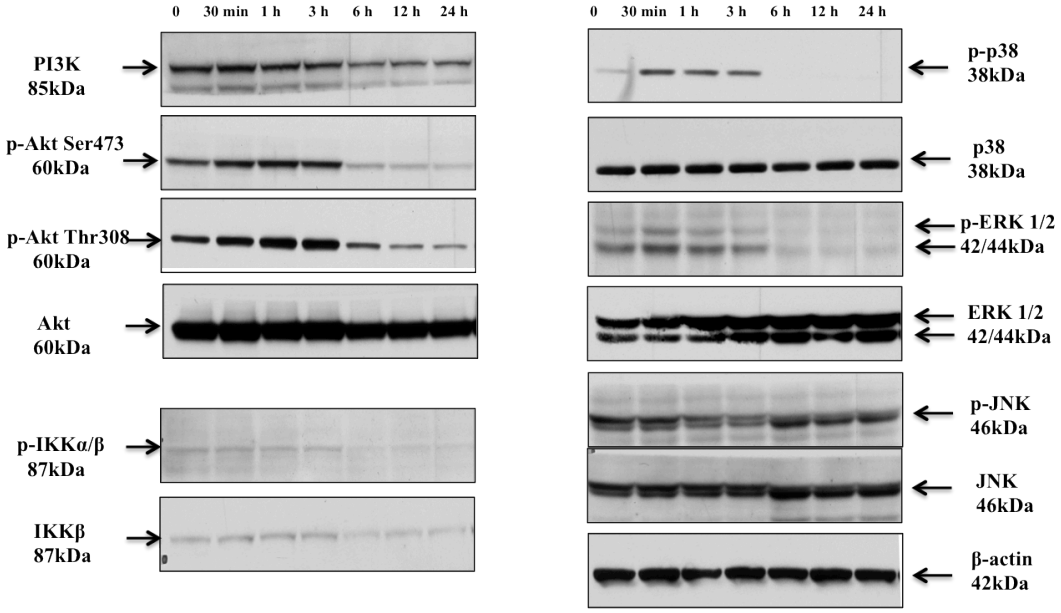
IR-induced TNF-α secretion in prostate cancer treatment is not only involved in modulating redox balance, but is also subjected to regulation by various oxidative

stresses. Compared to normal cells, tumor cells are usually under higher oxidative stress and secrete more pro-inflammatory mediators. Under sustained oxidative stress within the tumor microenvironment, TNF-α further amplifies IR-induced oxidative stress, leading to downstream signaling activation, such as rapid RelA-mediated canonical NF-κB pathway and subsequent RelB-mediated noncanonical NF-κB pathway. In androgen-responsive LNCap cells, RelB inhibits AR expression and/or forms a RelB-AR axis that contributes to sustained PSA suppression. Prolonged oxidative stress and RelB overexpression facilitate prostate cancer progression to an aggressive stage and/or to radioresistance. The considerable uncertainty about the reliability of the prognostic capabilities of the PSA test makes this study highly significant for clinical practices. It provides a convincing mechanism to explain false negative PSA results for patients with prostate cancer that is growing and progressing.

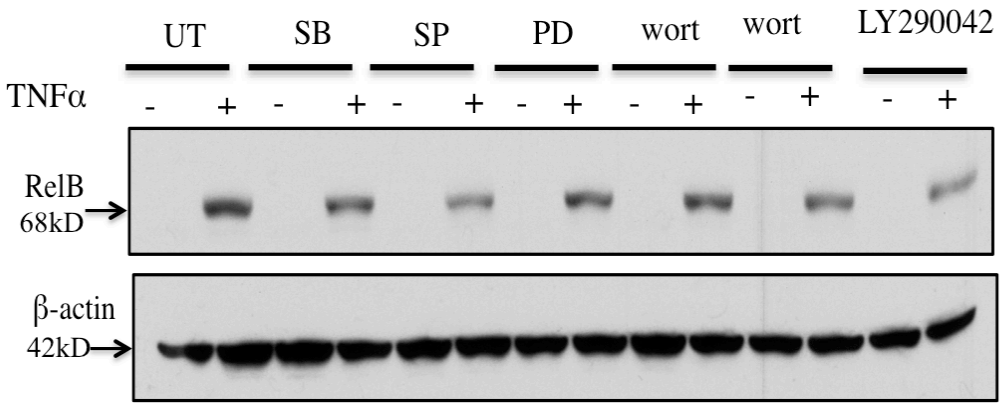
The findings of this study also indicate that modulation of IR-induced oxidative stress and inflammatory cytokine signaling may provide a better basis for enhancing radiation-mediated killing in prostate cancer treatment with minimal normal tissue damage. Inhibition of the RelB-AR axis and redox intervention, together with anti-TNF-α therapy, may be useful therapeutic approaches for the treatment of prostate cancer.

Figure 4.1. TNFα-regulated major cellular kinase signaling pathways in LNCap cells.

A. To determine the activation of multiple kinase signaling pathways in a time dependent manner after TNF-α treatment (0.5ng/mL), total cell lysate were prepared at the indicated time points and analyzed by Western blotting.



B. Addition of respective kinase inhibitors abrogated RelB induction to a certain extent, indicating theses kinase pathways are the upstream events contributing to activation of RelB-mediated noncanonical NF-κB pathway.



SB: SB203580 p38 inhibitor SP: SP600125 JNK inhibitor
PD: PD98059 ERK inhibitor
Wortmannin PI3K inhibitor LY290042 PI3K inhibitor

C. Summarized illustration of sequential activation of major cellular signaling pathways in LNCap cells after low dose TNF-α treatment

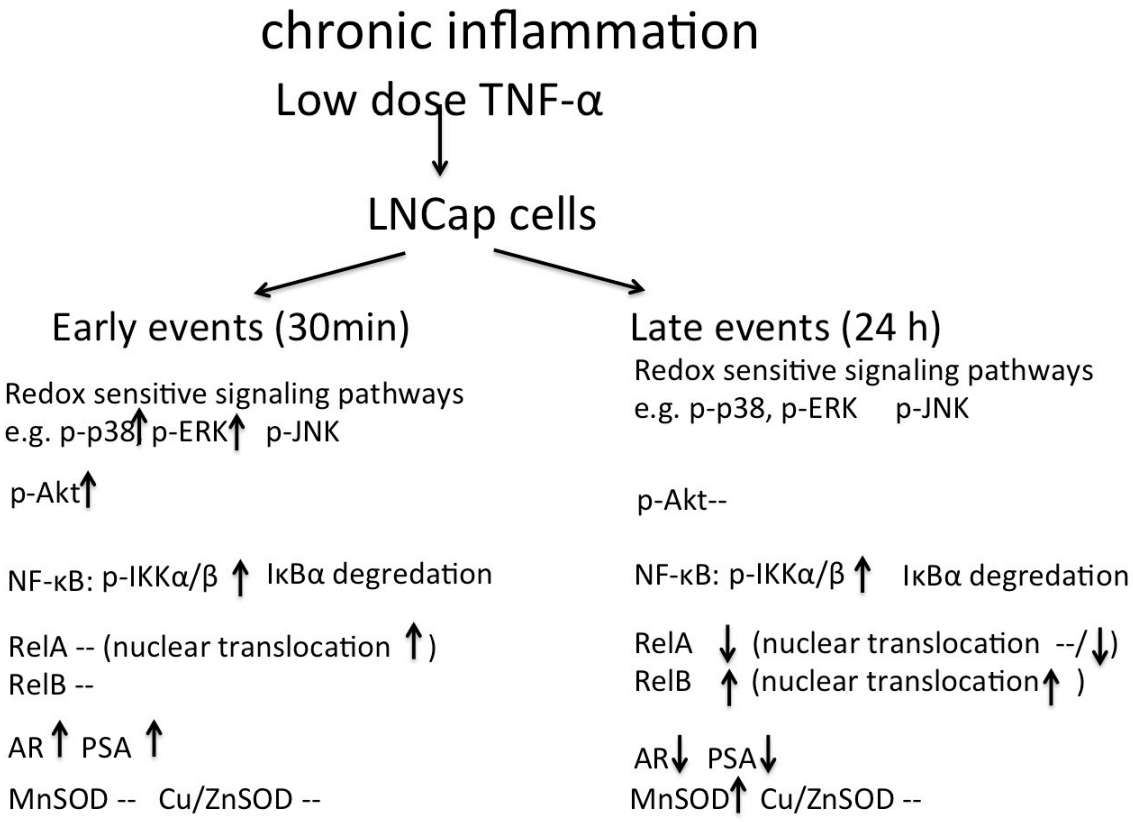
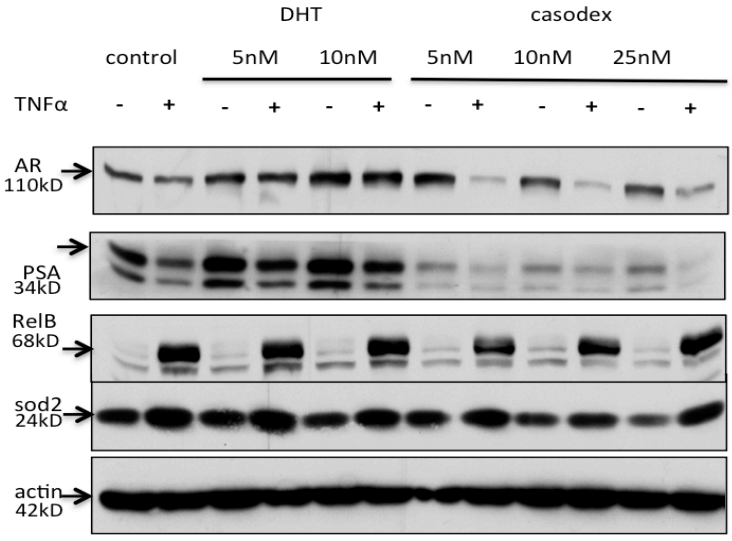


Figure 4.2. RelB plays a dominant negative role in PSA suppression.

TNF-α induces RelB and MnSOD but suppresses PSA expression in spite of the status of androgen receptor. LNCap cells were pretreated with different concentration of Dihydrotestosterone (DHT), a sex steroid and androgen hormone or different concentration of casodex (Bicalutamide) belonging to the group of medicines called antiandrogens, then exposed TNF-α (0.5ng/mL) for 24 h (upper panel). Overexpressing RelB suppresses DHT-induced AR and PSA elevation. LNCap cells were transiently transfected with RelB overexpressing plasmid or control vector for 12 h, then treated with or without DHT for 24 h. Total cell lysate was prepared for the Western blotting assay (bottom panel).



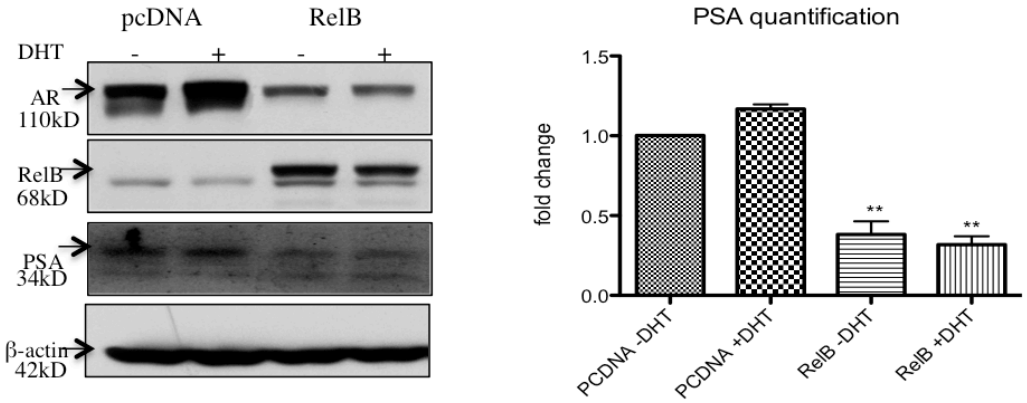
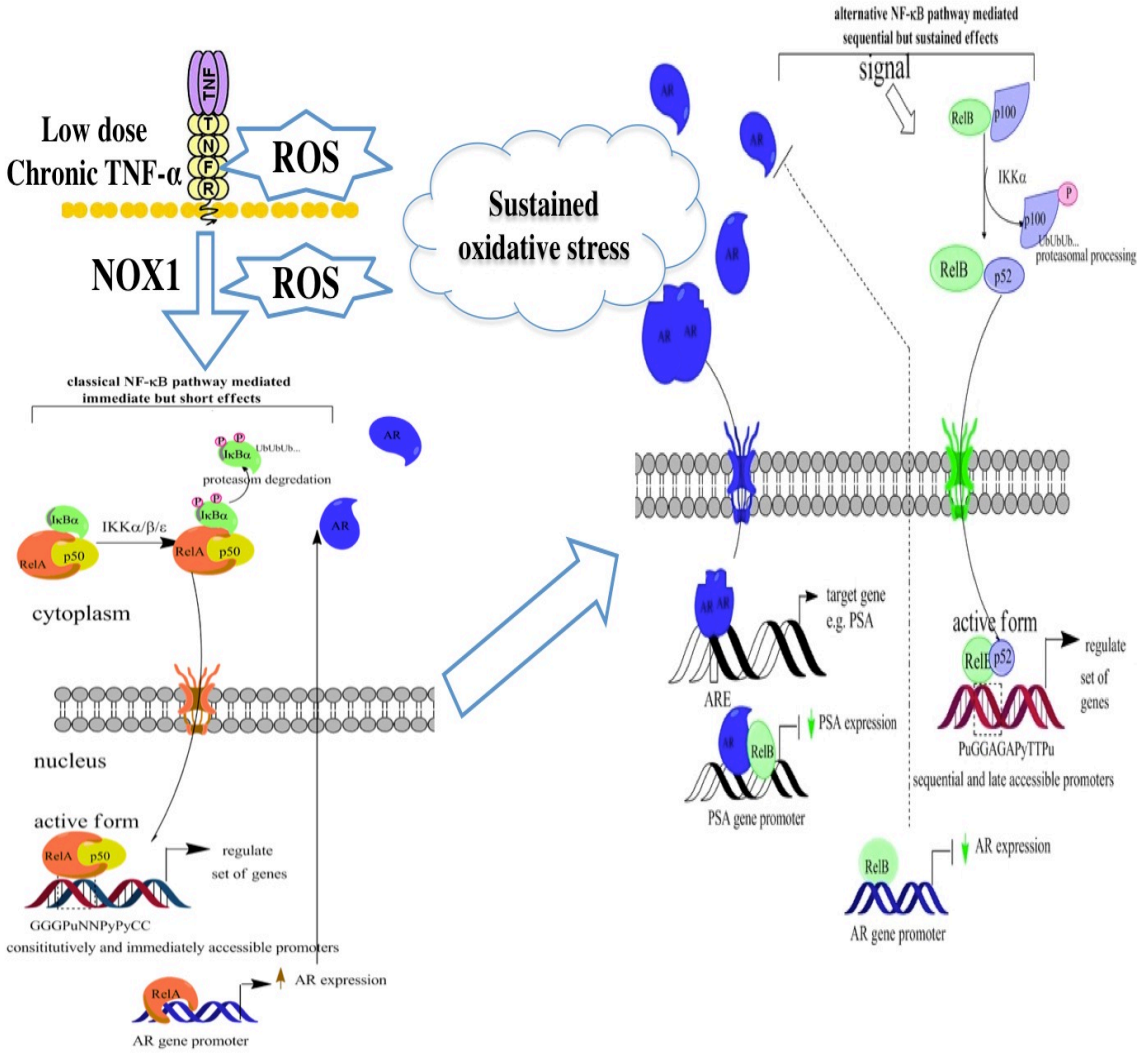


Figure 4.3. Overall summary

Schematic illustration of sequential activation of canonical and noncanonical NF-κB pathway, and sustained PSA suppression mediated by RelB-AR axis at advanced stage of prostate cancer in presence of sustained high level of oxidative stress.



Appendix: List of abbreviations

2-ME = 2-methoxyestradiol
3DCRT = 3-dimensional conformal radiation therapy
8OH-dG = 8-hydroxy-deoxyguanosine
8-oxoG = 8-oxoguanine
AP-1 = activator protein-1
APE1 = AP endonuclease 1
AP site = abasic site
AR = androgen receptor
ARE = androgen response elements
AhR = Aryl hydrocarbon receptor
ATF = activating transcription factors
BER= base excision repair
BPH = benign prostate hyperplastic
CAF = cancer associated fibroblast
CHIP = chromatin immunoprecipitation
CDK = cyclin-dependent kinases
COI = cytochrome oxidase subunit I
COX-2 = cyclooxygenase-2
CREB = cAMP response element binding protein
CRPC = castration resistant prostate cancer
CSC = cancer stem cell
Cu/ZnSOD = copper/zinc superoxide dismutase
DBD = DNA binding domain
DEP-1=density-enhanced protein tyrosine phosphatase-1
DHE = dihydroethidium
DHT = Dihydrotestosterone
DPI = diphenylene iodonium
DNTGFBRII = dominant negative mutant TGF-β type II receptor
EMT = epithelial mesenchymal transition
ETC = mitochondrial electron transport chain
ECSOD = extracellular superoxide dismutase
EGF = epidermal growth factor
EGFR = epidermal growth factor receptors
ESR = electron spin resonance
FGF = fibroblast growth factor
FUS/TLS= Fused/Translocated in LipoSarcoma
GSH = glutathione
GPx = glutathione peroxidase
GST = glutathione S-transferase
GSK = glycogen synthase kinase
GR = glutathione reductase
Grx = glutaredoxin
HIF = hypoxia-inducible factors
HO-1=heme oxygenase-1
HSP90 = heat shock protein 90
IGF = insulin-like type I growth factor
IGF-1R = insulin-like type I growth factor receptor
IL-1β = interleukin-1β

IL-6 = interleukin-6
IL-8 = interleukin-8
IMRT = intensity modulated radiation therapy
IFN- γ = interferon- γ
IR = ionizing radiation
Jak = Janus kinase
LBD =ligand binding domain
LPX = lipoxygenase
MAPK = mitogen-activated protein kinase
MCT4 = mono-carboxylate transporter 4
MEF = mouse embryonic fibroblast
MnSOD = manganese superoxide dismutase
mtDNA = mitochondrial DNA
NE = neuroendocrine
NED = neuroendocrine differentiation
NF-κB = nuclear factor kappa B
NOX = NADPH oxidase
NTR1 = neurotensin receptor 1
PI3K = phosphatidyl inositol 3-kinase
PIA = proliferative inflammatory atrophy
PIAS = protein inhibitors of activated STATs
Prostate cancer = PCa
Prx = peroxiredoxin
PSA = prostate specific antigen
PTP= protein tyrosine phosphatases
Rac1=ras-related C3 botulinum toxin substrate 1
RNS = reactive nitrogen species
ROS = reactive oxygen species
SASPs = senescence-activated secretory pathways
SH2 = *Src*-homology 2
SHP = small heterodimer partner
sIL-6R = soluble IL-6 receptor
SOCS = suppressor of cytokine signaling
SOD = superoxide dismutase
STAT = signal transducers and activators of transcription
TAM = tumor-associated macrophages
TACE = TNF-converting enzyme
TNAF4 = TNF-α receptor-associated factor 4
TNF-α = tumor necrosis factor-alpha
TGF-β = transforming growth factor-beta
TRADD = TNF receptor-associated protein with death domain
TRAIL= TNF-related apoptosis-inducing ligand
Trx = thioredoxin
TRXox = oxidized thioredoxin
TrxR = thioredoxin reductase
TRXre = reduced thioredoxin
VEGF = vascular endothelial growth factor
VEGFR2 = vascular endothelial growth factor receptor 2
WBC = white blood cells
XO = xanthine oxidase

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Publications

Miao L, Holley AK, Zeng Z, Wang C, St Clair DK, St Clair WH. Tumor necrosis factor alpha dependent redox signaling suppresses prostate specific antigen level by activating the RelB and androgen receptor axis. (under review)

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Presentations and Abstracts

Miao L, Holley AK, Zeng Z, Wang C, St Clair DK, St Clair WH. Tumor necrosis factor alpha dependent redox signaling suppresses prostate specific antigen level by activating the RelB and androgen receptor axis. Presented and **Awarded** at Cancer Research Day, Markey Cancer Center, University of Kentucky, 2013 (poster)

Zhao Y, Miriyala S, Mitov M, **Miao L**, Schnell D, Dhar S, Sultana R, Butterfield DA and St. Clair DK. Redox based protein identification of doxorubicin-mediated cardiac injury in mice. Presented at Cancer Research Day, Markey Cancer Center, University of Kentucky, 2013 (poster)

Dhar SK, Bakthavatchalu V, **Miao L,** Chen J, Zhu HN, St Clair DK. The nitration of DNA polymerase gamma (pol γ) that activates autophagy responses is a novel mechanism by which UVB induces skin cancer. AACR, Washington, DC, 2013. (poster)

Miao L, Aaron HK, St Clair DK, St Clair WH. Tumor necrosis factor alpha (TNFα) dependent changes in prostate specific antigen (PSA) reveal a novel mechanism for redox signaling Presented at the 19th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), San Diego, 2012. (poster)

Miao L, Aaron HK, St Clair DK, St Clair WH. Redox-mediated RelB-dependent regulation of Prostate Specific Antigen (PSA) gene expression in prostate cancer. Presented at the 18th annual meeting of SFRBM (Society of Free Radical Biology and Medicine), Atlanta, 2011. (poster)

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