RADIOSENSITIZATION OF BLADDER CANCER CELLS BY THE mTOR INHIBITOR, RAD001:

A NOVEL STRATEGY FOR TREATMENT

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"It is called research…

because after every search, we have to go back and re-search"

My mentor (retelling Albert Einstein)

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RÉSUMÉ

La radiothérapie pour le cancer invasif de la vessie permet la préservation de l'organe, mais la toxicité systémique et le contrôle local restent problématiques. En tant que tel, il existe un besoin d'augmenter la radiosensibilisation des cellules tumorales afin d'améliorer l'efficacité du traitement. Le but de cette étude était d'examiner si la protéine mTOR (cible de la rapamycine chez les mammifères), une kinase en aval de la voie de survie PI3K/AKT, peut être une cible pour la thérapie combinée du cancer de la vessie.

Les tests clonogéniques ont été effectués en utilisant six lignées cellulaires humaines de cancer de la vessie pour examiner les effets des radiations ionisantes sur la croissance, lorsqu'elles sont testées seules et en combinaison avec RAD001, un puissant inhibiteur de mTOR. L'analyse du cycle cellulaire a été effectuée en utilisant la cytométrie de flux et le buvardage de western. Dans l'étude *in vivo*, des souris nues ont subi une implantation sous-cutanée de cellules KU7 et 253J-BV. L'effet des traitements avec RAD001 (1,5 mg / kg, tous les jours), des radiations fractionnées (totale 9 Gy), et la combinaison de RAD001 et les radiations a été suivi pendant 4 semaines. La cinétique de croissance de la tumeur a été mesurée jusqu'au point limite expérimental accepté. Le niveau d'expression de p21 a été ciblé en utilisant un spécifique shRNA, puis l'effet a été suivi par test clonogénique en vue d'évaluer son rôle dans la radiosensibilité des cellules. L'autophagie et l'apoptose ont été évalués par immunofluorescence de la protéine LC-3 et l'immunobuvardage de la caspase-3, respectivement.

In vitro, une diminution significative de la formation de colonies a été observée dans le traitement combiné par rapport à RAD001 ou la radiothérapie seule ($p < 0,05$) dans toutes les lignées cellulaires. Un arrêt au stade G0/G1 du cycle cellulaire ainsi qu'une augmentation significative de l'arrêt en G2 ont été observés dans le traitement combiné par rapport aux traitements seuls. Des changements de niveaux de la cycline D1, de p27 et de p21 en corrélation avec les changements observés dans le cycle cellulaire ont aussi été observés. De plus, les radiations ont rapidement activé AKT alors que RAD001 a inhibé efficacement la signalisation en aval de mTOR, tel qu'indiqué par l'inhibition de la phosphorylation de S6. En outre, l'autophagie a été induite après le traitement avec RAD001 et en combinaison, tel qu'indiqué par la conversion de LC3-I à LC3-II, un marqueur protéique de l'autophagie. Nos données *in vivo* ont confirmé les données *in vitro* : une diminution significative du poids des tumeurs a été observée dans le groupe de traitement combiné (90 % de diminution, $p < 0,001$) par rapport à un ou l'autre traitement seul (60 % de diminution pour RAD001, $p < 0,05$; 77 % de baisse pour les radiations, $p < 0,05$). En l'absence de p21, les cellules sont devenues plus sensibles à la radiation ; on a observé une augmentation de l'autophagie et d'apoptose. Ces résultats montrent un effet additif de la combinaison de RAD001 et des radiations pour contrôler le cancer de la vessie, en agissant sur diverses voies, y compris p21.

L'inhibition de la signalisation de mTOR semble prometteuse comme modalité thérapeutique pour le cancer de la vessie, en particulier dans le contexte de la combinaison avec la radiothérapie.

ABSTRACT

Radiation therapy for invasive bladder cancer allows for organ preservation but systemic toxicity and local control remain problematic. As such, there is a need to increase radiosensitization of tumor cells to improve efficacy. The aim of this study was to investigate if mTOR (mammalian target of rapamycin), a downstream kinase of the PI3K/AKT survival pathway, may be a target for combined bladder cancer therapy.

Clonogenic assays were performed using 6 bladder cancer cell lines in order to address the effects of ionizing radiation (IR) on growth, when tested alone and in combination with RAD001, a potent mTOR inhibitor. Cell cycle analysis was performed using flow cytometry and Western blotting. In the *in vivo* study, nude mice were subcutaneously inoculated with KU7 and 253J-BV cells. Treatment with RAD001 (1.5 mg/kg, daily), fractionated IR (total 9 Gy), and the combination of RAD001 and IR was followed over 4 weeks. Tumor growth kinetic was measured and tumor weight at the experimental endpoint. Knockdown of p21 was performed using shRNA followed by clonogenic assays in order to assess the role of p21 in radiosensitization. Autophagy and apoptosis were assessed by LC3 immunofluorescence and Western blotting for caspase-3, respectively.

In vitro, a significant decrease in colony formation was observed in the combined treatment when compared to RAD001 or radiation alone ($p<0.05$) in all cell lines. A G0/G1 as well as a significant increase in G2 arrests was observed in the combined treatment compared to either treatment alone. Changes in the levels of cyclin D1, p27 and p21 correlated with the observed changes in the cell cycle. Moreover, IR rapidly activated AKT whereas RAD001 inhibited mTOR downstream signaling as shown by the inhibition of the S6 protein phosphorylation. Furthermore, autophagy was induced following the treatment with RAD001 and in combination, as indicated by the conversion of LC3-I to LC3-II, a protein marker for autophagy. Our *in vivo* data confirmed our *in vitro* data: a significant decrease in tumor weight was observed in the combined treatment arm (90% decrease, $p<0.001$) compared to either treatment alone (60% decrease for RAD001, $p<0.05$; 77% decrease for IR, $p<0.05$). In the absence of p21, cells were rendered more sensitive to IR, and an increase in autophagy and apoptosis was observed. These findings point to additive beneficial effects of the combined therapy on bladder cancer, mediated by various pathways including p21.

The inhibition of mTOR signaling appears promising as a therapeutic modality for bladder cancer, especially in the context of combination with radiation therapy.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis demonstrates for the first time a role for Everolimus (RAD001) in radio-sensitizing bladder cancer cells to treatment with ionizing radiation.

The observations presented in the early experiments are the first to demonstrate a wide range growth inhibition by RAD001 of different bladder cancer cell lines. The evaluation of the combinatory effects of RAD001 and ionizing radiation demonstrated that despite the different levels of sensitivities of the cell lines to radiation alone *in vitro*, they all showed a significantly lower proliferation rate; the radio-sensitization effects of RAD001 on bladder cancer growth *in vivo*, were further established. This is the first evidence of an additive effect of ionizing radiation and RAD001 in bladder cancer.

Dissection of the molecular pathways involved in the response to the combination treatment revealed the involvement of both autophagy and apoptosis, with p21 playing a potentially central role in balancing between the two processes. No previous work has clearly demonstrated such a role for either autophagy, or p21 in contrast to apoptosis, in bladder cancer response to treatment, which opens the way to novel therapeutic approaches.

This work has led to the publishing of two (2) peer-reviewed journal articles in the literature (annex A and B) and one article currently in the writing process. Furthermore, based on these findings, a phase I/II investigator-initiated clinical trial was opened at McGill evaluating the effect of combining RAD001 with radiation for the treatment of muscle invasive bladder cancer.

ABBREVIATIONS

4E-BP1: 4E Binding Protein 1

AMPK: AMP-activated Kinase

AP: Apurinic and/or Apyrimidinic

APC/C: Anaphase-Promoting Complex/Cyclosome

Atg: Autophagy-related genes

ATP: Adenosine Tri-Phosphate

ATPase: Adenosine Tri-Phosphatase

BER: Base Excision Repair

BCA: Bi-Cinchoinic Acid

BCG: Bacillus Calmette-Guerin

BSA: Bovine Serum Albumin

CAK: Cdk-Activating Kinase

Cdk: Cyclin-dependent kinase

CIS: Carcinoma In Situ

CKI: Cdk inhibitor

CREBBP: CREB- Binding Protein

CT: Computed Tomography

DAB: 3,3'-Di-Amino-Benzidine

DISC: Death-Induced Signaling Complex

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DNA-PK: DNA-activated Protein Kinase

DPC: DNA-Protein Cross-link

DR: Death Receptor

DSB: Double-Strand Break

EGFR: Epidermal Growth Factor Receptor

eEF2: eukaryotic Elongation Factor 2

eIF4E: eukaryotic Initiation Factor 4E

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FKBP12: FK506-Binding Protein12

GSTM1: Glutathione-S-Transferase Mu1

G0: Gap zero

G1: Gap one

G2: Gap two

GAP: GTPase Activator Protein

GI_{50}: 50% Growth Inhibition

Gy: Gray

H&E: Haematoxylin and Eosin

HR: Homologous Recombination

HRP: Horse Radish Peroxidase

IR: Ionizing Radiation

LET: Linear Energy Transfer

Mdm2: Murine Double Minute2

MDS: Multiply Damage Sites

MI-UBC: Muscle Invasive-Urolthelial Bladder Cancer

MMC: Mytomycin C

MMR: Mismatch Repair

M-Phase: Mitosis Phase

MRI: Magnetic Resonance Imaging

mTOR **:** mammalian Target of Rapamycin

MTT: 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide

Na_3VO_4: Sodium Orthovanadate

NAT-2: *N*-Acetyl Transferase-2

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

NMI-UBC: Non Muscle Invasive-Urothelial Bladder Cancer

PARP-1: Poly (ADP-Ribosyl) Polymerase-1

PBS: Phosphate Buffer Saline

PCNA: Proliferating Cell Nuclear Antigen

PI: Propidium Iodide

PI3K: Phospho-Inositide-3 Kinase

Polβ: Polymerase β

Rb: Retinoblastoma Tumor

PUNLMP: Papillary Urothelial Neoplasia of Low Malignant Potential

REF: Rat Embryo Fibroblast

Rheb: Ras-homologue enriched in brain

ROS: Reactive Oxygen Species

RIPA: Radio ImmunoPrecipitation Assay

Ser: Serine

S phase: Synthesis phase

SSB: Single Strand Break

Thr: Threonine

TBS: Tris-Buffered Saline

TNF-α: Tumor Necrosis Factor α

TUR: Transurethral Resection

TUNEL: Terminal deoxynucleotidyl transferase (TdT)-Mediated dUTP Nick End-Labeling

UBC: Urothelial Bladder Cancer

VEGF: Vascular and Endothelial Growth Factor

Tyr: Tyrosine

CHAPTER I: INTRODUCTION

1.1 The bladder

The bladder is a hollow muscular organ whose main function is that of a urine reservoir.[1] In the female, the superior part of the bladder (apex) is apposed to the uterus and ileum, while it is apposed to the ileum and pelvic portion of the colon in the male. The base of the bladder faces posteriorly and is separated from the rectum by the uterus and vagina in the female, and by the vasa differentia, seminal vesicles and ureters in the male. The anterolateral surface on each side of the bladder is apposed to the pubic bone and the anterior bladder is separated from the pubic bone by the retropubic space. The neck of the bladder, its most inferior part, connects with the urethra.[1, 2]

1.1.1 Development of the bladder

The bladder derives initially from the urogenital sinus, which is the ventral division of the cloaca. The urethral portion of the cloaca will later become the prostatic and membranous urethra in the male, and the entire urethra in the female.[2]

After 8 weeks of gestation, the ventral part of the urogenital sinus expands to form an epithelial sac, the apex of which tapers into an urachus. By the 10^{th} week, the bladder is a cylindrical tube lined with a single monolayer of cuboidal cells surrounded by loose connective tissue. As the fetus enters its 12^{th} week of gestation, the bladder lining is already a bilayer of cuboidal cells that start acquiring urothelial characteristics between week 13 and 17. It is only by the 21^{st} week that the bladder lining becomes 4 to 5 cells in

thickness and begins differentiating into a proper urothelium composed of different layers.[1, 2]

1.1.2 The mature bladder

1.1.2.1 Anatomy

The bladder consists of two parts: the body, which lies above the ureteral orifices and the base, consisting of the trigone and bladder neck. The bladder outlet is composed of: the base, urethra, and external urethral sphincter. The urethra begins at the internal meatus (opening) of the bladder and extends to the external meatus. The bladder neck is important for reproduction: in male, closure of the bladder neck enables antegrade (forward) ejaculation.[3]

The bladder is an abdominal organ, which wall consists of 3 layers: the mucosa (inner urothelium lining), detrusor and adventia (outermost layer consisting of connective tissue). As mentioned earlier, the detrusor is the layer consisting of a meshwork of smooth muscle fibers that orchestrates the contraction of the bladder during voiding, and its relaxation during filling. These smooth muscle fibers have the ability to elicit an important active tension that allows the bladder to fill with urine at low pressures. The bladder fulfills its urine reservoir function through the parallel activity of the detrusor muscle and the bladder outlet. The bladder sphincter plays a major role in urinary continence *via* control of the bladder neck and proximal urethra closures. It consists of a cylindrical structure that is thicker anteriorly giving it a characteristic shape of a horseshoe. [1]

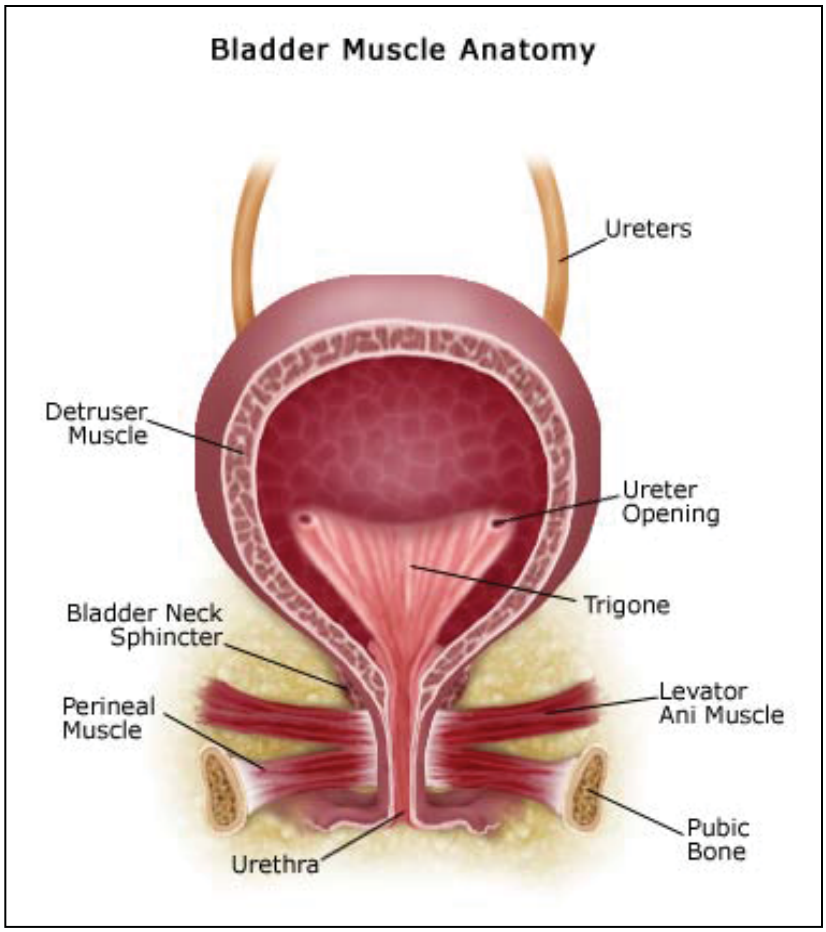

Figure A: Anatomy of the bladder [4]

Innervation of the bladder. The innervation of the bladder is complex: it involves both the central somatic and autonomic nervous systems *via* three sets of peripheral nerves: sacral parasympathetic (aka pelvic), thoracolumbar sympathetic (hypogastric and sympathetic chain) and sacral somatic nerves. [5, 6]

Parasympathetic ganglia are found within vesical plexuses and in the bladder wall. The parasympathetic nerve fibers run in the pelvic nerve (S_2-S_4) emerging at the spinal cord vertebrae, and supply the pelvic and vesical plexuses before entering the bladder. Sympathetic nerves arise from the spinal cord (at segments T10-L2), and go through the sympathetic trunk to the inferior mesenteric ganglion. From there, it reaches the bladder through hypogastric nerves. The detrusor and the urethral sphincter are also innervated by sympathetic nerves that originate from T10-L2 of the spinal cord. The somatic (voluntary) nervous system on the other hand, supplies the pelvic floor musculature.

The three systems (parasympathetic, sympathetic and somatic) carry sensory and motor nerves and innervate both the bladder and urethral sphincter. These systems originate from parasympathetic ganglia located in the second, third, and fourth segments of the sacral spinal cord.[1] Within the spinal cord, information from bladder afferents is integrated with that from other viscera and somatic sources and projected to the brainstem centers that coordinate the micturition (urination) cycle.[7]

Smooth muscles of the bladder. Smooth muscles are a crucial component of the detrusor muscle: unlike skeletal muscles, smooth ones allow for a greater length change (75% compared to 30% in skeletal muscles), which serves the bladder well in terms of

elasticity and accommodates the changes in bladder volume between the filled/empty states.[8]

During filling, the myocytes get stretched and trigger an activation of cation channels that permit rapid entry of sodium and calcium. Cations then depolarize the smooth muscle membrane potential, and if the stretch is significant, an action potential is initiated.[1, 9]

The capacity of the bladder is maintained *via* the smooth muscle ability to keep a steady level of contracture and tone. Tone depends on intrinsic factors such as the response to stretch, locally secreted agents like nitric oxide and temperature. It also depends on extrinsic factors that include circulating hormones and autonomic nerve activity. In general, smooth muscles take a longer time to contract/relax than do skeletal muscles, making contractions slow but sustained and resistant to fatigue. The ability of the detrusor smooth muscles to change length to such a large degree allows the bladder to adjust to wider variations in volume.[8-11]

The bladder wall stroma. The bladder wall stroma is mainly constituted of collagen and elastin connected through a proteoglycan matrix, and contributes to the wall viscoelastic properties.[12] Collagen is found outside the muscle bundles as types I, III and IV. The role of collagen in bladder storage function was emphasized when a study found that the ratio of connective tissue in the stroma to smooth muscle was significantly increased in poorly compliant *vs* normal bladders. An increase in the ratio of type III to type I collagen was also noted.[13] Furthermore, aging which is strongly associated with poor bladder compliance, is also associated with an increase in the ratio of collagen to

smooth muscle content, in both men and women.[14] Compared to collagen, elastin fibers are relatively sparse in the bladder. Unlike collagen, elastin can be detected in all layers of the bladder wall.[15] Elastin is also thought to play a role in modulating bladder compliance. In fact, experiments on rats with spinal cord-induced injuries showed a decrease in elastin to collagen ratio that correlated with a reduced bladder compliance caused by the emergence of detrusor over-activity 10 weeks post-surgery.[16]

Bladder vascularization. The human bladder is supplied with blood *via* the superior and inferior vesical arteries that are branches of the internal iliac artery. Vesical veins drain blood off the bladder into the internal iliac veins.[17] Internally, the bladder is carefully designed with vascular grooves under the urothelium. These grooves are filled by a dense network of blood capillaries, and allow these capillaries to be in close proximity to the epithelium, at a distance of tenths of a micron.[18] The grooves are also thought to play a role in maintaining a barrier function.[19] As the bladder is a relatively flexible organ with a significant change in surface area between filling and voiding, the blood vessels must have the ability to lengthen considerably in order to maintain good blood flow. Thus, the overall resistance of the vessels, as they lengthen, should not increase in order to prevent reduction in effective perfusion of the tissue. This is only possible because of the adaptability of blood vessel's intramural tension: the blood flow is able to adapt to the large increase in surface area until the pressure increases in the bladder. In fact, if pressure is high enough, resistance to blood flow increases and blood supply decreases, eventually causing the detrusor to be deprived of oxygen.[1, 20]

1.1.2.2. Function

The urinary bladder has several important functions. First, it is the organ that collects urine excreted by the kidneys before disposal by urination: urine enters the bladder *via* the ureters and exits *via* the urethra. It is able to store an adequate volume of urine, and for that it is able to stretch and rearrange itself for proper bladder volume control. Second, the urothelium acts as a protective layer and prevents the smooth muscle and intrinsic nerves from exposure to urine. The urothelium also expands readily during filling. It is highly compliant as it stores variable volumes of urine, dilating and shrinking to control its volume. Lastly, the bladder emptying (voiding) requires a synchronous activation of all the smooth muscles of the bladder body.

Bladder filling mechanics and urine storage: As mentioned the bladder is an organ that can undergo large changes in volume, going from empty to full. This change in volume is possible due to its viscoelastic behavior accommodated by both the urothelium and the underneath wall smooth muscle and connective tissue, [11] displaying both neuromuscular and mechanical properties. The bladder viscoelasticity is highly dependent on the muscular part of the bladder wall, the detrusor muscle: while the bladder is filling, the detrusor muscle is relaxed so the bladder wall can expand, and the sphincter muscles are contracted to keep the urethra closed.[1] When contractile protein content (such as elastin) exceeds collagen, greater distensibility is achieved (compliance). Conversely, when collagen levels increase, compliance falls. Bladder compliance (C) is defined as the change in volume (V) relative to the corresponding change in intravesical pressure (P): $C=\Delta V/\Delta P$. [1, 11]

The urothelium: The urothelium refers to the inner lining of the bladder, facing the lumen. Historically, it was thought that the urothelial function was restricted to acting as a passive barrier between urine in the bladder and plasma. However, it is now known that it is physiologically involved in the bladder basic functions: storage of urine, maintenance of urine composition and facilitation of voiding.[1] The urothelium has 3 distinct layers: a 5-10 μm in diameter thickness basal cells, on top of which lie the intermediate cells (20 μm in diameter). Umbrella cells are at the luminal surface of the urothelium and constitute the largest epithelial cells in the body, being 100-200 μm in diameter. Umbrella cells are polyhedral/hexagonal and have the ability to flatten and increase in surface area with stretching. The surface of umbrella cells is covered by a layer of Glycosaminoglycans (CAG), thought to play antibacterial adherence functions, to provide charged surface area and to prevent urothelial damages.[21]

The urothelium is not a simple barrier. It was shown to have a slight permeability through active transport of ions, osmosis and passive diffusion. Furthermore, it acts as a neural interface and is able to detect chemical, mechanical and thermal stimuli *via* sensory neurons (nocireceptors/mechanoreceptors).[22, 23]

Urine voiding: Urine voiding occurs as a result of both the abdominal pressure and the detrusor pressure of a filled bladder along with simultaneous relaxation of the external urinary sphincter. The velocity of urine flow depends on the urethral resistance, in an fashion proportionate to diameter.[1]

1.1.2.3 Diseases of the bladder

Besides neoplasm of the bladder, which will be described in the next section, most diseases of the bladder are disabling rather than lethal. These abnormalities of bladder function can be categorized as: i) congenital anomalies, ii) inflammation and iii) metaplastic lesions.[24]

Congenital anomalies of the bladder. Congenital anomalies are those that arise at birth and are often structural in nature. In bladder, congenital anomalies are the result of a focal failure of the normal musculature development but can also be caused by some urinary tract obstruction during development.[25] A *bladder diverticulum* is a pouch-like evagination of the bladder wall that varies in size from less than 1 cm to 10 cm in diameter. It arises in two forms: congenital or acquired (hyperplasia/neoplasia). In either case bladder diverticulum causes an obstruction to urine outflow and a noticeable thickening of the bladder muscle wall. Most diverticula are small and asymptomatic, but the presence of a diverticulum renders urine relatively static, and thus predisposes to infections.[1, 2, 25]

Exstrophy of the bladder is a failure, of developmental origins, in the formation of the anterior bladder wall, causing the bladder to lie as an opened sac or to fuse with the surface of the body. The exposed bladder mucosa is often subject to infections that spread to the upper urinary system through the ureters. Exstrophy is managed *via* surgical correction with a long-term positive prognosis.[25] Adult patients suffering from exstrophy are at higher risk of adenocarcinoma.[26] Other anomalies include: vesicoureteral reflux, the retrograde flow of urine from the bladder to the upper urinary tract[27], congenital vesicovaginal, vesicouterine and vesicocolonic fistulas, which result

from abnormal connections between the bladder and the vagina, rectum or uterus,[28] and urachal cysts that arise from the persistence of urachus, the canal that connects the fetal bladder with the allantois. Carcinomas can arise from those cysts. [29]

Inflammation of the bladder. Acute and chronic cystitis are the most common forms of bladder inflammation of bacterial etiology. The common agents of cystitis are: *Escherichia coli*, followed by *Proteus, Klebsiella,* and *Enterobacter.*[24] Females are more likely to develop cystitis than men. Of note, some men with chronic cystitis are often diagnosed with chronic non-bacterial prostatitis and show a great overlap of syndromes. Other less common causes of bladder cystitis include: *Tuberculosis, Candida Albicans, Schistosoma Haematobium, Chlamydia* and *Mycoplasma*. Of note, irradiation of the bladder region also causes cystitis, known as radiation cystitis (reviewed in [24]). Different cystitis patients share the same symptoms: frequent urination, localized lower abdominal pain around the bladder/suprapubic region and dysuria, which refers to burning/pain on urination. [30, 31] There are also other forms of cystitis that are less common; variants include: Interstitial Cystitis [32], Malacoplakia [33] and Polypoid Cystitis.[34] Although there is no treatment to reliably eliminate cystitis, medications offer relief.

Metaplastic lesions. Metaplasia refers to the reversible transformation from one differentiated cell type to the other. For the bladder, metaplastic lesions occur in the urothelium where epithelial cells undergo transformations into other, differentiated cell types.[35] Cystitis Cystica and Glandularis refer to common lesions in which cells from the urothelium grow downward into the lamina propria and transform from epithelial cells into cuboidal/columnar epithelium lining (glandularis) or form cystic spaces lined by the

urothelium (cystica).[36] The two types of metaplastic transformations often co-occur;[37] however, they are not associated with an increased risk of adenocarcinomas.[38]

Another common metaplastic lesion of the bladder is squamous metaplasia. Normally, and in response to bladder injury, the urothelium is replaced by squamous epithelium, a more durable lining. Squamous metaplasia mimics this “repair” without prior injury however. It often occurs on the anterior wall of the bladder and can be divided into keratinizing and non-keratinizing types.[37] Whether squamous metaplasia is a precursor of cancer is still a matter of debate. When examined under a microscope, the mucosa of the bladder wall is thickened and typically exhibits white or gray-white coloration.[35, 37]

1.2 Urothelial Bladder Cancer (UBC)

UBC is one of the most frequently occurring cancers worldwide and constitutes a heavy burden with significant morbidity and mortality: it is the 7th most common cancer in men, and the 17th most common in women.[39, 40] In Canada, it is the 4th most commonly diagnosed cancer in men, and the 12th in women, while ranking as the 7th leading cause of cancer death in men, and 11th in women.[41]

1.2.1 Associated Risk factors

UBC is not a familial disease: high-risk families are rare and no Mendelian inheritance patterns have been identified. UBC is mainly a cancer of the environment and age.[1] Besides age, established risk factors are: gender, ethnicity, geographical location, tobacco smoking, occupational hazard, dietary intake, and to a lesser degree, genetic predisposition (reviewed in [40]).

Age, gender and ethnicity. The incidence and mortality rates of UBC increase with age with a median age of 70 at diagnosis. Patients younger than 40 years old are rare, and often present with less aggressive and well-differentiated cancer.[40, 42] Males are three to four times more at risk of developing UBC than female, and this has been explained in part by a higher prevalence of smoking and exposure to environmental toxins in male populations. Unlike other cancers, UBC is twice as high in Caucasians than in African-Americans (reviewed in [40, 42, 43]).

Geographical location. UBC incidence varies across the world, with the highest rates reported in Southern and Eastern Europe, parts of Africa, the Middle-East and North America, while the lowest occur in Asia and under-developed Africa.[44] Different

histological cell types of UBC are geographically dependent although the urothelial type is the most common (different UBC types will be described later): Around 96% of UBC in North America and Europe are urothelial carcinoma; in Africa 60% to 90% are urothelial and 10% to 40% are squamous; of note, Egypt has the highest rate of squamous cell carcinoma because of endemic infections with *Schistosoma* species, namely *S. haematobium*.[45]

Tobacco smoking. Tobacco smoke contains known carcinogenic chemicals, mainly aromatic amines (β-naphtylamine) and polycyclic aromatic hydrocarbons, which are renally-excreted substances with carcinogenic effects on the urinary system.[40] Thus, tobacco, and cigarette smoking more specifically, is a main cause of urothelial cancer formation, and has been reported to account for 46% of UBC deaths in developed countries, and 28% in developing ones.[45, 46] In terms of incidence, tobacco smoking contributes to 60% of UBC incidence in males and 30% in females. For smokers, and depending on the intensity and duration of smoking, there is 2 to 6 times more chance of developing UBC, and the intensity and duration of smoking has been shown to be linearly related to the increased risk of the disease.[46-48] The risk of second-hand smoke in UBC is low, and not statistically different from that of non-smokers.[47] In UBC patients treated by radical cystectomy (bladder ablation), smoking status was reported to be associated with shorter disease-free survival after surgery.[49]

Occupational Hazards. Occupational exposure to carcinogens is an established risk factor of UBC, and is considered as the most important after tobacco smoking.[50] Although establishing causative relationships between occupations and risk of UBC is challenging due to a long latency between exposure and UBC formation, some

occupations have been associated with exposure to carcinogens *via* inhalation or through skin absorption.[51-53]

Compared to agriculture workers, industrial workers showed an overall increased risk of UBC by 30%, with rubber workers being at the highest risk.[54] Carcinogens tightly linked to occupations are mainly the aromatic amines (including benzidine, 4-aminobiphenyl, 2-naphtylamine and 4-chloro-o-toluidine), polycyclic aromatic hydrocarbons and chlorinated hydrocarbons.[40, 50-52, 54] In fact, 20% of all UBCs were suggested to be related to such exposures, mainly in areas processing paints, dye, metal and petroleum.[1, 40, 43, 54]

Dietary intake. Several nutritional aspects have been linked to UBC. High fluid intake has been associated with a lower risk of UBC; in fact, most nutrients/metabolites are excreted in the urine and thus are in prolonged contact with the urothelium of the bladder during storage. High fluid intake reduces storage time and exposure to such metabolites.[55] While a Mediterranean diet rich in vitamins A, C, E, fruits and vegetables (notably citrus, berries, tomatoes and carrots) has been linked to an effective detoxification of the bladder and thus a decreased risk of UBC [56-58], prolonged intake of fried food, fat, processed meats, spices and barbequed meat was shown to increase the risk of UBC.[1, 59] Furthermore, water with high arsenic content was also associated with increased risk of UBC.[40] Whether selenium and zinc content of the diet lowers the risk of UBC is still unclear: Michaud *et al.* have reported such intakes to be associated with a decreased risk [56] while a more recent study reported no significant association between selenium and UBC incidence.[60]

Genetic susceptibility. As stated earlier, UBC is not a familial disease despite the increase in risk of UBC diagnosis by two-folds in a first degree relative of UBC patients.[39] Several genes/polymorphisms have been related to the formation of UBC *via* increasing susceptibility to environmental carcinogens.[40] A study of a Spanish population of UBC patients identified polymorphisms (deletions) of *N*-Acetyl Transferase-2 (NAT-2) and Glutothione-S-Transferase-1 (GST1), and showed an increase in overall risk of UBC in those patients.[61] NAT-2 is an *N*-acetyl transferase enzyme that detoxifies nitrosamines, a known bladder carcinogen. Specifically, NAT-2 regulates the rate of acetylation of compounds such as caffeine, which are related to bladder cancer formation. The slow NAT-2 polymorphism renders acetylation less effective, and thus the bladder more susceptible to carcinogenic effects.[62] GSTM1 is an enzyme that conjugates several reactive chemicals, including arylamines and nitrosamines. The null GSTM1 polymorphism is associated with an increased bladder risk as well.[61] Both the null GSTM1 and slow NAT-2 lead to high levels of 3-aminobiphenyl and higher risk of bladder cancer. These polymorphisms are present in 27% of white, 15% of African-American, and 3% of Asian males, thus partially explaining the different bladder cancer incidence rates across ethnic groups.[1, 43] Overall, heredity is not an established risk factor for UBC (no gene(s) alterations directly linked to UBC) although genetic disposition affects the individual susceptibility to carcinogens such as tobacco smoke, caffeine, dyes, etc. Thus the genetic influence on incidence is mainly *via* impacting on susceptibility of other risk factors.[40]

1.2.2 Natural history of bladder cancer

The natural history of UBC is challenging to predict, in terms of disease recurrence and progression, and this is due to the biological heterogeneity of the disease.[43] UBC displays two main features and is thus classified into two categories: Non-Muscle Invasive UBC (NMI-UBC) and Muscle Invasive-UBC (MI-UBC). Around 70% of all UBC are NMI and present superficial lesions that are often indolent, low-grade and confined to the superficial mucosa.[63] Despite this indolent aspect, it is reported that 70% of superficial UBC will recur, making long-term follow up of UBC patients necessary.[43, 64] NMI-UBC includes Carcinoma In Situ (CIS; 25%) and Papillary Urothelial Neoplasia of Low-Malignant Potential (PUNLMP; 75%) [65]. CIS is a non-papillary, flat, high-grade tumor of the surface of the epithelium, characterized by the loss of umbrella cell shape, cell enlargement with chromatin clumping and loss of normal mitotic figures.[66] PUNLMP, on the other hand is a papillary growth, generally solitary, composed of thin papillary stalks.[65-67] PUNLMP has a low proliferation index and is not associated with invasion or metastasis. [43]

MI-UBCs are invasive by definition and considered as potentially life threatening. As mentioned, they account for around 30% of UBC at diagnosis.[63] Invasive UBC is divided into two groups, characterized by the extent of invasion at diagnosis. Lamina propria invasive tumors are high-grade cancers detected in clusters of single cells within the lamina propria of the bladder. Invasion and metastasis is possible due to the large vascular and lymphatic network within the tissue layer.[1, 63, 64] Deep-muscle invasive UBC represents cancerous cells that extend through the lamina propria into the deep muscle.

Primary cancers of the bladder arise from the urothelium and the most common type is transitional cell UBC. Other uncommon/rare types of UBC include adenocarcinomas and small cell neuroendocrine UBC.[68] Approximately, 75% of patients diagnosed with UBC initially present with painless, intermittent hematuria (blood in urine) [69]. However, besides hematuria, UBC is often relatively asymptomatic; and given the intermittent and painless aspect of hematuria, UBC can go clinically undiagnosed in its early stages as a single negative urine analysis cannot exclude the possibility of cancer. [70-72] The remaining 25% of patients will present with irritative voiding symptoms such as urgency, frequency and dysuria, and these symptoms can be often mistaken for urinary tract infection or benign prostatic hypertrophy.[73]

Once diagnosed and treated for superficial NMI-UBC, it is estimated that around 80% of the patients will have recurrence within one to two years of initial treatment, if treated by Trans-Urethral Resection alone (TUR; described below).[74, 75] Whether UBC will recur post-treatment is dependent on grade, depth of invasion, multiplicity, tumor size/morphology, and presence or absence of CIS.[75] Approximately 25% of those patients with recurrence will eventually progress to MI-UBC.[76] Ultimately, and even after treatment by radical cystectomy for progression of MI-UBC (described below), it is estimated that 50% of patients will develop metastasis and die of their disease.[77] MI-UBC most common sites of metastasis are: lymph nodes, bone, lung, liver, and peritoneum.[78]

1.2.3 Bladder cancer diagnosis

Clinical presentation. Most patients diagnosed with UBC initially present with hematuria, the most common sign of bladder cancer. In fact, it was estimated that around

80% of all patients with UBC would be diagnosed with either gross (blood in urine visible to naked eye) or microscopic hematuria (red blood cells detected under microscopy following urinalysis).[79, 80] While gross, painless hematuria was found to be indicative of UBC in 15% of patients,[81] asymptomatic microscopic hematuria is a more common symptom of UBC, with around 3%-5% of patients with microscopic hematuria found to have a malignancy.[82]

Of note, hematuria is not strictly indicative of UBC, as it can be associated with nonmalignant disorders such as stone disease and inflammatory conditions. However, hematuria is still indicative of further bladder evaluation for cancer. Although UBC is usually asymptomatic, some patients (those with high-grade CIS) present with significant symptoms of bladder irritability such as frequent urination, urgency and dysuria.[79]

Cytoscopy. Cystoscopy is performed in order to determine the presence of a lesion in the urothelium, and is considered the cornerstone for diagnosis of UBC.[80, 83] The advantages of flexible cystoscopy are the minimum discomfort it causes given the use of flexible instruments with high quality optics, as well as its requirement of only topical or local anesthesia.[80] Cystoscopy allows the complete visualization of mucosal surfaces, and thus the identification of tumors and their classification into either papillary or sessile (attached and flat tumors).[84, 85]

Radiologic imaging. Currently, Computed Tomography (CT) with intravenous pyelography (CT urogram) is the preferred initial imaging modality to assess upper tract carcinoma once the diagnosis of UBC is suspected.[83] Intra-Venous Pyelography (IVP) allows the visualization of the upper urinary tract system and relies on evaluating defects

in the urothelium filling to detect a malignant disease. Although unable to either detect tumors smaller than 1 cm or differentiate superficial from intramural invasion, CT scan is still able to detect < 1 cm tumors in the upper tract as well as extra-vesical tumor extension, with 80% accuracy. It is also useful to rule out lymphatic involvement and node metastasis.[86, 87] However, at this point, the cystoscopy along with radiologic imaging would be informative of the i) presence/absence of UBC, ii) its papillary/sessile state, iii) the presence or not of invasion and (iv) the presence of concomitant upper tract tumors.[79, 84]

Urine cytologic study. Urine cytology is a non-invasive adjunctive diagnostic approach used to detect UBC, and later in follow-ups.[80, 84] Cytology studies can be performed on either urine voided from the bladder, or following bladder washing. A positive urine cytology is highly predictive of the presence of malignant cancers.[80, 83, 85] Urine cytology is less sensitive in general for low-grade cancers where cells of tumors closely resemble the normal urothelium.[88]

Other diagnostic tests. The first commercially available diagnostic test for UBC was based on the detection of Bladder Tumor Antigen (BTA), and was reported to be more sensitive, but less specific than cytological examination. Other markers have also been evaluated such as the Nuclear Matrix Protein 22 (NMP22) which levels in the urine are assessed *via* immunoassays.[89, 90]

Following lesion detection, TUR is performed in order to confirm diagnosis and determine the extent of the disease within the bladder: the tumor is resected, while the

muscle within the area of the resected tumor is also sampled to assess if invasion has occurred.[91, 92] Of note, a sessile tumor on cystoscopy usually suggests a high-grade tumor; in that case, CT or Magnetic Resonance Imaging (MRI) of the abdomen and pelvic area is recommended before TUR.[83, 91] In the case of a papillary appearance that suggests CIS or NMI-UBC, CT scan/ MRI can be deferred and TUR performed. Bone scan should also be performed if elevated levels of alkaline phosphatase are detected in blood.[83, 91, 92] The management of the disease will then be based on the extent of UBC, within three categories: i) non-muscle invasive, ii) muscle-invasive, and iii) metastatic.

1.2.4 Clinical management of bladder cancer

Follow-up in UBC. Follow-up of patients diagnosed with UBC is a crucial component of disease management, as UBC's main feature is its high recurrence rate. [84] Follow-up consists of cystoscopy and urine cytology every three months for two years, then every six months for the next two years, and then once yearly indefinitely.[93] Recurrence could be the result of incomplete tumor resection by TUR, or tumor cell re-implantation.[94]

1.2.4.1 Management of superficial NMI-UBC

1.2.4.1.1 Management of low-risk patients

TUR is the gold standard for initial diagnosis and treatment of superficial, NMI-UBC.[95-99] It is estimated that the 10-year disease-specific survival after TUR for low-grade tumors to be 85%.[100] With TUR, appropriate resection is important to avoid residual tumor, as a study reported that 70% of patients (n=47) had incomplete resection.[101] It is also recommended to repeat TUR, 2-6 weeks after the initial

procedure in cases of high-grade tumors, incomplete initial resection, or if no muscle tissue was initially sampled.[98]

Intravesical Chemotherapy. The International Bladder Cancer Group (IBCG) recommends for low-grade NMI-UBC an immediate single post-operative (post-TUR) chemotherapy instillation.[92] A meta-analysis has shown that immediate intravesical chemotherapy after TUR resulted in a 12% reduction in tumor recurrence.[102] However, the benefit of such intervention is limited to low-grade NMI-UBC.[103] Agents used for intravesical treatment are the immune-modulator Bacillus Calmette-Guerin (BCG) and interferon-alpha, and chemotherapeutic agents such as mitomycin C (MMC), doxorubicin, thiotepa and gemcitabine. Complications and side effects associated with intravesical chemotherapy include: irritative voiding symptoms, fever, arthritis, granulomatous prostatitis, BCG sepsis, myelo-suppression, skin rash and irritation of the gastro-intestinal tract (reviewed in [84]). Of note, MMC is the most effective post-operative agent, as BCG is not appropriate for immediate post-op administration.

1.2.4.1.2 Management of intermediate-risk patients

Intermediate-risk patients are at high-risk of recurrence, but low risk of disease progression. There is no consensus regarding the management of patients with superficial, intermediate-risk disease. Although most guidelines recommend adjuvant therapy of BCG or chemotherapy (post-TUR), these recommendations vary. The European Association of Urologists (EAU) outlines adjuvant BCG with maintenance for at least a year, or instillation of chemotherapy for 6-12 months as a treatment modality of intermediate risk disease.[98] The International Consultation on Urologic Disease (ICUD) on the other hand, recommends chemotherapy (less than 6 months) as a first line treatment, and BCG

as second-line therapy.[95] The National Cooperative Cancer Network (NCCN) suggests observation, or treatment with BCG or MMC,[99] whereas the American Urological Association (AUA) recommends BCG or MMC treatments.[97, 104]

1.2.4.1.3 Management of high-risk patients

High-risk patients tend to develop rapid recurrence of the disease after BCG therapy and are at an increased risk of progression to MI-UBC.[105] For those, BCG induction with maintenance after complete TUR is the current recommendation. When disease recurs (those with multiple high-grade tumors, or CIS), immediate radical cystectomy is offered.[98, 106] The First International Consultation on Bladder Tumors (FICBT) however, considers radical cystectomy to constitute over-treatment, and rather, recommends a 6-weeks induction course of BCG, with a one-to-three years maintenance.

1.2.4.2 Management of MI-UBC

Localized muscle-invasive bladder cancer invades the muscularis propria, without metastasis (T2, N0, M0).

Radical Cystectomy. Radical cystectomy is the standard of care for MI-UBC. For men, radical cysto-prostatectomy is performed, whereby both the bladder and the prostate are completely resected. In women, an anterior exenteration is performed, whereby the bladder, urethra, uterus and anterior ventral vaginal wall are resected.[84] In all cases of cystectomy, the removal of pelvic lymph nodes (pelvic lymphadenectomy) is also standard practice. Lymphadenectomy's limits are the genito-femoral nerve laterally, the bladder medially, the bifurcation of the common ileac artery cephalad and the endo-pelvic fascia caudal. Extended lymphadenectomy to the aortic bifurcation is also recommended,

as it not only provides more precise staging, but also potentially improves survival.[107-110] After cystectomy, urinary diversion is done.

Neoadjuvant Chemotherapy. Operable patients with MI-UBC can receive chemotherapy before the planned surgery.[91]

The rationale behind administering neoadjuvant chemotherapy relies on the fact that early intervention would benefit patients with micro-metastatic disease, and would lead the down-staging of the tumor pre-operatively.[84] Furthermore, tolerability of chemotherapy is expected to be better before cystectomy rather than after it.[91] However, neoadjuvant chemotherapy has disadvantages, including risk of overtreatment, and a delay in cystectomy for patients who do not respond to chemotherapy.[111-113] Neoadjuvant chemotherapy is associated with an absolute 5-year survival benefit of 5% in patients with muscle-invasive, clinically node-negative and non-metastatic disease.[114, 115] Generally, chemotherapy alone is not recommended as the primary treatment for MI-UBC.

Pre-operative radiation therapy (RT). Pre-operative RT is administered to patients prior to surgery, and was shown to potentially down-stage the cancer in 40-65% of patients, and improve local control in 10-42% of patients (reviewed in [91]). However, the studies have several limitations,[116, 117] rendering pre-operative RT not recommended for operable MI-UBC.

Adjuvant chemotherapy. Adjuvant chemotherapy is administered post-operatively. The availability of the pathological staging constitutes an advantage to adjuvant chemotherapy, as better accuracy in patient selection is achieved and less risk of

overtreatment.[84] The role and benefits of adjuvant chemotherapy remain uncertain, as insufficient evidence exists to support the use of chemotherapy post-surgery in increasing survival and/or preventing progression.[84, 91, 116]

Definitive RT. RT, instead of cystectomy, is a bladder-sparing procedure carried *via* external beam radiation. The Radiation Therapy Oncology Group (RTOG) has investigated the use of RT in the treatment of localized MI-UBC, along with the use of paclitaxel or cisplatin for radio-sensitization.[118-120] The study reported an 81% complete response rate, and a 5-year overall and disease-specific survival rate of 56% and 71%, respectively.[120, 121] A good candidate for RT has a solitary tumor that is less than 6 cm with no extensive CIS, and an adequate renal function. The target dose is 60-66 Gy, while the target field comprises the bladder only.[92] Recent studies reported an overall 5-year survival for RT, of 30-60%.[122-124] Others have suggested daily fractions of radiations to the bladder and pelvic lymph nodes of 40 Gy, with a boost to the bladder tumor to a total of 64 Gy. Furthermore, concurrent chemotherapy is recommended with either one of two established radio-sensitizers: cisplatin or paclitaxel. More recently, the combination of MMC plus 5-fluorouracil was shown to be an effective radiosensitizer in bladder cancer.[84, 125, 126]

1.2.4.3 Management of metastatic UBC

Metastasis is the ultimate cause of death from UBC.[41] The initial metastatic spread is to the pelvic lymph nodes, and later to other organs *via* lymphatic and hematogenous routes: lungs, bones, liver and brain.[43] With metastatic UBC, prognosis is poor and cure is rarely achieved, with a median survival of 12 months.[78]

Metastatic UBC cells that are chemo-sensitive, cisplatin remains the most effective combined agent.[84, 91, 120] The main prognostic factor is the suitability of patients for treatment with cisplatin-based combination chemotherapy. Several factors would prevent some patients from receiving cisplatin-based chemotherapy, and these include: age, chronic upper-tract inflammation, obstructing upper tract disease, general poor health status and poor renal function.[91]

For fit patients, first-line treatment is cisplatin-containing combination chemotherapy with either gemcitabine, or methotrexate, vinblastine, and adriamycin (M-VAC).[91, 109, 111, 127-129] If patients progress through platinum-based combination therapy, palliative care is then offered, with vinflunine as one of the options.[130] For patients who develop bone metastasis, bisphosphonate (zoledronic acid) treatment may be administered, along with vitamin D and calcium, as a palliative measure.[130-132]

For unfit patients, ineligible for cisplatin-combination treatment, carboplatin-containing chemotherapy is administered in combination with gemcitabine, or methotrexate, vinblastine, and adriamycin (M-VAC).[91]

1.3 Radiation and radio-resistance

Ionizing radiation (IR) refers to either particulate or electromagnetic (EM) radiation that, with sufficient energy, is able to eject one or more orbital electrons from the atoms/molecules, causing the atom to be ionized.[133] Whether of natural source or man-made, the biological effects of irradiation are the late effect of a series of events triggered by the passage of radiation through the medium.

1.3.1 Physiochemical basics of radiation

Ionization refers to the displacement of an orbital electron, an electron on the outermost shell of an atom, when the radiation is of sufficient energy. The key characteristic of ionizing energy is the large amount of energy it releases locally. Excitation on the other hand involves the transfer of energy between radiation and the atom without actual ejection of an electron. Most clinical applications involve either x-rays or gamma (γ) rays, which are two forms of EM radiation. While x-rays are produced by the acceleration of electrons to hit a tungsten target (the Bremsstrahlung effect), γ-rays are produced spontaneously by radioactive isotopes, as a result of an excess energy given off when an unstable nucleus breaks up and decays to a more stable form.[134] The dose of radiation to biological material is defined in terms of the amount of energy absorbed per unit mass. One Gy is equivalent to 1 J/kg.

Ionizing radiation represents the extreme end of the EM spectrum and is characterized by short wavelength and high frequency, making it extremely high in energy. The energy is computed as the frequency multiplied by a constant known as Plank's constant. For radiation energy to be ionizing, and thus displace an electron, it has

to carry enough energy to overcome the binding energies of electrons. In a biological system, the typical binding energy for electrons is around 10 electron-Volt (eV).

Other forms of radiation, particulate radiations, can also occur naturally or experimentally, and these include electrons, protons, α-particles, neutrons, negative π-mesons, and heavy charged ions.[133] Of these particulate radiations, electrons are the one of most clinical relevance; small and negatively charged, they can be accelerated to high energy particles and are used in the treatment of cancer.[135]

Direct/Indirect action of radiation. Radiation interacts with target structures either directly or indirectly to cause ionization. Particles with high Linear Energy Transfer (LET; to be described later) such as neutrons and α-particles act dominantly through the direct pathway that initiates the chain of events, leading to biological changes. Radiation can also interact with the target indirectly, *via* the production of free-radicals. The latter confer even a wider effect of radiation as they are able to diffuse over distances sufficient enough to interact with and damage critical cellular targets.[136] Free radicals are produced as a result of the radiolysis of water; most of the energy deposited in cells is absorbed in water, leading to the rapid production of reactive radical intermediates that in turn can react with other molecules in the cell. The most important products of the radiolysis of water are hydroxide (·OH) and hydrogen (·H) radicals. It is estimated that around two-third of biological damages induced by ionizing radiation are due to indirect action, with ·OH radicals being the most damaging.[137] From a clinical perspective, X-rays and γ-rays are indirectly ionizing.

Linear Energy Transfer (LET). LET is an important tool to describe the quality of different types of radiation; as a particle moves through matter, it loses energy towards its surrounding, along the path of the particle. LET is the average energy lost by a particle over a given track length, and is measured in terms of energy lost per unit path length (keV/μm). Thus, the higher the energy of a certain type of particle/ray, the lower the LET, as less energy will be deposited in the surrounding over the same track length. Conversely, as LET increases, more energy will affect the surrounding; in cancer, higher LET radiation causes more cell kill per Gy, and thus the Relative Biological Effectiveness (RBE) of the radiation also increases. RBE does not only depend on the radiation ray itself, but also varies according to the tissue.[138]

1.3.2 Radiation-induced DNA damage

Cellular deoxyribonucleic acid (DNA) is the main target of ionizing radiation. Cellular DNA can be damaged in several ways: i) *via* direct ionization of the DNA, ii) *via* reaction with electrons/solvated electrons in the milieu, iii) *via* reactions of ·OH and last iv) *via* reactions with other radicals.[139] The chemical mechanism of DNA damage includes the addition of ·OH radicals to unsaturated bonds of the DNA bases, abstracting hydrogen atoms from all sites on the deoxyribose sugar moiety of DNA, and subsequent reactions with oxygen and other radicals. As stated earlier, 60-70% of the cellular DNA damage caused by ionizing radiation is caused by hydroxyl ·OH radicals.[140]

1.3.2.1 Types of DNA damage

Ionizing radiation produces a wide array of damages to cellular DNA. Simple damages to DNA include base damages and Single-Strand Breaks (SSB), at rates of 2500 damaged bases/cell/Gy and 1000 SSB/cell/Gy, respectively.[141] Extensive *in vitro*

studies have identified more than 100 radiation-induced base damages including to purine and pyrimidine rings and sites of base loss. For pyrimidines and purines, the C5-C6 double bond is the major site of ·OH radical attack. Base loss, due to destabilization of the glycosyl bond linking the base to the sugar moiety in the DNA results in apurinic and/or apyrimidinic (AP) sites. SSB DNA breaks result mainly from damages to the deoxyribose sugar moiety of the DNA (reviewed in [133]). These damage sites are readily repaired post-radiation *via* several mechanisms that will be examined later.

More significant radiation produces more complex damages such as Double Strand Breaks (DSBs) and other Multiple Damage Sites (MDS), and these are more challenging for the cell to repair. MDS refer to localized damages by high LET radiation that involves one or more DSBs, several SSBs, and as well as clustered base damages.[142] Unlike other types of DNA damages, these lesions may fail to repair and can be potentially lethal. More recently, it was shown that ionizing radiation can also cause DNA-protein cross-links (DPC), even at a clinically significant dose range of 1-4 Gy. Such cross-linking of nuclear proteins does not cause DNA breaks, but rather impedes DNA processing mechanisms such as replication, transcription and repair. [143]

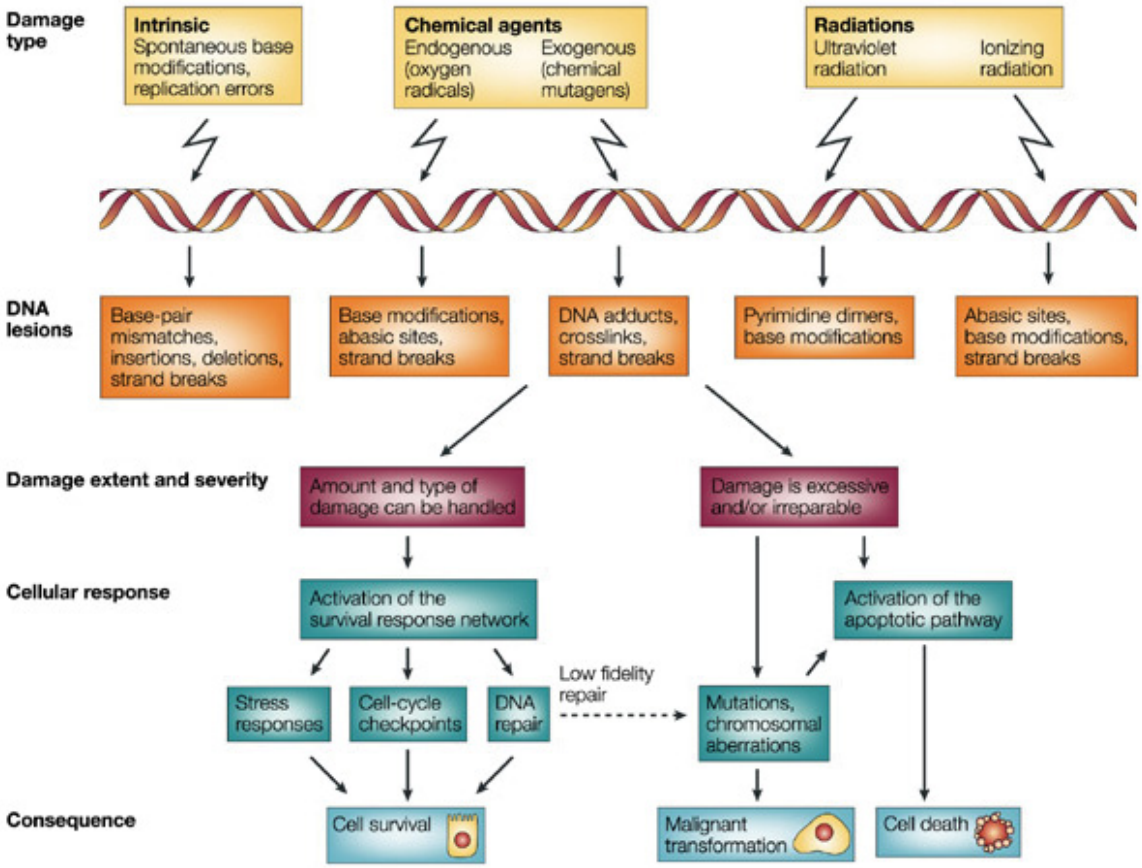

Figure B: Different types of DNA damages [144]

1.3.2.2 Repair mechanisms of radiation-induced DNA damages

Overview of DNA repair mechanisms. Different DNA repair mechanisms keep the human genome under constant surveillance, and allow the removal or tolerance of pre-cytotoxic and pre-mutagenic lesions, often in an error-free or sometimes error-prone way. More than a 130 DNA repair genes have been documented, and these encode a wide array of enzymes specialized in repairing damages to DNA.[145, 146] These different DNA-repair genes are often categorized into genes associated with signaling and regulation of

DNA repair on the one hand, and genes associated with distinct repair mechanisms such as Base Excision Repair (BER), Nucleotide Excision Repair (NER), DSB repair, and Mismatch Repair (MMR), on the other hand.

In eukaryotic cells, seven mechanisms allow the restoration of the structural DNA integrity (reviewed in [133] and [147]). Direct repair of defects of O^6-alkylguanine, O^4-alkylthymine and alkyl-phospho-triesters in DNA involves several protein activities that are able to recognize very specific modified bases, typically those methylated, and transfers the modifying group from the DNA to themselves. NER works on bulky lesions such as base dimers and chemically induced intra-strand crosslinks. It acts *via* endonucleolytic cleavage near the dimer, followed by a polymerase with an exonucleolytic activity that cuts out the thymine dimer while simultaneously synthesizing an appropriate matching strand. BER removes aberrant bases that could result from the deamination of normal bases, and plays an important part in the defense against radiation-induced damages. MMR refers to a multi-enzyme system that recognizes inappropriately matched bases in DNA and replaces one of the two bases with a matching one. Homologous Recombination (HR) is the principal method for repair of radiation-induced DSBs in lower eukaryotes, and acts by strand exchange from the other daughter chromosome, making the process largely error-free. In mammalian cells, the main mechanism of DNA DSB repair is Non-Homologous End-Joining (NHEJ*)*. Poly (ADP-Ribosyl) Polymerase-1 (PARP-1) activity is specialized in the SSB repair mechanism.

Of note, not all these mechanisms are involved in the repair of radiation-induced DNA damages.

1.3.2.2.1 Repair of radiation-induced DNA Damages

Among the seven mechanisms listed above, only three specific pathways are involved in repair of radiation damages: BER, HR and NHEJ each acting on specific types of lesions. HR and NHEJ are the main pathways of DNA DSB repair.

During BER, damaged DNA bases are cut-out from the genome and replaced by the normal nucleotide sequence. BER acts mainly on base damages, but also repairs AP sites and SSBs. Briefly, BER is initiated when the N-glycosyl bond on the damaged purine or pyrimidine is cleaved by a DNA glycosylase. This is followed by the generation of a nucleotide gap in the DNA complex *via* the activity of a 5'AP endonuclease and a DNA deoxyribophosphodiesterase. The gap is then filled by DNA Polymerase β (Polβ) that uses the opposite strand as the template for gap-filling synthesis. Ligation is last performed by the DNA ligases I and III.[148] PARP-1 belongs to a family that consists of 18 others members; it plays an important role in DNA repair, mainly in BER. PARPs are chromatin-associated enzymes that modify several proteins by poly-(ADP-ribosylation). PARP-1 is considered the master switch between apoptosis and necrosis and is involved in DNA repair *via* three possible mechanisms. First, it interacts with Polβ during BER, allowing DNA strand-break resealing. Second, PARP-1 remodels the chromatin structure of DNA upon detection of DNA damages, giving DNA repair enzymes access to the site of DNA damages. Last, PARP-1 interferes with several proteins involved in DNA repair *via* a conserved sequence motif. The protein list includes: p53, WAF1, XPA, and MSH6.[149]

DNA DSBs are a common form of DNA damages that result from ionizing radiation. DSBs are highly potent inducers of genotoxic events such as chromosome

breaks and chromosomal exchanges, and of cell death. As mentioned earlier, the two main pathways of DSB repair are HR and NHEJ. While HR is considered to be error-free, NHEJ is the main pathway in mammalian cells and is error-prone.[150]

HR repairs a DSB by using the undamaged sister chromatid as a template resulting in an accurate repair of the DSB. The process is mediated through the RAD52 group of proteins that includes RAD50, RAD51, RAD52, RAD54 and Meiotic Recombination 11 (MRE11). HR is initiated by a nucleolytic resection of the DSB by the MRE11-RAD50-NSB1 complex. The resulting 3' single-stranded DNA is protected against exonucleolytic digestion by binding to other RAD52 proteins. RAD52 and RAD51 interact together with Replication Protein A (RPA) to stimulate the DNA strand-exchange activity of RAD51, with the complementary DNA strand. After strand exchange, repair is completed by DNA polymerases, ligases and Holliday junction resolvases. Of note, BRCA2 also plays a role in HR mechanisms through complexing with RAD51 to assemble DSB repair proteins required to remove breaks that accumulate during DNA replication. In fact, it was reported that BRCA2-deficient cells have 10-fold lower levels of HR compared to normal cells. Although mammalian cells do not rely much on HR, they do employ the mechanism in late S and G phases of the cell cycle for mitotic recombination if an undamaged sister chromatid is available.[150, 151]

Unlike HR that uses a DNA template for DSB repair, NHEJ ligates the two ends of a DSB without the requirement of sequence homology between the DNA ends. NHEJ starts by the binding of a large complex, XRCC5 (consisting of the proteins Ku70 and Ku80), to the damaged DNA, conferring protection against exonuclease digestion. Following binding, XRCC5 associates with the DNA-activated Protein Kinase (DNA-PK)

to form an active DNA-PK_{cs} holoenzyme. The latter will be activated *via* interaction with a single-strand DNA at the site of DSB. The active enzyme will later bind to Artemis, a protein involved in processing over-hangs during NHEJ, thus degrading single-strand overhangs and hairpins once ligation is terminated. Ligation of the two ends is assured by the XRCC4-ligase IV complex that binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but non-ligatable ends.[152]

DNA repair and cellular sensitivity to radiation. In the scope of radiation therapy, the ultimate goal of ionizing radiation is to cause the death of cancer cells. There is no clear relationship between the expression levels of different DNA repair genes and the relative radio-sensitivity among cells. However, several models act in support to targeting DNA repair genes in order to increase cell radio-sensitivity. For example, cells from patients with ataxia telangiectasia, a DNA repair deficiency syndrome, exhibit an extreme radio-sensitivity.[153] Furthermore, cells defective in the expression of the BRCA1 and BRCA2 proteins have decreased HR-related repair of DSBs, and thus a decreased radiation cell survival.[154] Thus, several strategies have been designed to radio-sensitize human cancer cells by targeting the DSB repair mechanism.

Table 1: Summary of major DNA repair mechanisms

DNA Repair Mechanism	Description
Direct DNA defects repair	- Involves repair of: O^6-alkylguanine, O^4-alkylthymine and alkyl-phospho-triesters. - Repair proteins recognize very specific modified bases and transfer the modifying group from the DNA to themselves.
Nucleotide Excision Repair (NER)	- Works on bulky lesions: base dimers and chemically induced intra-strand crosslinks. - Acts *via* endonucleolytic cleavage near the dimer, followed by a polymerase with an exonucleolytic activity to cut out the thymine dimer while simultaneously synthesizing an appropriate matching strand.
Base Excision Repair (BER)	- Removes aberrant bases resulting from the deamination of normal bases.
Mismatch Repair (MMR)	- Multi-enzyme system. - Recognizes inappropriately matched bases in DNA and replaces one of the two bases with a matching one.
Homologous Recombination (HR)	- Main method for repair of radiation-induced DSBs in lower eukaryotes. - Acts by strand exchange from the other daughter chromosome. - Processes largely error-free.
Non-Homologous End Join (NHEJ)	- Main mechanism of DSB repair in humans. - Ligates the two ends of a DSB without the requirement of sequence homology between the DNA ends.
Poly (ADP-Ribosyl) Polymerase-1 (PARP-1)	- Is a chromatin-associated enzyme. - Activity is specialized in the SSB repair mechanism.

1.3.3 The cellular response to radiation-induced DNA damages

Once DNA has been damaged by radiation, the cell responds by initiating different DNA-damage-response pathways that lead to repair, growth arrest or cell death if repair is not possible.

1.3.3.1 DNA-damage response pathways

The first step in cellular response to DNA damages induced by radiation is the activation of DNA-damage response pathways that transduce a signal from a damage sensor, often a DNA-binding protein, and triggers the activation of a signaling cascade that targets a series of downstream effectors of the DNA-damage response.[155] Specific proteins are known to bind preferentially to certain types of DNA lesions; for example, mismatched bases are bound by the MSH2/3/6 proteins, while the Ku dimer binds to DSBs and the Xeroderma Pigmentosum group C (XPC) protein and selectively recognizes UV-induced damages. These involved signaling proteins are very sensitive and have the capacity to largely amplify the initial stimulus.

In mammalian cells, the ATR/ATM network is the main radiation-induced DNA damage signaling pathway. The ATM protein kinase was initially characterized in patients with the ataxia telangiectasia syndrome. ATM was mutated in these highly radiosensitive patients reflecting the importance of ATM in the cellular response to DSBs. ATM mediates the response to damages by phosphorylating several substrates, and is necessary for the immediate, rapid response to repair damages. In un-damaged cells, ATM is thought to be sequestered as an oligomer, a dimer of two ATM molecules; in this oligomer, ATMs are contained and inactive, unable to phosphorylate their substrates. Once damages occur, the kinase domain of one ATM molecule phosphorylates a

neighboring ATM, which disrupts the ATM oligomers. Phosphorylated ATM dissociates from the complex and is free to phosphorylate substrates in the cell.[156]

The role of ATM is to phosphorylate and potentially activate Artemis that plays an important role in NHEJ, as mentioned earlier. ATM also targets the MRN complex formed by nibrin and Mre-11. The complex is phosphorylated by ATM in response to ionizing radiation, allowing nibrin to translocate the MRN complex to the nucleus, and to re-localize it to the sites of DSBs following irradiation.[144]

ATM also controls the tumor suppressor gene *p53*, which encodes the transcription factor p53, described as the "guardian of the genome" due to its important role in eliminating cells with damaged DNA.[157] Under normal conditions, p53 is constantly regulated by the Murine double minute-2 protein (Mdm2) that keeps p53 under tight control by blocking its transcriptional activity, and thus its ability to induce growth arrest and apoptosis. As a result, the p53 protein has a very short half-life under normal conditions. Of note, Mdm2 itself is a direct partner of p53, and together, they form an auto-regulatory feedback loop. After radiation-induced DNA damage, p53 is stabilized *via* different mechanisms. By-passing the Mdm2-p53 is one major mechanism whereby Mdm2 expression is down-regulated, the Mdm2-p53 interaction is prevented and the Mdm2-mediated degradation of p53 is inhibited.[158] For example, the $p14^{ARF}$ stabilizes p53 by binding and antagonizing Mdm2. Following radiation, $p14^{ARF}$ is over-expressed and will activate p53-dependent cell-cycle arrest. Another mechanism of p53 stabilization is the hetero-tetramerization of p53 that masks the nuclear export signal of p53 and retains it in the nucleus, away from cytoplasmic degradation. Last, in response to stress

signal caused by radiation, p53 is subjected to post-translational modifications such as phosphorylation and acetylation, which modulate its stability.[159]

1.3.3.2 Radiation-induced growth arrest

Following radiation, the dividing cell responds by delaying/arresting its growth cycle. Radiation-induced delays in the G1-S and G2 phases of the cell cycle have been extensively described. The p53 protein plays a role in the radiation-induced G1 delay, and such delays at cell-cycle checkpoints are believed to prevent the replication of damaged DNA. These delays also allow periods of time during which cells could survey and repair DNA damages.[160] The cell cycle regulation and its response to radiation-induced DNA damages will be elaborated in more details *vide infra*.

1.3.4 Modulation of cellular response to radiation *via* signal transduction pathways

Ionizing radiation initiates signaling through different transduction pathways *via* the activation of specific trans-membrane receptors.

The epidermal growth factor receptor (EGFR) is the main receptor activated in response to irradiation, in several carcinoma cell lines. In fact, it was shown that a radiation exposure even in the range of 1-2 Gy can activate the pathway downstream of the EGFR in the absence of its ligand EGF.[161] The activation of trans-membrane receptors was shown to occur *via* the metabolic generation of reactive oxygen species (ROS) that result from ionizing radiation.[162] In addition to the EGFR family of receptors, other growth factors and cytokine receptors also play a role in the cell response to radiation. For example, interleukin-6 (IL-6) is a cytokine that confers epithelial cells with the ability to survive and proliferate. It was shown that IL-6 can generate anti-

apoptotic signaling in cells in a protective fashion against the toxic effects of radiation.[163] Radiation-induced cell signaling can proceed by several routes, including the Phosphoinositide-3 Kinase (PI3K) pathway that will be described later.

Understanding the pathways involved in response to radiation-induced stress could prove beneficial to radio-sensitizing cancer cells during radiation therapy. For example, anti-apoptotic signaling from the EGFR can be blocked by use of inhibitory antibodies such as AG1478.[164] Small molecule inhibitors of the tyrosine kinase domain of EGFR have also been used to block tumor cell survival and growth. The Farnestyl-Transferase Inhibitors (FTIs) block the processing of the Ras protein, downstream of the EGFR pathway, resulting in radio-sensitization. In fact, treating cells with FTIs prior to radiation was shown to provide a synergistic effect on radiation-induced cell killing in human cancer cell lines.[165-167]

1.3.5 Radiation-induced apoptosis

Apoptosis is a cellular programmed sequence of reactions, triggered by internal or external stimuli, eventually leading to cell death. Apoptosis occurs mainly *via* caspases, which are cysteine proteases that directly cause cell death, and are regulated at the translational level.[168]

Radiation induces apoptosis in several ways: i) the intrinsic apoptotic pathway is activated in response to internal signals such as DNA damages, and is mediated *via* the p53 protein that eventually lead to the permeabilization of the outer mitochondrial membrane, and the influx of Bcl-2 family of proteins; ii) the external apoptotic pathway is activated by external stimuli *via* the activation of cell surface receptors known as the

Death Receptors (DRs). Binding of ligands such as Tumor-Necrosis Factor α (TNF-α) to their receptors induces the formation of the Death-Induced Signaling Complex (DISC) that recruits caspase-8 and promotes the activation of the caspase cascade; iii) the interaction of the plasma membrane with ROS to trigger the initiation of extrinsic signaling mediated by ceramide. Ceramide is generated by hydrolysis of sphingomyelin *via* the activation of Sphingo-Myelinase (SMase). Signaling through ceramide eventually leads to apoptosis.[169, 170]

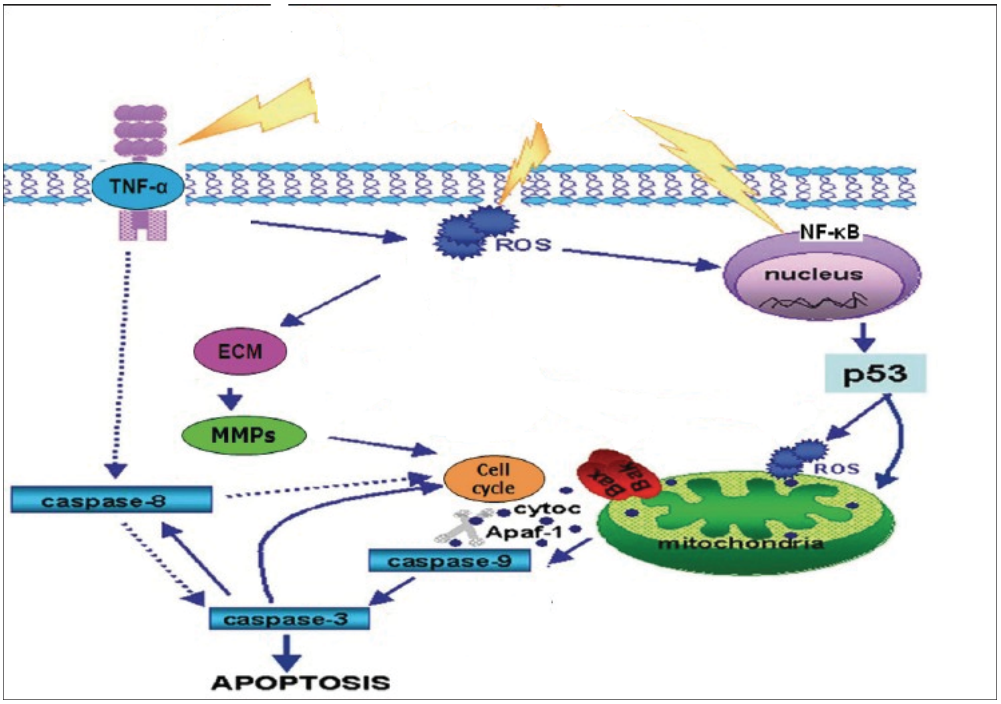

Figure C: Radiation-induced apoptosis [171]

In human cancer, the contribution of apoptosis towards radiation-induced cell death mainly depends on the tissue type. Thymocytes, lymphocytes and cells from the hematopoietic and germinal lineages are usually apoptosis sensitive. For solid tumors,

apoptosis may not be the primary reason for cell death. In these tumors, cell death is the result of mitotic catastrophe, irreversible cell-cycle arrest, and more recently autophagy has been documented as cell-death process in response to radiation. Controversy also exists on whether autophagy may not be a cell-survival process. (Reviewed in [133])

1.3.6 Radiobiology of solid tumors

Radiation and chemotherapy aim at killing cells, but generally do not have enough sensitivity to target tumor cells, leading to increased cytotoxicity and rendering the treatment options less favourable. For decades, scientists have studies the tumor microenvironement looking for ways to differentiate the tumor from the surrounding normal cell in the context of treatment. Differences in the tumor microenvironement were identified when compared to normal cells and they include: elevated interstitial fluid pressure, hypoxia (low oxygen pressure) and low extracellular pH. It has been extensively demonstrated that the presence of low oxygen foci in solid tumors has direct negative impact on treatment outcomes and this is mainly due to the disrupted tumor vasculature.[133, 172] These findings have focused the attention toward dealing with tumor hypoxia in the context of disease management.

As stated previously, many of the currently used chemotherapeutic drugs and radiation require a proper flow of oxygen for maximal effects. Studies have shown that decreased levels of oxygen can have a direct negative effect on chemotherapeutic drug and radiation efficiencies due to decreased cellular division and cycle arrests caused by low oxygen levels. Tumor vasculature, being the driving force behind low oxygen levels, differs from normal one by having an incomplete endothelial lining, lack of pericytes and

lack of an intact basement membrane.[133] This disorganization in tumor vasculature inhibits the ability to deliver nutrients and to remove waste products.

To overcome this, VEGF therapy in combination to chemotherapy or radiation was used to improve the much-needed reoxygenation in tumors.[173, 174] Other strategies for improving oxygen tension include decreasing the oxygen consumption of the cell [175] and inhibiting the Ras signaling pathway whose activation was shown to increase hypoxia in cells.[176] The success of these strategies highlighted the radiosensitizing role of oxygen, and provided tools to predict response to treatment by identifying the extent of hypoxia in the tumors prior to treatment.

1.4 The eukaryotic cell cycle

The cell cycle is an essential mechanism by which cells reproduce, and is required for a proper functioning of the organism, in order to replace dead cells by new ones. It is thus no surprise that cell cycle is impaired in cancer cells, conferring those cells with the continuous ability to proliferate.

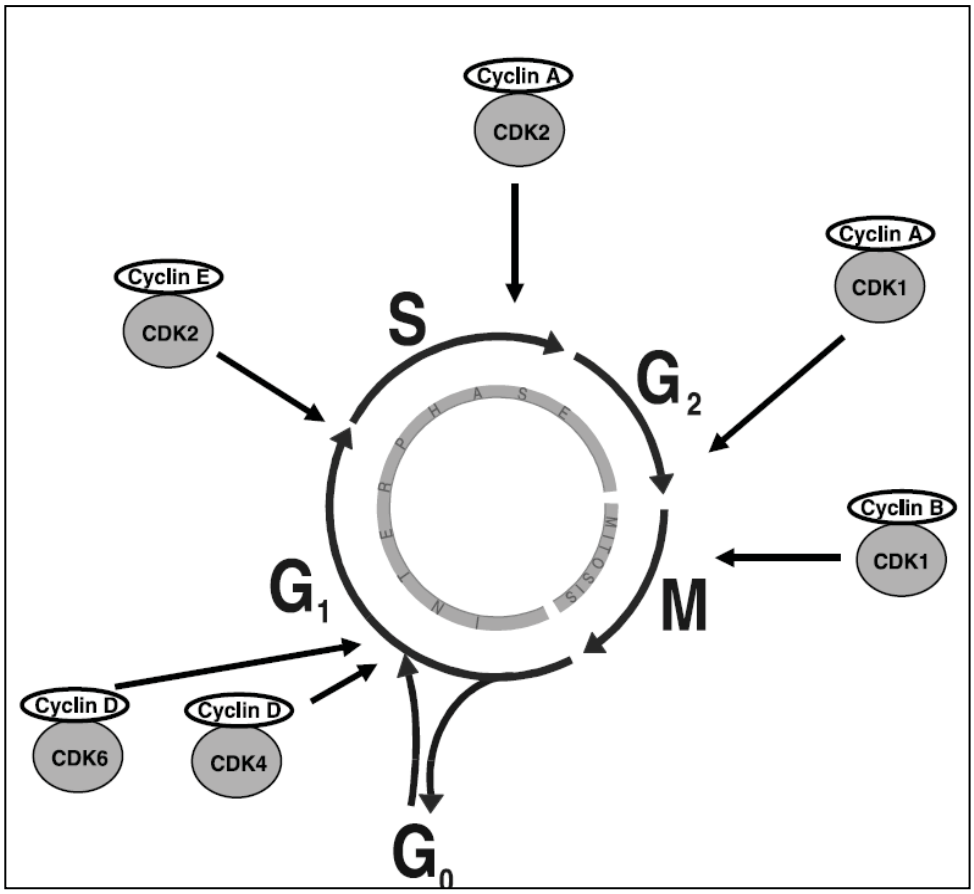

Figure D: The stages of the cell cycle [177]

1.4.1 Overview of the eukaryotic cell cycle

1.4.1.1 The different phases of the cell cycle

1.4.1.1.1 Cycling cells

The eukaryotic cell cycle comprises of four phases that present in the following sequence: Gap 1 (G1) → Synthesis (S-phase) → Gap 2 (G2) → Mitosis (M-phase). During the S-phase, chromosome duplication occurs *via* DNA synthesis, and takes 10-12 hrs, which occupy half of the cell cycle in rapidly dividing mammalian cells. The M-phase (1-2 hrs) is when mitosis *per se* takes place, a process by which nuclear and cytoplasmic divisions occur to generate two daughter cells following DNA replication. The G1 phase constitutes a phase between mitosis and the S-phase, while G2 separates S-phase from mitosis. The two gap phases provide the cell with the opportunity to sense internal and external stimuli that reflect suitable conditions for the cell, prior to the S-phase and mitosis. To divide, G1 is specifically very important to assess the suitability of external/internal stimuli prior to DNA replication in the S-phase. If the conditions are favorable, on the other hand, the cell enters a commitment point known as the restriction point, where it commits to DNA replication. (Reviewed in [178])

1.4.1.1.2 Non-cycling cells

If the conditions indicated in the prior section are not favorable, the cell enters a resting state known as G0, where it remains quiescent before resuming proliferation.

1.4.1.2 The cell cycle control system

The cell cycle is tightly controlled by a set of key proteins, cyclins, together with kinases, activators and inhibitors, that form the control system that either activates or

inhibits cell cycle progression as described below. The cell cycle operates like a timer that triggers the events of the cycle in a set of sequences; the basis of this control system is a connected series of biochemical switches that act in a binary fashion (on/off), and launch the events in a complete and irreversible fashion. In order to ensure proper functioning of the cell cycle resulting in division, the control system is highly reliable *via* different backup mechanisms. Adaptability is also critical for a balanced response to different intracellular and extracellular signals that inhibit or activate the cycle.[179]

In eukaryotes, the cell has to pass three major regulatory transitions, known as checkpoints, in order to complete a full cycle; i) the start checkpoint (restriction point) occurs in late G1, when the cell commits to go through the cell cycle and to duplicate its DNA at the S-phase. At this checkpoint, the control system triggers the entrance into the S-phase only if environmental conditions are favorable; ii) the G2-M checkpoint triggers the entry of the cell into mitosis. The cell would pass the checkpoint if favorable cues are detected, from both the environment, and internally, in reference to proper DNA replication; iii) Within the M phase, the metaphase-to-anaphase transition triggers the start of anaphase, a step of mitosis where sister-chromatids separate. The critical event of this transition is the proper attachment of chromosomes to the mitotic spindle during metaphase.[178, 180]

1.4.2 Cyclin-dependent kinases, cyclins, and major regulatory proteins of the cell-cycle control system

1.4.2.1 Cyclin-dependent kinases and cyclins

Cyclin-dependent kinases (Cdks) are a family of protein kinases that are central to the cell-cycle control system. It is the rise and fall in activities of Cdks that lead to

cyclical changes in the phosphorylation of intracellular proteins that regulate major events in the cell cycle. For example, an increase in Cdk activity at the G2/M checkpoint increases the phosphorylation of proteins that control events occurring at the onset of mitosis (such as chromosome condensation).[181]

As their name indicates, Cdks are dependent on a family of proteins known as cyclins: for Cdks to exhibit kinase activity, they need to tightly bind cyclins. While the latter undergo constant cycles of synthesis and degradation, depending on the stage of the cell cycle, Cdks levels are constant, and the cell cycle progression results from the cyclic assembly/activation of the cyclin-Cdk complexes.[182]

In terms of Cdks, there are four types in vertebrates: Cdk1, Cdk2, Cdk4 and Cdk6. Cdk1 interacts with cyclins A and B, Cdk2 with cyclins A and E, and Cdk4/6 with cyclin D. Of note, there are three D (D1, D2 and D3) cyclins in mammals.[182, 183]

Four classes of cyclins A, B, D and E act at different stages of the cell cycle: i) the G1/S-phase cyclins act in late G1 and help trigger progression through the start checkpoint, and activate Cdks resulting in the cell commitment into the cycle. In humans, cyclin E is the G1/S actin cyclin, and its levels decrease in the S-phase; ii) S-phase cyclins help stimulate DNA duplication soon after the start checkpoint. Cyclin A levels remain elevated until mitosis and further contribute to the control of some early mitotic events; iii) M-phase cyclin stimulates Cdks for entry into mitosis, at the G2/M checkpoint. Cyclin B is later destroyed in mid-mitosis; iv) G1-phase cyclin, or the cyclin D in humans, acts as support to the G1/S cyclins.[183]

Different cyclin-Cdk complexes are able to trigger different but specific cell-cycle events, and this is due to the dual ability of the cyclins to activate a proper Cdk partner, along with directing it to specific targets. As a consequence, each cyclin-Cdk complex will phosphorylate a different set of substrate proteins. The activity of the Cdks is further controlled at a different level, by other cell-cycle regulatory proteins.

1.4.2.2 Major cell-cycle regulatory proteins

The Cdk activity during the cell cycle is primarily determined by the rise and fall of cyclin levels. The level of cyclin is fine-tuned by a wide array of cell-cycle regulatory proteins. These proteins can be classified into three broad categories, based on their effect on Cdks activity: Cdk activators, Cdk inhibitors (CKI), and last ubiquitin ligases.[177]

Cdk activators. Cdk activators contribute to the full activation of Cdks *via* activating phosphorylation, the phosphorylation of the inhibitory sites on Cdks, and finally *via* phosphatase activity that further removes inhibitory phosphates from Cdks. To this class, belongs the Cdk-activating kinase that phosphorylates Cdks at threonine (Thr) 161 residue, thus allowing full activation of Cdk following cyclin binding.[184] Wee1 is a kinase that phosphorylates Thr located at the amino acid position 14 and tyrosine (tyr) 15, two inhibitory sites in Cdks, mainly involved in suppressing Cdk1 activity before mitosis.[185]

Cdk inhibitors. CKIs confer inhibitory regulation to the cyclin-Cdk complexes. The main mechanism of CKIs is the stimulation of rearrangements in the structure of the Cdk active site, rendering it inactive.[186] For example, p27 is a CKI that suppresses G1/S Cdk and S-Cdks activities in G1, helping cells to leave the cycle when they

terminally differentiate and go into quiescence. The protein p21 is of high importance in cancer, and in radiation-induced DNA damages more specifically. It acts by suppressing the G1/S-Cdk and S-Cdk activities following DNA damages; p21 is of major interest to the current work, and will be discussed in greater details later in the thesis.

Ubiquitin ligases. Ubiquitin ligases play a major role during M, in the metaphase-to-anaphase transition. At this checkpoint, progression is not triggered by protein phosphorylation, but rather by proteolysis, i.e. protein destruction. The Anaphase-Promoting Complex/Cyclosome (APC/C) is a key regulator of the metaphase-to-anaphase transition, and is a ubiquitin ligase that catalyzes ubiquitynation of proteins involved in exit from mitosis. It is regulated by association with activating subunits such as cdc20 and Cdh1.[187, 188]

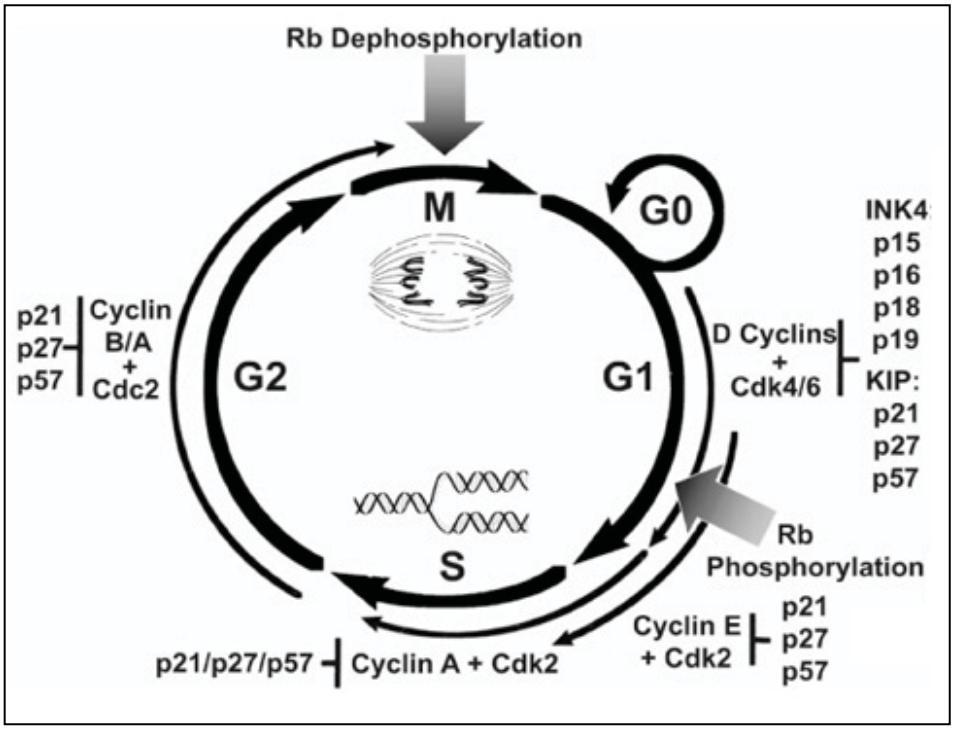

Figure E: The different cyclins, Cdks and inhibitors involved in cell cycle regulation [189]

1.4.3 Deregulation of the cell cycle in cancer

In cancer, alterations in the genetic control of cell division result in unrestrained cell proliferation. It is thus important first to describe the major targets of the different cyclin/Cdk complexes in order to better understand the hijacking of the system by cancer.

1.4.3.1 Major molecules involved in cell cycle regulation

The most frequently studied target of cyclin/Cdk complexes is the protein encoded by the retinoblastoma tumor suppressor gene (*pRb*). It is the substrate of cyclin D-Cdk4/6 complex. During G1, Rb is phosphorylated by the complex leading to the disruption of the Rb/E2F-1 complex and the release of the transcription factor E2F-1.[190, 191] Free E2F-1 positively regulates the transcription of different genes required for S-phase progression. The pRb remains hyper-phosphorylated *via* the action of cyclin E-Cdk2; this complex also phosphorylates p27, a cell cycle inhibitor and targets it to proteasomal degradation.[192] Besides p27, the complex phosphorylates the histone H1, leading to chromosome decondensation, an important step for DNA replication.[193]

During G1/S checkpoint, DNA damages are detected by ATM, which phosphorylates and activates p53 as *vide supra*. Active p53 then stimulates the transcription of different genes including p21 and Bax.[157, 194] The p21 will induce cell cycle arrest *via* Cdk inhibition, thus preventing the replication of damaged DNA; p53 induction of Bax induces cell death *via* the apoptotic pathway.[195]

During G2, cell cycle regulation seems to be independent of p53, and in case of DNA damages, the entry into mitosis is prevented by Cdk1 inhibition, *via* Chk1 and Chk2, two kinases activated following DNA damages.[177]

1.4.3.2 Cell cycle and cancer

The deregulation of the cell cycle that is associated with cancer occurs through mutations of proteins that act at different levels of the cycle. These genes can be proto-oncogenes that get activated by mutations or genomic amplifications, or tumor suppressor genes that get inactivated *via* inactivating mutations and/or deletions. For example, inactivation of tumor suppressor genes like pRb and p53 results in a loss of cell cycle inhibition, and thus continuous cell proliferation. Mutations have been reported in genes encoding Cdks, cyclins, CAK, CKI, Cdk substrates and checkpoint proteins as well.[196]

Cdks. Although infrequent, alterations of Cdk proteins have been reported in different cancers.[197] In sarcoma, glioma, and melanoma, Cdk4 is over-expressed *via* an amplification mechanism, thus allowing cells to pass the G1/S checkpoint, regardless of cue that might have instructed otherwise.[198] Cdk1 and Cdk2 are over-expressed in colon cancer, while mutations in Cdk4 and Cdk6 lead to the loss of CKI binding site on either of the two Cdks.[199-201]

Cyclins. Altered expression levels of cyclins have been more commonly reported than those affecting Cdks.[202] Both cyclins A and E are overexpressed in lung carcinoma, with the elevated expression of cyclin A alone, correlating with shorter survival.[203] Cyclin E is further overexpressed *via* genomic amplification in breast and colon cancer, as well as in acute lymphoblastic and myeloid leukemia.[204-206] High levels of cyclin B in breast cancer are independent prognostic markers of an aggressive disease.[207] Cyclin D has been the most extensively studied cyclin in cancer, given its crucial role in acting as a growth sensor. Aberrant levels of cyclin D1, which normally binds to Cdk4/6 in early G1, were found in parathyroid adenomas,[208] B-cell

malignancies (*via* chromosomal translocation of t(11; 14)),[209] breast, esophageal, bladder, lung, and squamous cell carcinomas (*via* gene amplification).(reviewed in [202])

Cdk inhibitors. The activity of CKI results in growth suppression through the activation of pRb. The p16 protein is a specific inhibitor of cyclin D-Cdk, preventing phosphorylation of the pRb protein, and thus arresting cells in G1. The *p16* gene is altered in different human tumors, and can be activated by a variety of mechanisms including deletions, point mutations and hypermethylations.[210] Deletions of *p16* exist in gliomas, mesotheliomas, nasopharyngeal, pancreatic tumors and acute lymphoblastic leukemias. (Reviewed by [202]). The *p19* gene resides besides the *p16* gene and acts independently of it in regulating p53. Large deletion of the *p19* gene results in mutated p19 and thus deregulation of p53.[211] Loss of p27 has been also reported for a number of human cancers. p21 is implicated in tumorigenesis through its regulation by p53, and a mutation in p53 causes a loss of p21 regulation in response to DNA damages.[212]

The importance of these markers has been specifically shown in UBC, especially for p53, p21 and pRb. In fact, an immunohistochemical (IHC) study performed on a large cohort of UBC patients (226 cystectomy samples, including 50 matched lymph node metastasis), has reported significant increase in p53 expression, a decrease/absence of p21, p27 and pRB. Furthermore, these alterations were shown to have prognostic, or biomarker value as they are independently associated with disease recurrence and bladder cancer-specific death in patients with organ-confined disease. The proportion of these biomarkers alterations (each alone, or in combination) was highest in lymph node metastases.[213]

1.4.3.3 The effect of radiation on cell cycle

As mentioned earlier, DNA is the critical target of ionizing radiation and its degradation is an early indication of cell death and/or cell cycle arrest. Earlier work on radiation effects on mammalian cells was done in HeLa cells using pulse labeling with tritium [^{3}H]- labeled thymidine to determine the rate of cell accumulation at different cell cycle checkpoints. It was shown that ionizing radiation affects the cell cycle progression differently, depending on the checkpoint.[214] Ionizing radiation degrades DNA *via* an enzymatic activity, first described in 1992 by Compton et.al,[215] to be an endonuclease dependent on Ca^{2+} and Mg^{2+}. This enzyme was later identified as PARP.[216, 217]

Table 2: Summary of major effects of ionizing radiation on cell cycle phases

Cell cycle phase	Effect
G1	Arrest
S	Delay
G2	Arrest
M	Delay

Ionizing radiation induces cell cycle arrest in G1, a delay in S-phase progression, and an arrest at G2 (summary in Table 2).[218] In G1 the arrest in response to irradiation results from signaling *via* p53. Once damage is detected, the level of p53 rises one to two hrs following irradiation, and remains high for up to 72 hrs.[219] The rise in p53 is associated with a G1 arrest in cells expressing wild type p53, *via* regulation of pRb, p21 and p27 as mentioned earlier. In cancer however, p53 is known to be mutated in most

types of tumors. In such cells, mutant p53 is unable to accumulate and activate cell cycle inhibitors. As a result, the cycle continues, leading to replication of damaged DNA; once DNA repair mechanism fails to fix damages, the cell ultimately die.[157]

Radiation-induced DNA damages cause a delay in progression through the S-phase, which is explained by a slowing of the DNA synthesis rate. The response of the DNA synthesis to the different doses of ionizing radiation is biphasic as cells exhibit a different behavior at low *vs.* higher doses of radiation.[218] At low dose, a radiosensitive component of the delay consists of a decrease in the replicon initiation,[220] while the radio-resistant component, at higher doses, is due to a reduction in the rate of DNA chain elongation.[221]

Unlike the G1 arrest, the G2 arrest and the delayed progression through the M-phase in response to radiation, is not dependent on p53, but rather on other oncogenes.[218] In fact, studies on rat embryo fibroblasts transfected by H-ras and c-myc oncogenes showed that the resulting cells exhibited longer G2 arrest at 1.9 Gy, compared to the parental cells.[222] More interestingly, the prolonged G2 arrest was associated with radio-resistance. In H-ras+v-myc transformed radio-resistant cells, the G2 delay was significantly greater at different examined doses (ranging from 5 to 20 Gy).[223]

This delay also affects the G2/M progression, and this was demonstrated to result from radiation-induced alterations in cyclin B expression. In HeLa cells, synchronization at the G1/S checkpoint, followed by irradiation with 10 Gy in early S-phase showed that the non-irradiated cells entered the G2 phase at 9-12 hrs, and exited from M-phase at 15 hrs. In parallel, the levels of cyclin B mRNA rose at 9h, peaked at 12h, and decreased as

the cells re-entered the G1 phase. In irradiated cells however, there was no exit from the M-phase until 24h; the cells remained in G2 for at least 9 hrs longer than the non-irradiated cells. Concurrently cyclin B mRNA remained at low levels.[224, 225]

Cyclin B expression is also associated with radio-resistance: in radio-resistant REF cells, a 10 Gy irradiation induced a 10 hrs G2 delay with a marked decrease in cyclin B mRNA level as observed with HeLa cells. In radio-sensitive cells, the G2 delay was limited to two hrs with no significant inhibition of cyclin B accumulation.[226]

1.4.4 p21 $^{WAF1/Cip1}$

The p21 $^{WAF1/Cip1}$ is a cyclin-dependent kinase inhibitor, member of the large family of CKI. It is the most studied tumor suppressor gene in bladder cancer, along with p53 and p27.[227] p21 is a small 165 amino acid protein, encoded by *CDKN1A*, and acts mainly by preventing Cdks from phosphorylating key regulatory proteins of the cell cycle, thus inhibiting cellular proliferation.[228]

1.4.4.1 Discovery

The discovery of p21 dates back to 1993 as early work was focused on identifying p53 targets that mediate the tumor suppressor characteristics of p53 to downstream effectors. *p21* was then identified (initially named *WAF1*) as a gene located on chromosome 6p21.2, whose induction was associated with wild type p53 in human brain cancer cells. Introduction of *WAF1* cDNA into human brain, lung, and colon tumor cells *in vitro* inhibited cell growth, and further work identified a p53-binding site upstream of the *WAF1* coding region, in its promoter region.[229]

1.4.4.2 Role and mechanism of action

Initially, p21 function was thought to be limited to mediating p53-induced cell cycle arrest at G1, inhibiting cellular proliferation.[228, 230, 231] Upon p53 activation, *p21* transcription is up-regulated and the protein product translocates to the nucleus where it regulates the cell cycle progression at two levels: first, p21 binds and inhibits Cdk2 and Cdk1 activities, thus leading to growth arrest, through repressing the expression of cell cycle regulators such as cyclin B, Chk, cdc2 and cdc25c[227]; second, p21 binds to Proliferating Cell Nuclear Antigen (PCNA) and inhibits DNA replication by interfering with PCNA-mediated polymerase activity. In fact, it was demonstrated that p21 binds PCNA and competes with DNA polymerase δ.[232]

Later, p21 was found to be involved in more complex, p53-independent pathways: p21 associated directly with E2F1 and inhibits its transcriptional activity.[233] It also responds to notch1 signaling and suppresses wnt4 expression which is needed for controlling cellular growth.[234] The repression of cytokine-stimulated gene expression is also mediated *via* p21 which binds and represses the transcription factor signal transducer and activator of transcription 3 (STAT3).[235] As a tumor suppressor gene, p21 further represses the MYC oncogene-dependent transcription. Of note, p21 is part of a positive feedback loop that amplifies its own expression through the direct activation of the CREB-Binding Protein (CREBBP), which in turn induces *CDKN1A/p21* expression.[228]

The role of p21 in the cell extends beyond regulating the cell cycle, growth and progression towards the modulation of DNA repair: first, and by inhibiting the cell cycle progression, p21 allows time for the cell to undergo DNA repair. On the other hand, p21

can also compete for PCNA binding, with PCNA-dependent proteins that are involved in repair mechanisms, such as DNMT1. Recently, it was suggested that p21 also regulates NER directly, but the mechanism of regulation remains unclear, and the role of p21 in direct NER modulation is still controversial.[236, 237]

Although initially discovered to act as a cell cycle inhibitor, relatively more recent investigations have demonstrated a cytostatic role of p21, whereby it confers anti-apoptotic functions.[238] The shift from growth inhibition, tumor suppressor functions of p21 towards the anti-apoptotic, oncogenic ones occur through its selective inhibition of pro-apoptotic genes such as pro-caspase 3, caspase 8 and caspase 10.[239, 240] The dichotomy in p21 functions depends on its cellular sub-localization. While initial work had identified p21 as a nuclear inhibitory regulator of cell growth and proliferation, it was demonstrated that p21 also accumulates in the cytoplasm where it mainly carries anti-apoptotic, oncogenic functions.

The regulation of p21 expression occurs mainly at a transcription level, *via* either p53-dependent or p53-independent pathways. However, post-transcriptional control of p21 is also important especially with regards to p21 stabilization and cellular localization. Proteolysis of p21 is mediated through E3-ubiquitin ligase complexes such as APC/C; these complexes decrease the levels of p21 *via* proteasomal degradation, and it was shown that proteins involved in ubiquitin-dependent proteolysis of p21 are up-regulated in a variety of tumors.[241] Phosphorylation of p21 is another mechanism of prost-transcriptional regulation that sets p21 stability and localization in the cell.[228] For example, phosphorylation of p21 by cyclin E-Cdk2 promotes binding to SKP2, a protein responsible for its ubiquitination and degradation.[242] Phosphorylation of p21 by AKT1

stabilizes p21 and disrupts its binding to PCNA leading to its cytoplasmic accumulation. In the cytoplasm, p21 promotes survival by inhibiting apoptosis, and promotes cellular proliferation through its inability to inhibit Cdk2 and PCNA, a function that requires p21 to be present in the nucleus.[243, 244] The role of AKT in regulating p21 was further studied, and it was shown that inhibition of the PI3K/AKT pathway lead to loss of p21 expression, and that the rescue of the pathway resulted in the accumulation of p21 in the cytoplasm and an increase in cellular proliferation. More interestingly, p21 not only contributes to cellular proliferation, but also increased invasion/metastasis by modulating the expression of the ROCK/LIMK/Cofilin pathway, known to regulate cellular motility.[245] In another study, on UBC cells *in vitro* and in bladder cancer mouse models, it was shown that the up-regulation of p21 by the PI3K/AKT pathway occurs through the suppression of the Glycogen Synthase Kinase-3β (GSK-3β), and the activation of mTOR. The PI3K/AKT/mTOR pathway will be presented in further details.[246]

Of note, p21 mutation in bladder cancer is a rare event, and the modulation of its expression rather occurs through the modulation of the different pathways it partakes in.[247, 248] Clinically, UBC patients with p53/p21 negative tumors have higher rates of recurrence and a worse survival compared to those with p53 altered/p21 positive tumors.[249-251] Nevertheless, p21 alone has not shown additional prognostic information in predicting risk of progression of UBC although patients with normal p21 expression exhibited a better response to systemic chemotherapy compared to those lacking p21.[252, 253]

1.5 The PI3K/AKT/mTOR pathway

1.5.1 Overview of the pathway

PI3Ks phosphorylate membrane-embedded inositol phospholipid substrates, and they are implicated in regulation of cell survival, growth and migration through metabolism regulation and membrane trafficking.[254]

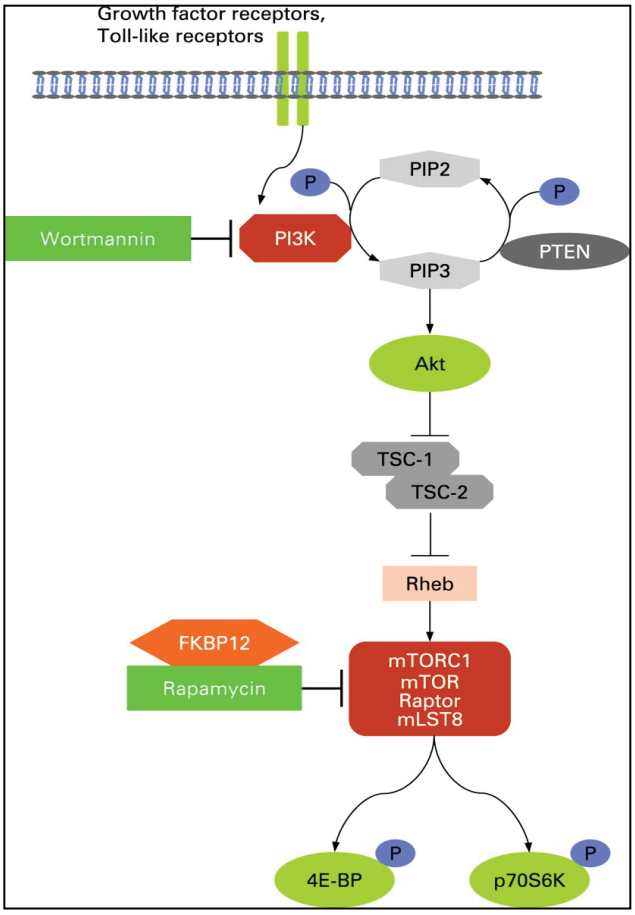

Figure F: The PI3K/AKT/mTOR signaling pathway [255]

Upon binding of growth factors such as platelet-derived growth factors (PDGF), insulin and epidermal growth factor (EGF) to their respective receptor tyrosine kinases (RTK), the RTK in question dimerizes and cross-phosphorylate.[256, 257] As shown in Figure F, the activated RTK recruits PI3K to the lipid membrane of the cell, where it phosphorylates Phosphat-Idylinositol-4,5-bis-Phosphate (PIP2) into Phosphat-Idylinositol-3,4,5-tri-Phosphate (PIP3).[256] PIP3 then recruits and activates proteins containing Pleckstrin homology (PH) domains, and this includes i) Guanine Exchange Factors (GEFs) that activate the Rac/Rho pathway involved in actin cytoskeletal rearrangement and cell migration, ii) Phosphoinositide-Dependent Kinase 1 (PDK1) and AKT (also known as protein kinase B; PKB), resulting in the activation of AKT involved in cell survival and proliferation [254] and last iii) the TSC2 (Tuberin Sclerosis Complex 2) and mTOR resulting in activation of mTOR, a kinase that promotes protein translation and cellular growth.[258]

1.5.2 The activation of AKT and the involved cellular functions

AKT is fully activated following phosphorylation at two residues: Thr308 and Ser473.[259] Although the kinase that phosphorylates the Ser473 residue is still unknown, the Thr308 residue is phosphorylated by PDK1 upon PI3K activation.[260] On the other hand, the activation of the AKT pathway is inhibited upstream by the Phosphatase and Tensin homolog on chromosome 10 (PTEN), a lipid phosphatase that removes the phosphate from the D3 position of PIP3, thus antagonizing the effect of the AKT pathway; the *PTEN g*ene acts as a tumor suppressor.[261]

Upon activation, AKT induces cellular proliferation *via* the inhibition of GSK-3β, thus preventing the degradation of cyclin D1,[262, 263] and inhibits cell cycle inhibitors

$p21^{WAF1}$ and $p27^{Kip1}$. [264] AKT further promotes cell survival by inhibiting apoptosis *via* inactivation of prop-apoptotic BAD, and inhibition of the caspase cascade by stabilizing PED/PEA 15, an inhibitor of caspase-3.[265] In terms of metabolism and cellular growth, AKT mediates the membrane translocation of Glucose transporter GLUT1 and GLUT4 upon phosphorylation,[266] and activates Phosphor-Fructo Kinase 2 (PFK2), thus stimulating glycolysis. AKT also regulates cellular growth *via* mTOR that restricts cell cycle progression when growth conditions are not favorable; it further increases mTOR activation by phosphorylating TSC2 and disrupting its interaction with TSC1.[267] Of note, and besides activating AKT, PDK1 also phosphorylates p70 ribosomal S6 kinase (S6K), which controls protein synthesis and is required for cell growth and metabolism *via* the storage of amino acids. [268, 269]

A recent and very important molecular characterization of UBC published by the The Cancer Genome Atlas Research Network in Nature in early 2014 highlights various molecular alteration involved in the progression of the disease. Of note, 69% of the tested samples showed molecular alterations, including 42% within the PI3K/AKT/mTOR pathway. About 9% of these alterations involved mutations or deletions of TSC1 or TSC2, rendering the tumors sensitive to mTOR inhibition. Thus, the need for further investigation using modulators of mTOR functions. [270]

1.5.3 The mTOR pathway and regulation of protein synthesis

1.5.3.1 Overview

The multi-domain protein mTOR is a serine/threonine kinase, member of the PI3K related kinases. mTOR plays a central role in the regulation of cell growth,

elegantly shown by genetic *mTOR* knockouts in mice and leading to embryos death in utero shortly after implantation.[271]

mTOR acts downstream in the PI3K/AKT survival pathway, and is activated by hormones and growth factors: upon insulin stimulation, AKT phosphorylates TSC2 that acts as a GTPase Activator Protein (GAP) and inhibits it, allowing the Ras-homologue enriched in brain (Rheb) to accumulate in a GTP-bound, active form. The accumulated Rheb interacts with mTOR and activates it.[272] The hyper-activation of mTOR favors cell and tissue growth, as shown in the *Drosophila melanogaster*, mice and humans. (reviewed in [273]) For example, in tuberous sclerosis, a human cell growth disorder, mutations in TSC1 or TSC2 (two inhibitors of mTOR) lead to hyper-activation of mTOR signaling, thus increasing cell growth *via* increased protein synthesis. In cardiac hypertrophy, over-activation of mTOR is responsible for the increased cell mass and volume.[274]

Besides hormones and growth factors, mTOR is further regulated by amino acids and by the overall cellular energy status. Leucine is the most effective amino acid, as removing it from the growth media *in vitro* caused a rapid inactivation of mTOR signaling, and made mTOR unresponsive to further stimulation by peptide hormones such as insulin.[275] On an energy level, depletion of ATP also impairs mTOR signaling *via* the AMP-activated Kinase (AMPK), an important sensor of cellular energy status.[276]

The major role of mTOR is the regulation of mRNA translation into proteins.[277] At the translation initiation level, mTOR phosphorylates regulatory sites of the 4E Binding Protein 1 (4E-BP1) that, in human, regulates the activity of the eukaryotic

Initiation Factor 4E (eIF4E).[278] mTOR also regulates translation elongation: the Eukaryotic Elongation Factor 2 (eEF2) is essential for the elongation phase of translation, and is regulated by eEF2 kinase, a Calcium/Calmodulin (CaM)-dependent enzyme.[277] The eEF2 kinase inactivates eEF2 by phosphorylating Thr56, and mTOR regulates eEF2 *via* phosphorylation at several sites: i) at Ser366, by S6K1, a direct target of mTOR, leading to inhibition of the kinase activity, and the release of eEF2 inhibition, allowing elongation to proceed,[279] ii) at Ser78, which impairs the binding of CaM to eEF2 kinase, thus inhibiting its activity,[280] and iii) at Ser359, which directly inhibits eEF2 kinase activity. mTOR also controls translation by modulating the ribosomal activity, and this is mediated by multiple phosphorylation of S6K by mTOR, at its phospho-regulatory region, leading to full activation.[281] Of note, the full activation of S6K also requires the phosphorylation of S6K catalytic domain by PDK1, in response to the insulin-activated PI3K/AKT pathway. The active S6K will phosphorylate and activate S6, increasing the translation of short mRNA with pyrimidine motifs, that encode ribosomal proteins, needed for the ribosomal assembly.[269]

The activity of mTOR is inhibited by the rapamycin:FKBP12 complex (as presented later), and the effects of inhibition are mediated by binding of mTOR to two partners: raptor and rictor and forming the mTORC1 and mTORC2 complexes, respectively. mTORC1 mediates the effects of mTOR inhibition on protein synthesis, and the best known targets of mTORC1 are proteins involved in the translational machinery.[273]

1.5.3.2 The effect of radiation on the mTOR pathway and protein synthesis

Protein synthesis is a highly regulated process and is accurately responsive to growth and stress stimuli. Ionizing radiation alters mRNA translation more profoundly than transcription [282] and protein synthesis response to genotoxic stress caused by ionizing radiation seems to be a biphasic response, in a time and dose-dependent manner.[283] At early, low dose exposure (less than 2Gy), ionizing radiation stimulates a rapid increase in protein synthesis *via* an increase of S6K and mTOR activities, eventually leading to an increase in 4E-BP1 phosphorylation and an increase in eIF4 levels needed for cap-initiation of translation.[284] This radiation-induced activation of growth factor signaling, and mTOR in particular is thought to provide radio-protective effects following radiation. This is commonly observed in cancer, where the activation of multiple survival signaling pathways occurs in response to radiation to maintain cell viability and increase protein synthesis associated with the production of DNA damage response proteins. It is thus a rational approach to attempt to target mTOR as a strategy to radio-sensitize cancer cells.[285, 286]

At a higher dose (20-50 Gy), ionizing radiation causes an inhibition of protein synthesis, and this happens through the inhibition of mTOR in a p53-dependent manner following AMPK activation. The inhibition of mTOR results in a hypo-phosphorylation of 4E-BP1, a subsequent inhibition of eIF4E, and a decrease in protein synthesis.[287, 288]

1.5.3.3 mTOR inhibition

1.5.3.3.1 Overview

Rapamycin is the first mTOR inhibitor, from which several other derivatives have been developed: RAD001, CCI-779 and Deforolimus.[289]

Rapamycin, also known as AY-022989 and Sirolimus was first isolated from a fungus, *Streptomyces hygroscopicus*, mainly for its immune-suppressive activity. It was initially discovered on Easter Island in search of rare molecules and has a molecular weight of 914.2 g/mol molecule with the chemical formula $C_{51}H_{79}NO_{13}$.[290] It was later discovered to have anti-tumor and anti-proliferative activities as well.Rapamycin was initially approved by the Food and Drug Administration for maintenance of immune-suppression in kidney allograft recipients.[291]

RAD001, also known as Everolimus is a 40-O-(2-hydroxyethyl)-rapamycin derivative with the $C_{53}H_{83}NO_{14}$ chemical formula and a molecular weight of 958.25 g/mol. The derivative was developed for improved pharmaco-kinetics for oral intake and was initially shown to inhibit T-cell proliferation between the G1 and S phases of the cell cycle.[292] The rapamycin analogue CCI-770 was designed as a pro-drug that is eventually metabolized to rapamycin in the body, while Deforolimus (known as A2357) is another derivative metabolized in the liver by CYP450 mechanism. It is known for its low toxicities/side effects, with mucositis being the major dose-limiting toxicity, along with myelo-suppression and skin toxicities reported in metastatic renal cell carcinoma.[293, 294]

1.5.3.3.2 Mechanism of action

Rapamycin and its derivatives act on mTOR through the same mechanism, the main difference being their bio-availability with CCI-779 and deforolimus administered intravenously, and the less soluble rapamycin and RAD001 given orally.[295] The mechanism of action of mTOR inhibitors is mediated by the formation of a ternary complex with FK506-Binding Protein (FKBP12) and mTOR; the complex does not affect the three-dimensional configuration of either mTOR or the inhibitors. It acts through: i) blocking Ca^{2+}-independent T-cell activation and proliferation, ii) hindering the ability of mTOR to activate $CD4^{+}$ helper cells and $CD8^{+}$ cytotoxic cells, as well as iii) inhibiting B-cell activation and differentiation into mature antibodies-producing cells, thus conferring immune-suppression and iv) blocking mTOR response to growth factors.(reviewed in [289] and [295])

1.5.3.3.3 mTOR inhibitors and cancer therapy

In cancer, rapamycin and its analogues were shown to inhibit proliferation in a wide range of cell lines, from different origins: rhabdomyosarcoma, neuroblastoma, glioblastoma, small-cell lung carcinoma, osteosarcoma, renal cell carcinoma, Ewing sarcoma, prostate cancer and breast cancer.[296-298] On a cellular level, the response to mTOR inhibition is mostly growth arrest. In dendritic cells and renal tubular cells, mTOR inhibition was shown to induce apoptosis *via* indirect inhibition of MYC and AKT expression, both involved in anti-apoptotic pathways. (Reviewed in [295])

Combining mTOR inhibitors with other targeted therapies was also assessed pre-clinically. In chronic myelogenous leukemia cells showing resistance to imatinib, combining imatinib with rapamycin or RAD001 resulted in a synergistic inhibition of

leukemic cell growth.[299] Rapamycin was also successfully tested in breast cancer when combined with Herceptin.[300] In multiple myeloma, the use of rapamycin sensitized cells to apoptosis induced by heat shock protein 90 inhibitors.[301] In pancreatic cancer, rapamycin combined with small inhibitors of the c-met oncogene product and Vascular Endothelial Growth Factor successfully inhibited primary and metastatic cell growth, as well as liver metastasis.[302]

The use of rapamycin and its derivatives has also been investigated in combination with chemotherapy and radiation therapy, as potent radio-sensitizers in the latter case. Combining rapamycin with vinblastine in orthotopic neuroblastoma-bearing mice inhibited tumor growth and angiogenesis, and further conferred an increase in survival compared to either drug alone. *In vitro* work has shown synergy when rapamycin was combined with paclitaxel, carboplatin or vinorelbine in hepatocellular carcinoma, and when RAD001 was combined with rituximab, doxorubicin and vincristine in lymphoma. Rapamycin and RAD001 were also studied as potent radio-sensitizers, and this was shown to happen *via* the PI3K/AKT/mTOR pathway, as RAD001 sensitized PTEN wild type cells to ionizing radiation and induced more cytotoxicity in these cells compared to the PTEN null cells. RAD001 also enhanced damages of the tumor vasculature *in vivo* through the induction of endothelial cell apoptosis. (Reviewed in [303])

1.5.3.3.4 mTOR inhibitors in UBC

In UBC, the use of RAD001 was first investigated by our laboratory, and was shown to inhibit cell growth in a dose-dependent manner, and dose arrest, with no clear evidence of apoptosis.[304] Work in mice showed the ability of RAD001 to inhibit the growth of tumors that were subcutaneously implanted. Molecular dissection revealed the

inhibition of protein synthesis through S6K and 4EBP-1 pathways to be the main mechanism for RAD001-induced growth inhibition, except for the UM-UC9 cell line where inhibition of angiogenesis was the main mechanism of action.[305, 306]

Of note, the PTEN status in UBC seems to have an important role in the inhibition of the PI3K/AKT/mTOR pathway, as PTEN loss was shown to associate with aggressive tumor growth: PTEN levels of expression were reduced in 94% of advanced stage UBC, while only 42% of superficial tumors, and 8% of CIS had a reduced PTEN expression.[307] This status of PTEN was reported to influence the response to treatment in UBC, as UBC cell lines with intact PTEN expression had a reduced sensitivity to PI3K/AKT/mTOR pathway inhibitors.[308]

1.6 Autophagy

Autophagy is a lysosomal catabolic pathway whereby cells recycle macro-molecules and organelles.[309] It is an evolutionary conserved pathway regulated by autophagy-related genes (atg) that encode the proteins needed for autophagosome formation.[310] As a process, it is thought to improve cell survival under starvation conditions by maintaining cellular metabolism and removing damaged cells. Yet, in cancer, autophagy shows an ability to suppress tumorigenesis. Autophagosomes are cytoplasmic vesicles with a double membrane surrounding a cargo. They fuse with lysosomes to form auto-lysosomes where the cargo gets digested into metabolites before being released back to the cytosol for recycling. (Reviewed in [309])

In cells stressed due to starvation, hypoxia and other stresses including genotoxic stress that results from ionizing radiation, autophagy is increased.[311] In unstressed cells, autophagic activity is kept low by mTOR.[312] However, autophagy also mediates a special type of cell death referred to as autophagic or type II programmed cell death. In fact, studies on atg gene depletion demonstrated an autophagy-dependent death of cultured cells exposed to stress.[313] Still, the mechanism that directs the switch in autophagy role from cell preservation under stress towards a death mechanism is still unclear. It is thought that it is the level of autophagy that determines the final outcome: a moderate, relatively short autophagy might be supporting cell survival, while a prolonged autophagy induction leads to the cell "eating itself", and thus cellular death.[314] The opposing views regarding the role of autophagy as a death or a survival mechanism stem from different observations. On the one hand, it was noted that in cells, autophagy is activated as a response to lack of nutrients, in order to recycle and supply the missing

components. In fact, in the mammalian liver, autophagy is switched on following starvation in order to produce amino acids after conversion into glucose, thus meeting the energy requirements of the brain. Interestingly, the addition of amino acids was shown to inhibit apoptosis, thus supporting the idea that apoptosis acts as a survival mechanism that allows the cell to survive in times of stress and nutrient starvation.[315] On the other hand, it was reported that the molecular machinery involved in autophagy is the same required during cell death. During apoptosis, an overlap between the mechanisms of cell death and autophagy is observed: whether apoptosis utilizes autophagy to induce cell death in response to certain factors (such as the lack of the main apoptotic machinery) is a probable hypothesis.[61] However, the complex interaction between the role of autophagy as a survival or death mechanism remains largely unknown.

The formation of the autophagosome is dependent on a set of atgs. Different Atg proteins would make two different ubiquitin-like conjugation systems: the Atg12 system and the LC3/Atg8 systems.[316] Atg12 is conjugated to Atg5 and forms a 800kDa protein complex with Atg16, while LC3-Atg8 is conjugated to a phosphatidylethanolamine and is associated with autophagosome formation.[317] LC3 is the mammalian autophagosomal ortholog of yeast Atg8.[318] Although these players have been identified, the exact mechanism of autophagosome formation is still poorly understood. However, it is well established that the extent of autophagy is regulated by proteins of the PI3K/AKT pathway such as PTEN, PDK1, AKT, TSC1/2 and mTOR, a negative regulator of autophagy.[315]

The mammalian gene encoding beclin 1 is a Bcl-2-interacting gene and is mono-allelically deleted in 40–75% of sporadic human breast cancers and ovarian cancers.

Beclin 1 was shown to promote autophagy in autophagy-defective yeast and in human MCF7 breast carcinoma cells. The autophagy-promoting activity of beclin 1 in MCF7 cells is associated with inhibition of their proliferation, *in vitro* clonogenicity and tumorigenesis in nude mice. The endogenous Beclin 1 protein expression level is frequently low in human breast epithelial carcinoma cell lines and tissues, but is expressed ubiquitously and at high levels in the normal breast epithelium.[319]

In cancer, the tumor environment is the main inducer of autophagy given the low levels of oxygen and nutrients. In several cancers, tumor suppressor genes stimulate autophagy, while oncogenes inhibit it, presenting evidence for a decrease in autophagy in cancers. But still, samples from cancers of different origins show autophagic vacuoles and activity, and this could be explained by the fact that autophagy diminishes necrotic cell lysis, and limits tumor inflammation, which is associated with increased tumorigeneis, in apoptosis-defective cells. (Reviewed in [320]) Furthermore, numerous evidence for anti-cancer activity of autophagy exist: i) Beclin-1, an important autophagy gene product is required for vesicle formation, and the gene was shown to be monoallelically lost in several tumors; ii) mutations in *p53* and *PTEN* genes induce autophagy and iii) oncogenic Bcl-2 interacts directly with Beclin-1 to inhibit autophagy. It thus seems that autophagy can both stimulate and prevent cancer, depending on the context.[321]

Different anti-cancer agents were shown to induce autophagy and these include: tamoxifen, rapamycin, arsenic trioxide, temozolomide, histone deacetylase inhibitors and most importantly in the scope of this work, ionizing radiation.[322] The question of whether autophagy is a survival mechanism or cell-death mechanism remains unclear,

especially that autophagy is detected in cells treated with anti-cancer agents prior to death.[313]

Still, manipulation of autophagy might be promising especially in sensitizing apoptosis-resistant cells to cancer therapy, whether chemotherapy or radiation therapy. For example, a mechanism of response to ionizing radiation *via* autophagy has been reported, in human breast, colon and prostate cancer cell lines. The controversial effects of radiation on autophagy depend mostly on cancer type, and even on the cell type within the same cancer. In prostate cancer, PC3 and DU145 cells were treated with radiation with or without RAD001 for radio-sensitization, along with different combinations of drugs, and autophagy was shown to increase with the addition of the mTOR inhibitor; autophagy was associated with cell death, and this is in contrast to the LNCaP prostate cancer cells, where radiation increases autophagy, but associates with increase cell survival. Radiation-associated increase in autophagy was observed in glioma, breast and non-small cell lung carcinoma. This increased autophagy was associated with cell death.[323]

CHAPTER II: HYPOTHESIS AND OBJECTIVES

As demonstrated in the literature review above, signaling through the PI3K/Akt/mTOR pathway plays an important role in tumorigenesis. Studies have shown that the dysregulation of PI3K/Akt/mTOR pathway generates a favorable oncogenic environment, and has been documented in a variety of human transformed cells and tumours. Furthermore, radio-resistance and disease recurrence are widely attributed to the activated PI3K/Akt/mTOR pathway. Our working hypothesis is that mTOR inhibitors alone and combined with radiation in bladder cancer will have significant antitumor activity with improved local and distant control of disease.

The aim of this research program was to target mTOR signaling pathway for bladder cancer therapy through various objectives:

i. Characterize a panel of bladder cancer cell lines, *in vitro*, representing different stages of the cancer with respect to expression levels of mTOR and its associated upstream/downstream signaling partners as well as pursue studies on effects of mTOR inhibition alone and in combination with radiation on growth of human urothelial cancer cells;

ii. Confirm the in *vitro* findings *in vivo*;

iii. Evaluate the effects of the combination therapy on the cell cycle;

iv. Investigate the role of p21 in the radiosensitization role of RAD001;

v. Interrogate the role of apoptosis and autophagy following a combined treatment.

CHAPTER III: MATERIALS AND METHODS

3.1 Cell lines

The following human bladder cancer cell lines: UM-UC1, UM-UC3, UM-UC5, UM-UC6, UM-UC13, RT4 and KU7 were obtained from the Specimen Core of the Genitourinary Specialized Programs of Research Excellence in bladder cancer at M.D. Anderson Cancer Center, where they had been previously characterized.[324] The metastatic variants 253-JP and the 253J-BV cell lines were kindly provided by Dr. Colin P.N Dinney from M.D. Anderson Cancer Center, Houston, Texas.[325] All cell lines were cultured in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich®, Canada) supplemented with L-glutamine, 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin) mixture. All products were obtained from Invitrogen (Burlington, Ontario, Canada). Cells were routinely passaged when reaching 80% confluence. They were studied at passages ranging from 15-40, with the exception of UM-UC6, which had a passage range of 120-125. *(NB: KU7 cell line was recently reported to have been contaminated by HeLa cells. This report was published after the collection of our data. This finding, while important, does not change the significance of our conclusions as several other cells lines were tested in our experiments with similar observations).[326]*

3.2 Drug treatment

The mTOR inhibitor RAD001 (Everolimus), was obtained from Novartis (Basel, Switzerland) as a dry powder and microemulsion for oral use. A 10 mM stock solution was prepared in sterile DMSO (Sigma-Aldrich®, Canada) and stored at -20°C for *in vitro* studies. The final concentrations (ranging from 0.001-100 nM depending on cell lines and

assays) were prepared in complete culture media just before use, filtered on a 0.22 μm filter for sterilization, and administered to cells in a dose dependent manner. The vehicle contained less than 1% DMSO.

3.3 Proliferation and survival assays

3.3.1 MTT Assays

In order to assess the effects of RAD001 on bladder cancer cell survival and proliferation, and for initial GI_{50} determination (drug concentration required to inhibit cancer cell growth by 50%), the different cell lines were treated with various concentrations of the drug (0.001-1000nM) for 72h. MTT assays (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Canada) were carried out *as per* the manufacturer's instructions. Briefly, MTT was dissolved in Phosphate Buffer Saline (PBS) and stored at 4°C at a stock solution of 5 mg/mL. 20 μL of the MTT solution was added to each well along with 80 μL of growth media, and the plates were incubated at 37°C. After 4 hrs, the media was removed and replaced with 200 μL of DMSO in order to solubilize the Formazan and allow color development. The absorbance was read at a wavelength of 550 nm using a microplate reader. Each measurement was performed in six replicates and each assay repeated at least twice. The percentage of growth inhibition was calculated as: [(T-Ct24)/(C-Ct24]x100, where T is the reading from the cells treated with RAD001, Ct24 the reading at baseline and C the reading from control cells.

3.3.2 Clonogenic Assays

Based on RAD001 GI_{50} obtained from the MTT results, we selected the following cell lines (where a clonogenic assay seemed more appropriate) for assessing the

radiosensitizing effect of RAD001 (at concentration indicated in parenthesis) on cell survival: UM-UC3 (75nM), UM-UC13 (75nM), KU7 (75nM), 253J-BV (8nM), UM-UC5 (0.5 nM) and UM-UC6 (0.5nM). Briefly, cells were seeded in a 6-well plate (surface area 9.5 cm^2 per well) at a density of 200 cells per well and maintained in the growth medium. Once attached, they were treated with RAD001 for 12 hrs at doses equivalent to the GI_{50} for each cell line as described above. Drug treatment was followed by radiation at different dosages, with and without RAD001. Controls included untreated cells along with cells treated with each of radiation and RAD001 treatment alone. Cells were further cultured at 37°C and allowed to form colonies for 10-14 days. The cells were washed with PBS and fixed for 15 min using 3.7% formaldehyde in PBS. After a second PBS wash, cells were stained with crystal violent (0.4% w/v; Fisher Scientific, Canada) and left to air dry before colonies counting. A cutoff of 50 viable cells per colony was chosen. Each treatment consisted of duplicate wells of a 6-well plate and the experiment was performed twice. The surviving fraction was calculated as: (mean colony count at the end of the experiment)/(cells initially plated) x (plating efficiency). The plating efficiency was defined as: (mean colony count)/cells plated in the non-irradiated control). All data points were adjusted to the non-irradiated control.

3.4 Immunohistochemistry

IHC was performed on formalin-fixed paraffin embedded sections of human bladder tumors in mice xenografts (described below). Tissue sections were treated *as per* standard IHC protocol. Briefly, formalin-fixed paraffin-embedded sections were deparaffinized in xylene and hydrated with changes of 100% ethanol, followed by 95% ethanol and distilled water. Antigen-retrieval was performed by heating the slides with 5%

citrate buffer solution (pH 7.0). Peroxidase blocking was done in 3% H_2O_2/PBS solution for 10 min followed by washes in PBS-Tween. Slides were then incubated in the primary antibody of interest followed by a secondary antibody. After washing, reactions were revealed by incubating sections with 3,3'-Diaminobenzidine (DAB) substrate (Sigma-Aldrich, Canada) according to manufacturer's instructions, and slides viewed by light microscopy under a Leica Diaplan inverted microscope (Leica, Inc.) equipped with a Leica DFC300FX camera (Leica Inc.).

To assess the levels of p21 expression, sections were incubated overnight at 4° C, with primary specific antibodies against p21 (Cell Signaling Technology, New England MA; 1:200 dilution). HRP-conjugated goat polyclonal anti-rabbit IgG secondary antibody was added and incubated at room temperature for one hr. Analysis of staining was based on an average of 5 foci, at 40X magnification, showing viable cells, given a H-score (minimum 100 cells, in 5 different foci at 40X magnitude), calculated by summing the products of the percentage cells at a given staining intensity (0-100) by the staining intensity (0 for negative, 1 for low and 2 for high staining).[327] Our H-score analysis follows a modified calculation to the ones often used, whereas the maximum staining intensity is 2, as opposed to 3, with a maximum H-score of 200, as opposed to 300.

3.5 Protein extraction and quantification

Untreated (negative control) and treated cells (RAD001, ionizing radiation, and both in combination) were washed with cold PBS on ice and scraped to then be re-suspended in cold RIPA (or lysis) buffer (50nM Tris-HCl pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150nM NaCl, 2nM EDTA, and 50 mM NaF), containing a cocktail of phosphatase and protease inhibitors (Roche Diagnostics, Canada) for 30 min

at 4°C. Cellular extracts were then centrifuged at 14000g for 10 min at 4°C. The supernatant containing the extracted proteins was collected for future analysis.
Protein quantification of cell lysates was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Scientific, IL), as per the manufacturer instructions.

3.6 Protein separation by electrophoresis

Whole cell lysates (40-60 µg proteins) were dissolved in Laemmli protein loading buffer and heated for 5 minutes at 100°C. Proteins were next separated by electrophoresis by running them on 10% polyacrylamide gels at 120V.[328] Gels were stained with Coomassie Blue in order to visualize the different protein bands present with respect to the molecular weight standards run in parallel (Invitrogen, Burlington, Ontario, Canada).

3.7 Western Blotting

Following electrophoretic separation, proteins were transferred onto nitrocellulose membranes (BioRad, California, USA) by electroblotting overnight at 30 mV at 4°C. Membranes were then washed 3 times with Tris-Buffered Saline (TBS) buffer, followed by membrane blocking using a 5% non-fat milk and/or 5% bovine serum albumin (BSA) in TBS solution, depending on the antibody, at room temperature for 1hr on a shaker, to reduce non-specific antibody binding.

The following rabbit monoclonal antibodies were used at a 1:1,000 dilution: total mTOR, phospho-mTOR (Ser2448), PTEN, total S6, phospho-S6 (Ser240/244), total AKT, phospho-AKT (Ser473), p21, p27kip1, cyclin D1 and β-actin (Cell signaling Technology, New England, MA). Membranes were then incubated with anti-rabbit secondary antibodies to reveal protein bands of interest using an ECL chemiluminiscence detection

system (Amersham Biosciences, Canada). Bands were scanned and protein levels were normalized against β–actin as an endogeneous 42kDa internal control protein band present at similar levels in all samples. Specific activation of phosphoproteins were also determined and expressed by the ratio between levels detected with antibodies against phosphor-epitopes *vs*. the protein itself. Density histograms were created using NIH ImageJ software.

3.8 Cell cycle analysis

In order to assess cell cycle distribution following treatment (RAD001 with or without ionizing radiation), Fluorescence-Activated Cell Sorting (FACS) was used. Cells were first allowed to attach in culture plates for 24 hrs before addition of RAD001 for 12 hrs before irradiation, at a dose equivalent to the GI_{50} determined for each cell line. RAD001 was followed by a dose of 4Gy of ionizing radiation and cells were further cultured for 48 hrs. Cells were trypsinized, washed once with PBS and fixed with absolute cold ethanol for 60 min at 4°C. They were centrifuged and the pellets re-suspended in a solution of 50 μg/mL Propidium Iodide (PI) in PBS, supplemented with RNase (100μg/mL; Invitrogen, Canada), and next transferred to FACS tubes and incubated in the dark for 30 min at 4° C to allow PI intake by DNA in the cell nucleus. PI intake was assessed using a Coulter Flow Cytometer (BD Biosciences, Canada). Cell cycle distribution was analyzed, with calculations done using Cell Quest© software.

3.9 Immunoflorescence

In order to assess the activation of autophagy, cells were first allowed to attach in Labtek© chamber slides (Fisher Scientific, Canada). Following the treatment with

RAD001 or IR, they were treated for 6 hrs with 25μM Chloroquine (Sigma-Aldrich®, Canada) diluted in complete growth medium with FBS in order to inhibit the degradation of the LC3 protein and allow for autophagy assessment. The cells were then washed with 100% ice-cold methanol and fixed for 15 min at -20°C, followed by a 3 wash cycle with PBS. Cells were blocked with 5% BSA in TBS solution, washed with PBS and incubated with LC3 antibody (Cell signaling Technology, New England, MA, 1:50 dilution) overnight at 4°C. This was followed by a wash with PBS and incubation with fluorochrome-conjugated secondary antibody. Cells were rinsed with PBS, the slides carefully dried and mounted with a coverslip using an immunofluorescence–mounting medium (MP Biomedicals, Santa Ana, CA).

3.10 Bladder tumor xenograft: in vivo model

The research protocol was approved by the Animal Care Committee of McGill University Health Center, in accordance with institutional and governmental guidelines and Declaration of Helsinki. Female nude mice, 4-6 weeks old (Nu/Nu strain; Charles River Laboratories, Wilmington, MA) were used to generate two xenograft bladder cancer models. Mice were implanted subcutaneously with the urothelial carcinoma KU7 and 253J-BV cells (10^6 cells). In order to facilitate tumor take, cells were suspended in 200 μl of Matrigel (BD Biosciences Co., Franklin, NJ) All mice developed tumors, which were allowed to grow for one week –time by which they reached 4-5 mm in diameter- prior randomization into four groups corresponding to the different treatment arms. Each group consisted of 14 mice: the first group was treated with placebo (5% glucose solution in water). The second group received RAD001 orally (microemulsion, diluted in 5% glucose solution) at a daily dose of 1.5 mg/kg for the entire period of the experiment (Of

note, in cancer patients, doses of RAD001 can vary from 2.5 mg to 200 mg in clinical trials). The third group consisted of tumor exposure to ionizing radiation at a fractionated dosage totaling 9 Gy (3x3 Gy) every second day, for the first week of the treatment. In the fourth group, mice were administered RAD001 at the above-mentioned dosage 1 day before the start of the tumor radiation treatment and daily afterwards for the entire period of the experiment. Mice were followed for one month following the onset of treatments. Tumor growth was monitored twice a week by a Vernier Caliper. Tumor volume was calculated as $V= [(Length \times Width^2) \times (\Pi/6)]$.[329] Body weight and animal behavior were monitored throughout the experiment. By the end of the four weeks, mice were euthanized in a CO_2 chamber and tumors were harvested, immediately weighed and fixed in 100% formalin solution and paraffin-embedded for histopathological evaluation (H&E staining) and IHC.

3.11 shRNA

The *CDKN1A* mRNA gene product was targeted with a p21-specific short hairpin RNAi (shRNA) using the Lenti-Pac HIV Expression Packaging kit as described by the manufacturer. (as described in [330]) Briefly, the first step consisted in producing lentiviral particles by co-transfecting the p21-specific HIV-based lentiviral expression plasmid together with the GeneCopoeia Lenti-Pac HIV Expression Packaging kit using the third generation HIV-based lentviral vector system, inside the 293Ta packaging cells. Following successful transduction, the pseudo-virus containing lentiviral expression construct were isolated and purified, prior to targeting the KU7 target cells in order to down-regulate p21 expression.

Lentivirus production: The p21-specific lentiviral expression clone was purchased from GeneCopoeia (OmicsLink™ shRNA Expression Clone; catalog No.HSH000331-HIVH1) and consisted of a p21-specific double strands, separated by a hairpin loop sequence.

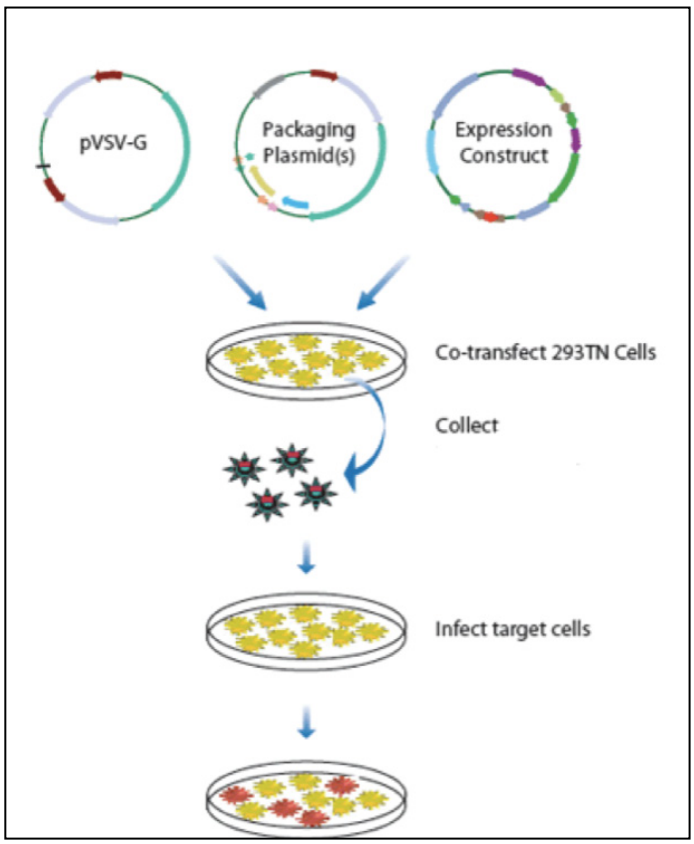

Figure G: The 3rd generation lentiviral packaging system and virus production

Both the sense strand and the antisense strand are part of a psiHIV-H1 vector with an HIVH1 promoter, the ampicillin resistance gene as well as elements required for viral packaging, transduction and stable integration into genomic DNA. The Lenti-Pack packaging kit (which includes two packaging plasmids, pMDLg/pRRE and pRSV-Rev

and the envelope plasmid pMD2.G) provides the elements essential for transcription and packaging of RNA copy into recombinant pseudo-viral particles (Fig. G).

The control shRNA consisted of a scrambled control clone in a psiHIV-H1 vector with the ampicillin resistance gene and eGFP reporter gene. Two days prior to transfection, $1.5x10^6$ 293Ta GeneCopoeia packaging cells were plated in a 10-cm dish, in 10 ml of Dubelcco's Modified Eagle Medium (DMEM, Invitrogen #11995) supplemented with 10% heat-inactivated FBS (Invitrogen #16000), and cultured at 37°C with 5% CO_2 until 70-80% confluence was reached. At transfection day, the DNA/Endofectin Lenti complex was prepared in a sterile polypropylene tube by diluting 2.5 µg of the lentiviral expression plasmid and 5.0 µl of Lenti-Pac HIV mix into 200 µl of Opti-MEM® I serum free media (Invitrogen #31985). To this DNA mix, we then added the EndoFectin Lenti reagent as provided by the manufacturer, drop-wise, while gently vortexing the DNA-containing tube. The mixture was then left to incubate for 10-25 min at room temperature to allow complex formation. Following the incubation, the DNA-EndoFectin Lenti complex was directly and evenly added to the cell culture dish to distribute the complex, and cells were further cultured in 5% CO_2 at 37°C, overnight. The next day, the culture medium was replaced with fresh DMEM medium supplemented with 5% heat-inactivated FBS and penicillin-streptomycin. In order to boost the titer of the lentivirus products, the TiterBoost reagent was added to the media at a 1/500 volume as per the manufacturer's instructions. After 48 hrs of incubation, the pseudo-virus containing culture medium was collected and centrifuged at 500 x g after which the supernatant was filtered through a 0.45 µm polyethersulfone low protein-binding filters.

Transduction of KU-7 target cells with the p21 and control Lentiviruses:

KU7 cells were plated in a 24-well plate 24 hrs prior to viral infection at a density of $6x10^4$ cells per well, with 0.5 ml of DMEM supplemented with 5% heat-inactivated FBS and penicillin-streptomycin. They were left to incubate at 37°C with 5% CO_2 overnight. At transduction day, cells were at 70% confluence; media was aspirated from each well and 0.5 ml of virus suspension, combined with TransDux (2.5 µl) viral transfection agent was added to a 1X final concentration. Plates were cultured overnight in a 37°C incubator with 5% CO_2. The next day, the medium was replaced by 0.5 ml fresh complete medium and left to incubate for another 48 hrs, after which proteins from the infected target cells were analyzed by Western blot to ascertain stable knock-down of p21 through the probing of transferred proteins with p21 antibodies, as described above.

3.12 Statistical Analysis

All statistical data analyses were carried using the Student's t-test (unpaired, two-tailed), and significance was set as $p < 0.05$. Data points were obtained from at least duplicate measurements, and each experiment was performed twice at least.

CHAPTER IV: RESULTS

4.1 The effects of RAD001 and IR on UBC cell lines

In order to assess the *in vitro* baseline response of UBC cells to RAD001, we selected a panel of nine cell lines that were treated with different drug concentrations, ranging between 0.001-1,000 nmol/L. Cell proliferation or growth was measured by MTT assays 72 hrs after treatment as described in Material and Methods. Cell lines of different origins, stages and grades of UBC were chosen in order to capture the effects of the mTOR inhibition on a wide, representative spectrum of bladder cancer cells; UM-UC3, UM-UC5, UM-UC6, 253-JP and 253J-BV cell lines were isolated from invasive urothelial carcinoma,[324] while UM-UC1 and UM-C13 originated from lymphatic metastases of bladder cancer.[331] The RT4 cell line however was derived from a well-differentiated superficial bladder cancer, and thus represented a lower-stage, less aggressive model of UBC.[332] As shown in Figure 1, we established dose-response curves for all these bladder cancer cell lines in terms of growth inhibition *vs*. RAD001 concentrations. Results reflect various growth sensitivities or responses to RAD001, which we categorized into highly sensitive, moderately sensitive and relatively resistant, based on the GI_{50} values, defined as the mean concentration of drug that generated 50% of growth inhibition. Cell lines with a GI_{50} less than 0.5 nmol/L were considered to be highly sensitive and included the UM-UC5 (0.1 nmol/L), UM-UC1 (0.17 nmol/L) and UM-UC6 (0.2 nmol/L). Three cell lines, RT4, (1.4 nmol/L), 253-JP (2.7 nmol/L) and 253J-BV (5.3 nmol/L) were considered to be moderately sensitive; while the last three, KU7 (76 nmol/L), UM-UC13 (77nmol/L) and UM-UC3 (86 nmol/L) were relatively resistant with GI_{50} values higher than 50 nmol/L.

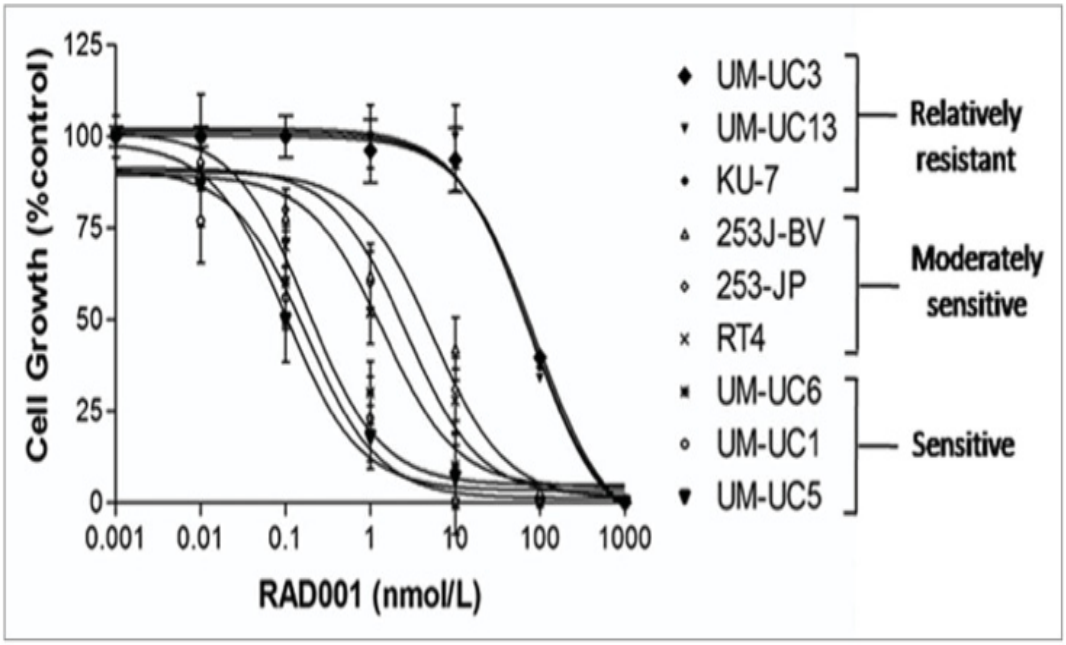

Figure 1: Dose-response of bladder cancer cell lines to the mTOR inhibitor, RAD001. Proliferation assays were performed after treatment with RAD001 at concentrations ranging from 0.001–1,000 nmol/L for 72 h. The GI_{50} value was defined as the mean concentration of drug that generated 50% of growth inhibition. RAD001 sensitivity was referred to as high at a GI_{50} <0.5 nmol/L (UM-UC1, UM-UC5, UM-UC6), moderate at 0.5 nmol/L < GI_{50} < 50 nmol/L (RT4, 253-JP, 253J-BV) and relatively resistant at GI_{50} >50 nmol/L (KU7, UM-UC3, UM-UC13).

Once the relative growth responses of the different cell lines were established and categorized in terms of relative sensitivity to RAD001, we next investigated the effect of IR on cell growth using clonogenic assays as described before. Of the nine cell lines assessed for sensitivity to RAD001, the RT4 cells were excluded based on the columnar-like growth of the colonies, which hinders our ability to properly assess colony formation by this assay. Similarly, 253-JP and UM-UC1 were also excluded given the very slow growth rate of these cells.

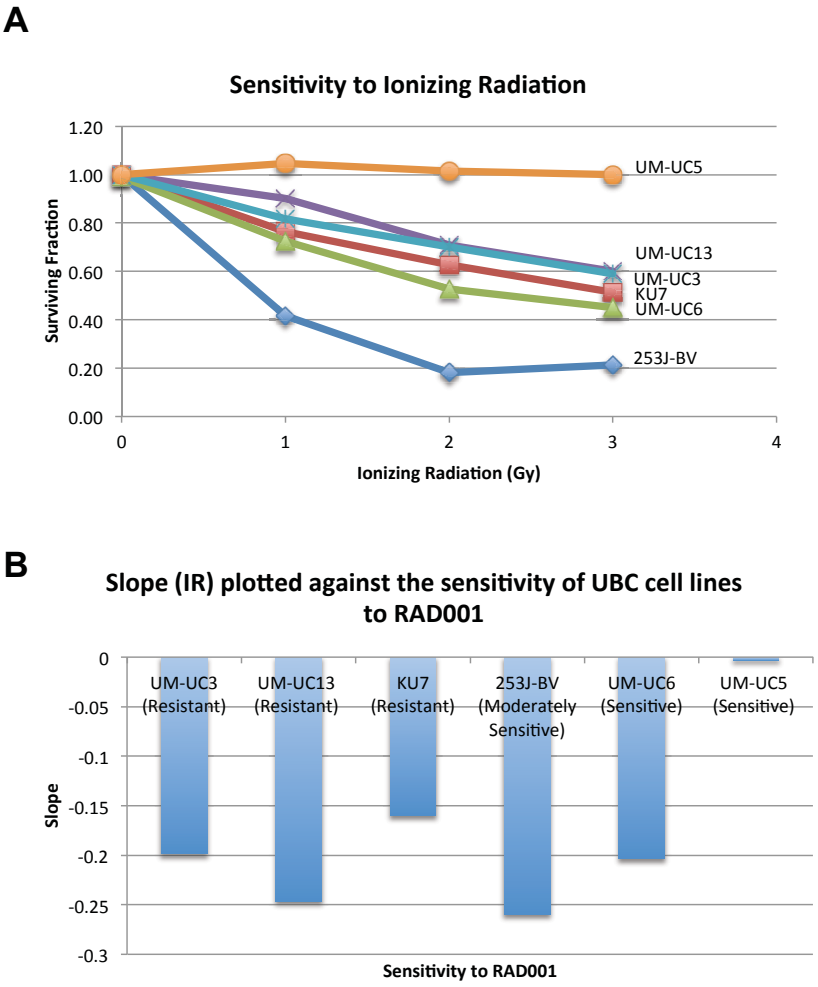

Figure 2: Response of a panel of bladder cancer cell lines to ionizing radiation. Plated cells were exposed to ionizing radiation and further cultured to measure growth by clonogenic assays, as described in Methods. (A) Based on the gathered results, we were able to classify these cell lines as radiation–sensitive, moderately sensitive and -relatively resistant. (B) The RAD001 GI_{50} was plotted against the slope of each cell line growth curve generated by colony formation assays once treated with IR.

As shown in Figure 2A, the six retained cell lines exhibited different growth response to radiation as a function of applied doses measured in Gy: UM-UC5 was the most resistant, while 253J-BV was the most sensitive. KU7, UM-UC3, UM-UC6 and

UM-UC13 were moderately resistant to radiation. Interestingly, when we plotted the RAD001 GI_{50} values against the slope of the curve for each cell line in the clonogenic assay post-IR (Fig. 2B), we found no correlation between growth sensitivity to RAD001 on one hand, and to IR on the other hand. A summary of these results is shown in Table 3.

Table 3: Classification of bladder cancer cell lines based on their relative RAD001 and ionizing radiation responses

	Radiation		
mTOR inhibition	Sensitive	Moderately resistant	Resistant
Sensitive		UM-UC6	UM-UC5
Moderately sensitive	253J-BV		
Relatively resistant		KU7; UM-UC3; UM-UC13	

No correlation was noted when RAD001 responses were compared to radiation responses.

Previous work has established the importance of the AKT survival pathway in conferring resistance and survival capabilities in different cancers, including bladder. (reviewed in [257, 333, 334]) In order to understand the molecular mechanism underlying the effect of RAD001 and/or IR on the panel of UBC cell lines, we first characterized the base-line expression levels (no treatment) of different players in the AKT survival pathway, including p-mTOR / mTOR and its close upstream and downstream signaling partners (PTEN, AKT, and p-S6/S6) by Western blot. Figure 3 shows high base-line expression levels of these signaling proteins; however, once normalized relative to β-actin

levels of expression, we found no clear association with the origin, stage or aggressive features of these nine cell lines.

We then asked how treatment with IR and/or RAD001 would affect the expression levels of the AKT survival pathway players.

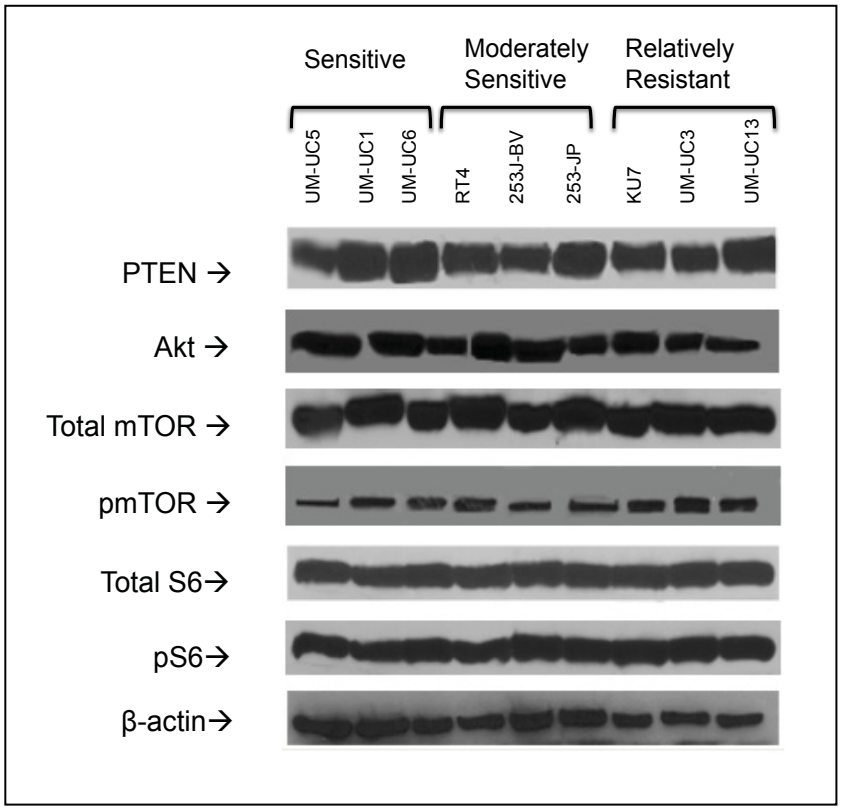

Figure 3: Expression of upstream and downstream targets of mTOR. Total protein levels of PTEN, AKT, mTOR, and its activated form, phospho-mTOR, S6 and phosphor-S6. β-actin was used as a loading reference

The effects of IR alone on AKT/p-AKT levels were assessed in the moderately resistant KU7 and the sensitive 253J-BV cell lines. Briefly, cells were treated with 4 Gy of ionizing radiation and proteins were extracted at 0, 15, 30 and 60 minutes post-

irradiation to submit lysates to Western blots analysis of AKT. In both cell lines, we found that IR induced a rapid increase in AKT activation, measured by higher p-AKT levels at 15 and 30 minutes of exposure in KU7 (~6 fold) and 253J-BV (~2.5 fold), respectively relative to constant levels of total AKT (Fig. 4). Furthermore, the response of AKT activation to radiation was dose-dependent, as evaluated in KU7 treated with 4, 6 and 10 Gy of radiation (~1.75 to 2 fold increase). Proteins were extracted at 15 minutes post-irradiation based on data shown in Fig. 4. Based on this demonstration of IR-induced AKT activation in a dose-dependent manner (Fig. 5), we used 4 or 5 Gy of radiation in subsequent experiments carried out using the KU7 cell lines.

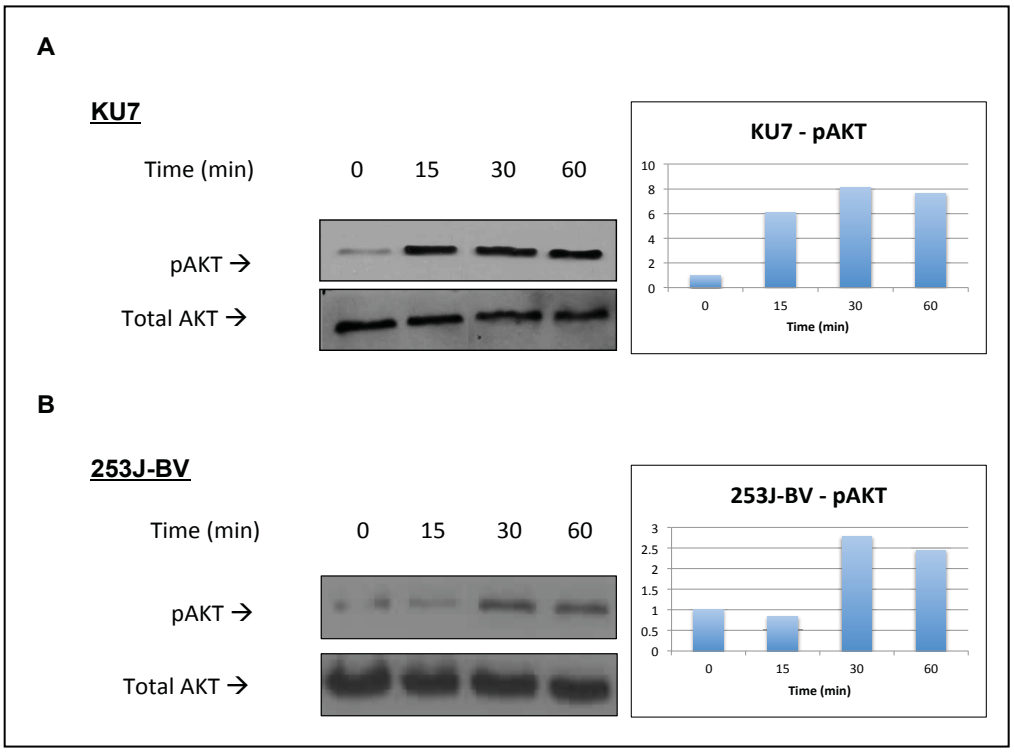

Figure 4: AKT phosphorylation following ionizing radiation. KU7 and 253J-BV cells were treated with 4 Gy of ionizing radiation. Cells were lysed 15, 30 and 60 minutes following treatment to analyze AKT activation (p-AKT; upper row) by Western blot. Total levels of AKT are shown in the lower row. Histograms represent the ratio between levels of activated AKT at each time point *vs.* overall levels of AKT protein.

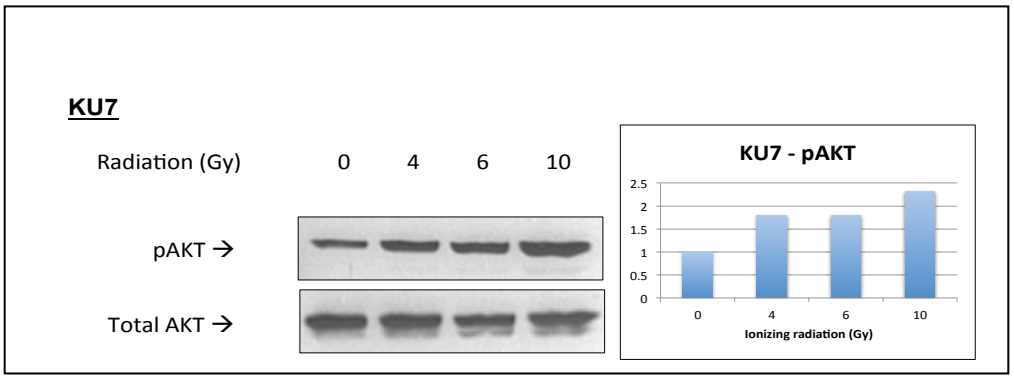

Figure 5: Dose-response of AKT activation by radiation. KU7 cells were exposed to 4, 6, and 10 Gy of ionizing radiation. Cells were lysed 15 minutes following treatment to analyze AKT activation (p-AKT; upper row) by Western blot, reported in histogram (right panel) on total levels of AKT, shown in parallel blots (left panel, lower row).

We then evaluated the time-course effects of RAD001 alone on the activation of S6, a direct downstream target of mTOR at 0, 2, 6 and 24 hrs' time points. For that purpose, levels of p-S6 compared to total S6 protein levels were assessed by Western blot in KU7 and 253J-BV, as well as in UM-UC3, selected for its aggressive behavior and resistance to RAD001, and in UM-UC6, selected for its high sensitivity to RAD001 (Table 3). For each of these cells, the respective GI_{50} dose was used depending on the sensitivity levels determined above (Fig. 1). As such, UM-UC6 was treated with 0.5 nmol/L RAD001 concentration, 253J-BV at 5nmol/L and KU7 and UM-UC3 at 75nmol/L. Results in Figure 6 show that RAD001 was effective at inhibiting p-S6 and the observed effect was time-dependent with the most sensitive being more rapidly inhibited and at a higher extent at 2 hrs. A more pronounced inhibition was observed with longer treatment time, across the four cell lines. Of interest, we also observed that the sensitivity to RAD001 positively correlated with the degree of p-S6 inhibition with the highest percent inhibition observed in sensitive and moderately sensitive UM-UC6 and 253J-BV cell

lines respectively, and a lower extent of inhibition in the relatively resistant UM-UC3 and KU7.

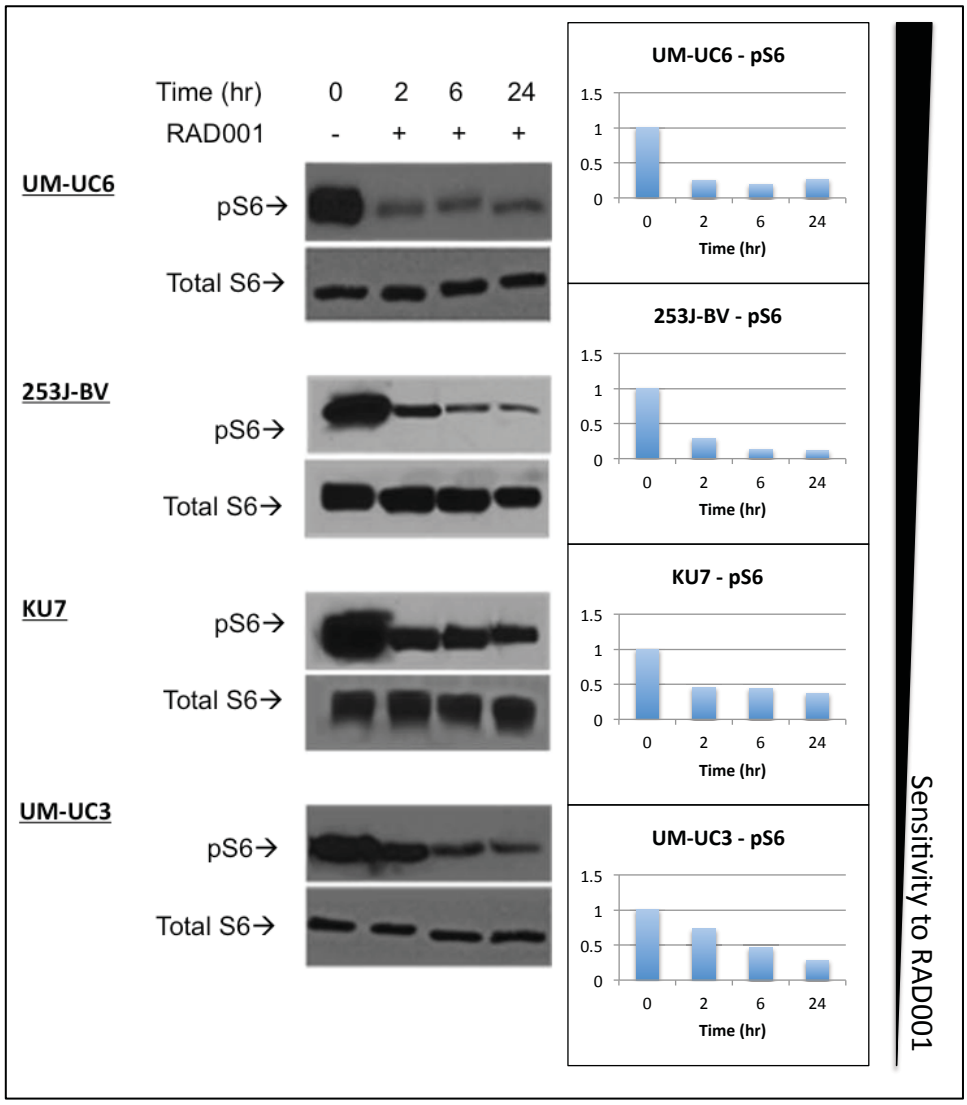

Figure 6: Expression of downstream targets of mTOR and effects of RAD001 on S6 activation. The activation S6 by phosphorylation of Ser240/244 (upper row, left panels) and total S6 levels (lower row, left panels) were determined by Western blotting of proteins from RAD001-treated cells for various time periods. Levels were assessed by scanning bands and used to calculate the extent of S6 activation (% of controls) reported over total S6 (histograms in right panels, presented according to their relative RAD001 sensitivity. These time-course effects of RAD001 were carried out at the GI50 concentration, as determined in sensitive (UM-UC6 at 0.5 nmol/L), moderately sensitive (253J-BV at 5 nmol/L) and relatively resistant (KU7 a and UM-UC3 at 75 nmol/L) bladder cancer cell lines.

Given the observed AKT activation following IR, and the decreased p-S6 levels that resulted from treatment with RAD001, we investigated the effects of combining IR and RAD001 on bladder cancer *via* the six cell lines characterized above by colony formation assays: 253J-BV, UM-UC6, KU7, UM-UC3, UM-UC13 and UM-UC5. Briefly, they were treated with RAD001 at their respective GI_{50} values 12 hrs prior to IR exposure up to 4Gy as indicated in the Material and Methods. In all tested cell lines, we observed in Figure 7 that combining RAD001 and IR conferred a decrease in cell growth compared to IR exposure alone, and the observed difference was statistically significant ($p< 0.05$). The combination of IR and RAD001 thus seems to result in additive, growth inhibitory effects on bladder cancer cells, pointing to radio-sensitization of these cells by RAD001.

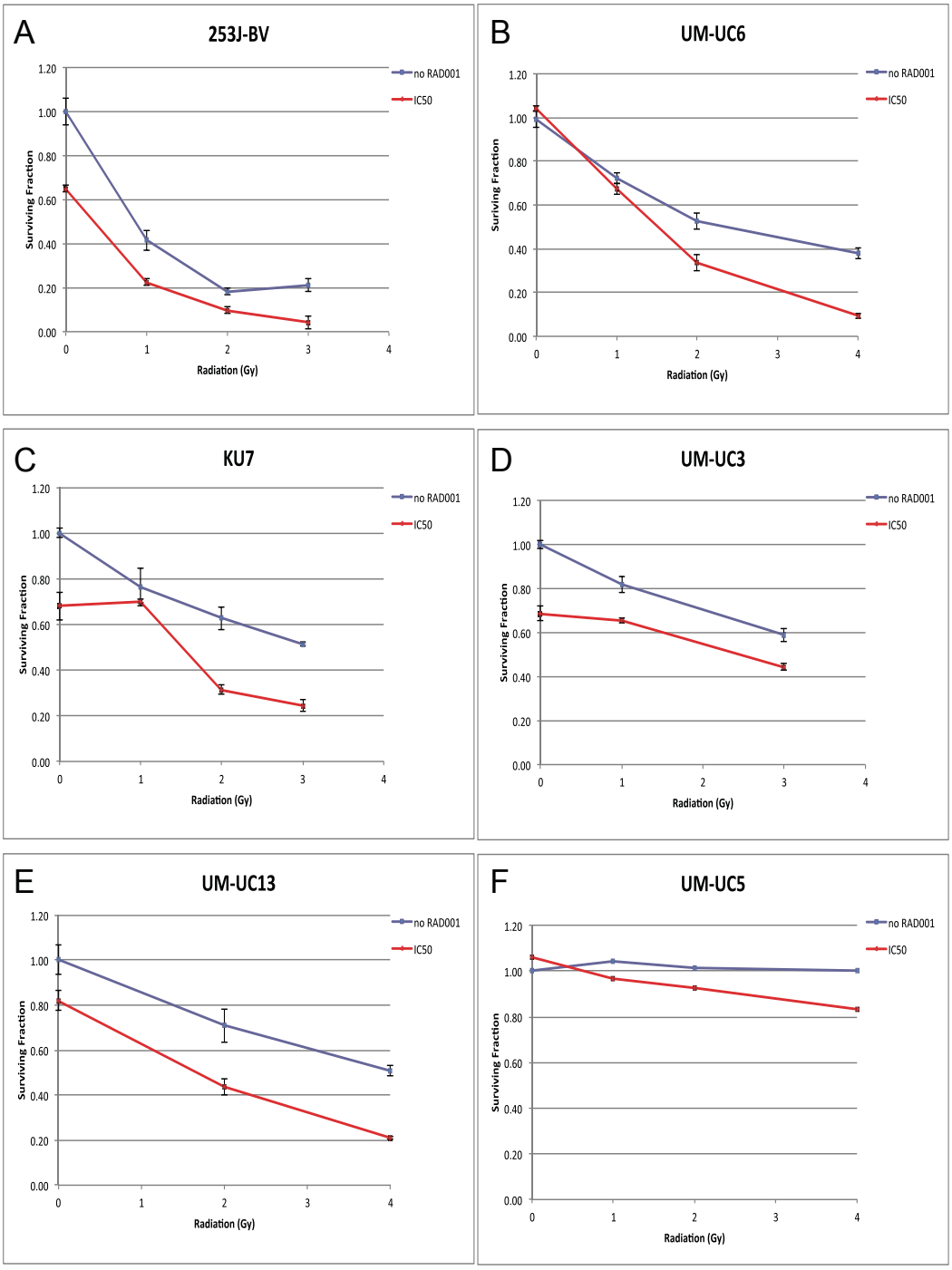

Figure 7: RAD001 combined with ionizing radiation in bladder cancer lines. Six cell lines (presented by increasing relative resistance to ionizing radiation (A) 253J-BV, (B) UM-UC6, (C) KU7, (D) UM-UC3, (E) UM-UC13, and (F) UM-UC5) were treated with RAD001 for 12 hrs before exposure to ionizing radiation and further grown as indicated in Methods. Colony formation was measured after cell fixation and staining with crystal violet, 10-14 days after treatment depending on cell lines. Results were statistically significant ($p<0.05$) in the combined treatment compared to either treatment alone in all tested cell lines.

To understand the effect of this combination on the underlying molecular pathway, we studied AKT and S6 protein expression and activation in KU7, 253J-BV, UM-UC6 and UM-UC3 cell lines using Western blot. The cells were treated with placebo, IR (5 Gy) alone, 0.5-100 nmol/L of RAD001 alone (depending on respective GI_{50}), or both. Exposure to IR was carried 12 hrs after RAD001 treatment, and levels of p-AKT and p-S6 were assessed at 30 minutes post-IR exposure. Results in Figure 8 show that across the four cell lines, IR alone had no effect on the inhibition of p-S6, while combination of IR and RAD001 were highly potent to lower the levels of p-S6 activation compared to unchanged total S6 protein due to RAD001. Furthermore, we observed that RAD001 alone could also contribute a slight increase in p-AKT compared to the control, similar to IR alone, and this could be explained by the existence of different negative control loops that will be described later on. Taken together, we conclude that the survival AKT pathway is turned on by ionizing radiation while protein synthesis is slowed down by RAD001 treatment due to inhibition of the key S6 protein activation.

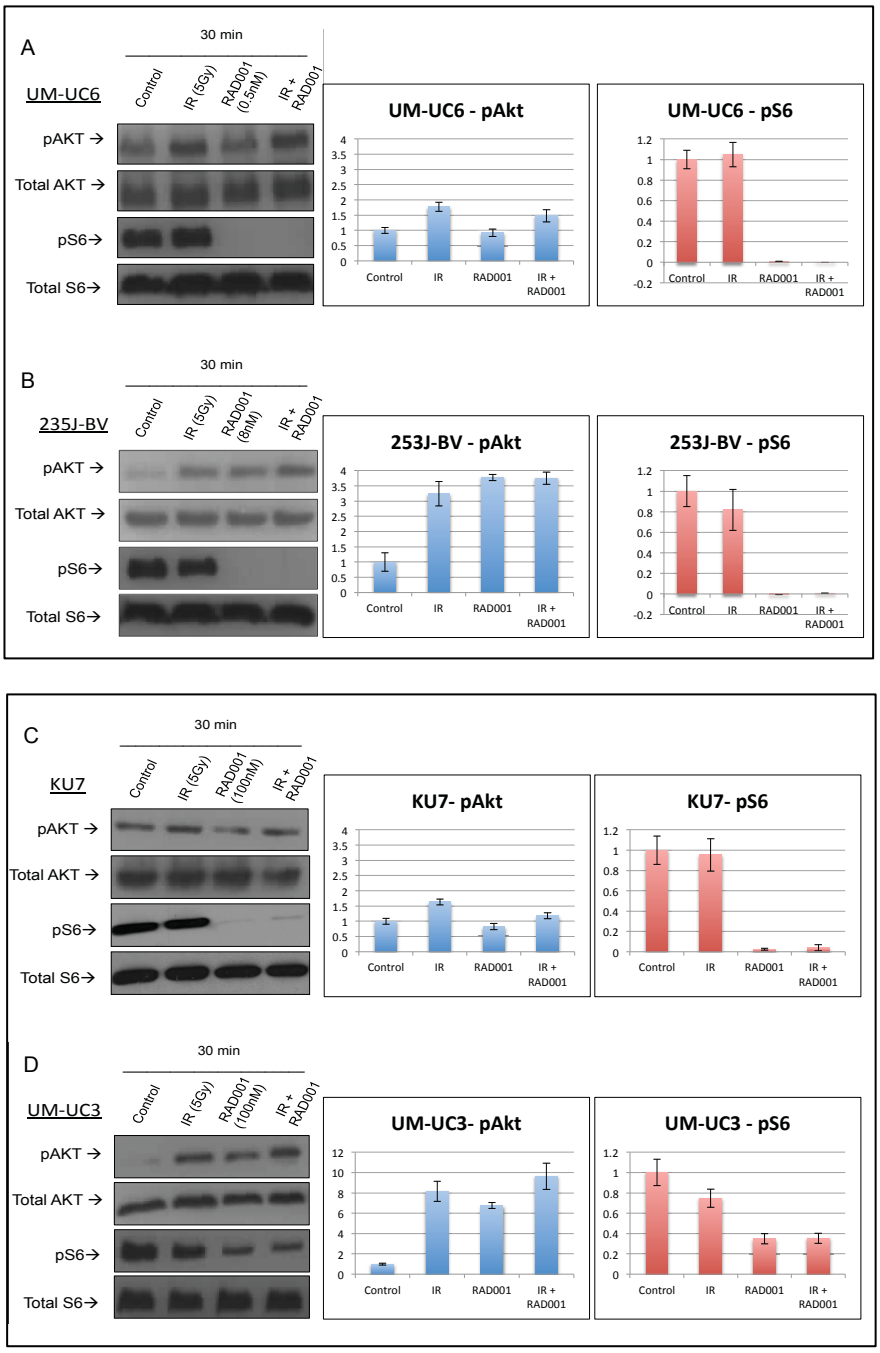

Figure 8: Effect of ionizing radiation and RAD001 alone on AKT and S6 phosphorylation respectively (A) UM-UC6 (B) 253J-BV (C) KU7 and (D) UM-UC3 cells were treated with 5 Gy of radiation, various doses of RAD001 (0.5 nM for UM-UC6, 8nM for 253J-BV, 75nM for KU7 and 100nM for UM-UC3) or both, and lysed. Levels of AKT and S6 activation by phosphorylation were analyzed by Western blots and reported to total levels of AKT and S6 levels. Levels of p-S6 activation were fixed at 1.0 for controls. ($p<0.05$).

Based on these promising *in vitro* results, we investigated whether the observed additive inhibitory effects of IR and RAD001 on cell growth inhibition through the clonogenic assays, could be observed in bladder tumors *in vivo*. For that purpose, female nude mice were subcutaneously implanted with either the relatively radio-resistant KU7 bladder cancer cells or the radiosensitive 253J-BV cells, as described in the Material and Methods. For both BC models, all mice (n=112 with 56 mice per cell line) developed tumors.

As shown in Figure 9, the periodic monitoring of KU7 bladder tumor development by measuring volume of subcutaneous tumors over time revealed a most rapid growth in controls, as expected and reaching the experimental points within 4 weeks (Fig. 9A).

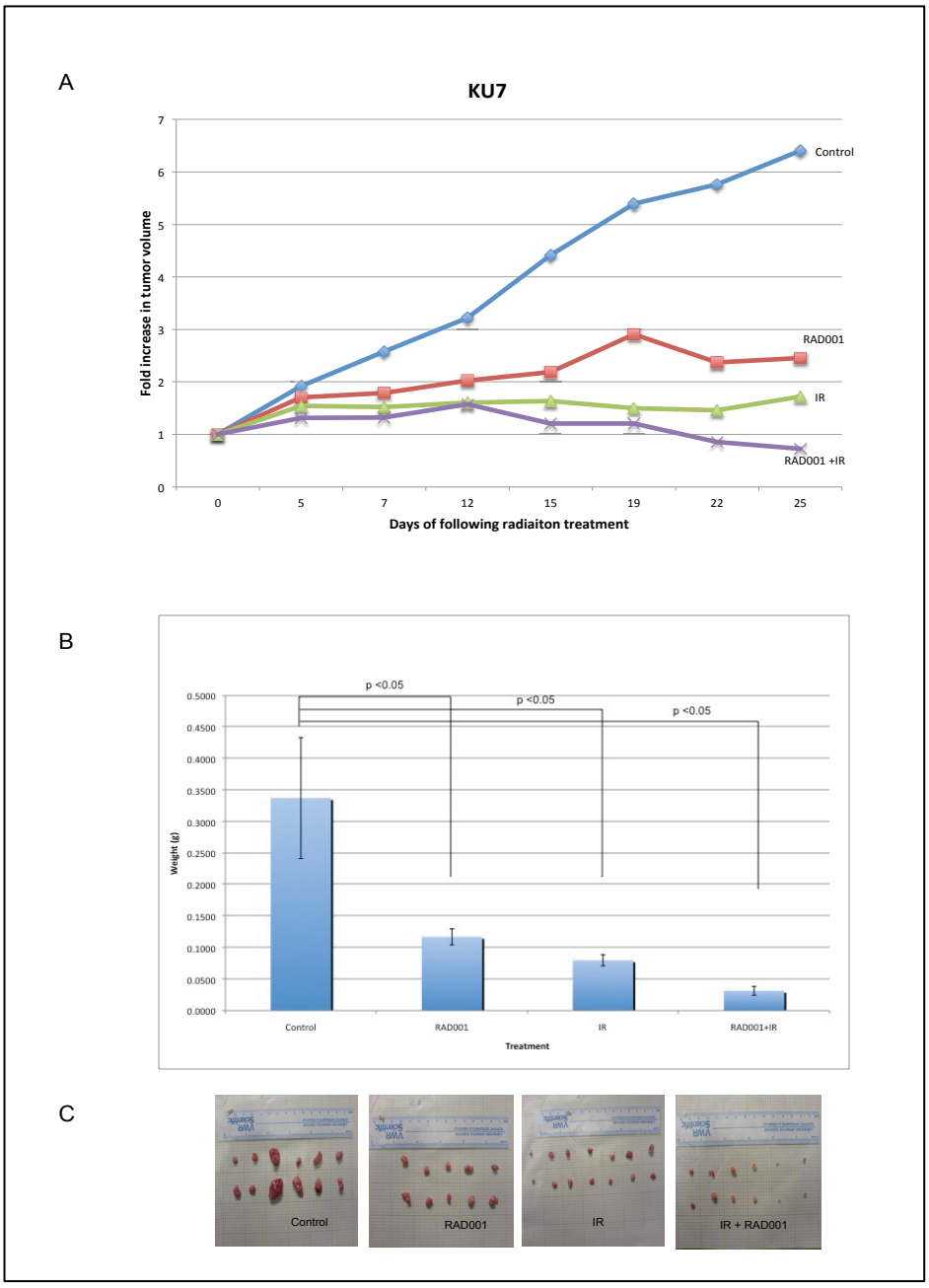

Figure 9: Effect of RAD001 and ionizing radiation on UBC tumors xenografts derived from KU7. Athymic (nude) mice were implanted with KU7 as described in Methods and randomized in treatments arms (n=14 / group): controls (glucose solution), IR (9Gy administered in 3 fractions over one week), RAD001 alone (oral dose of 1.5 mg/kg daily for 4 weeks) or combined with IR (RAD001 initiated one day prior to IR and continued over 4 weeks). (A) Increase in tumor volume over time. (B) Tumor weights (in grams) reached at the experimental endpoint and expressed as mean weight of tumors harvested for each group of mice in the 4 treatment arms, as indicated. (C) Macroscopic view of excised tumors. Statistical significance is indicated ($p<0.05$).

All treatments significantly slowed down the tumor growth rate ($p<0.05$), as indicated by change in tumor volume relative to the reference point at Day 0 and between treatments arms. Maximal inhibitory effect was observed at 5 weeks of treatment (1 week with RAD001 and 4 subsequent weeks following radiation). The difference between each treatment arm on the one hand, and with the control arm on the other was significant, with the combination therapy (RAD001 and IR), achieving the most effective or potent inhibition followed in decreasing order by IR and then RAD001.

These findings were supported by tumor weights, with intermediate size tumors observed upon administration of either of the two treatments alone (IR or RAD001) and smallest ones collected from in the combined therapy arm. The average tumor weight for the combination arm was 31 mg, compared to 117 mg for RAD001 alone, 80 mg with IR alone, and 340 mg for control treatment. Such decreases in tumor weights were statistically significant and also observed in 253J-BV xenografts (Fig. 10). Again the combination of RAD001 and ionizing radiation showed a maximal inhibitory effect on bladder cancer growth compared to control mice whereas treatment with either RAD001 alone or IR alone were less potent but still yielded a statistically significant inhibition of tumor growth compared to control mice.

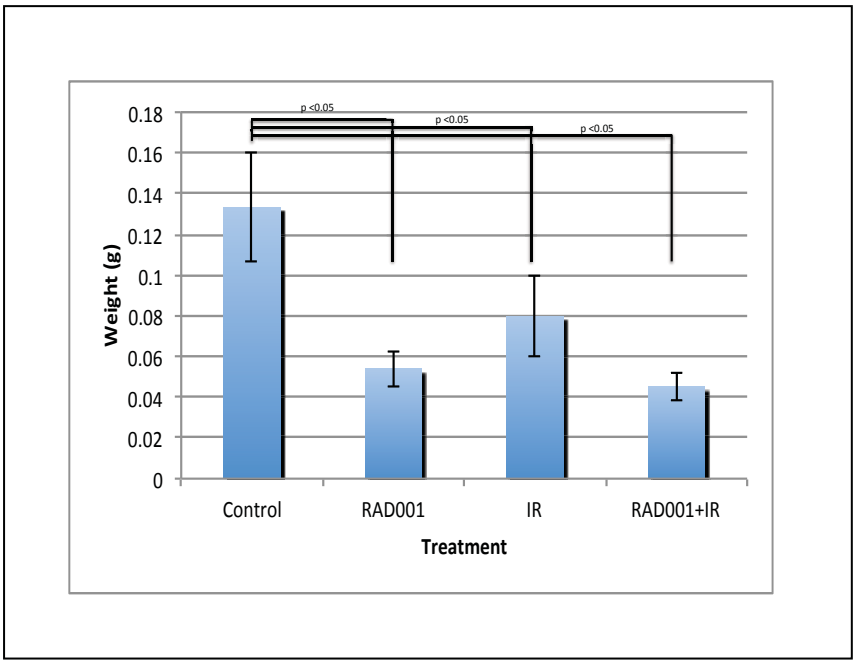

Figure 10: **Effect of RAD001 and ionizing radiation on UBC tumors xenografts derived from 253J-BV**. The bladder tumor model of 253J-BV was implanted in athymic mice as described in Methods. Tumor weights (in grams) at the experimental endpoint were determined for each group of mice in the four arms. Results are expressed as the mean weight of tumors for each group. Statistical significance is indicated ($p<0.05$).

4.2 The effects of RAD001 and IR on UBC cell cycle

The above findings of a combination of RAD001 and ionizing radiation causing an additive growth inhibition across a panel of UBC cell lines, compared to each treatment administered separately, prompted a study of the underlying mechanism by analyzing consequences on the cell cycle. Flow cytometry was performed (as described in Material and Methods) to determine the ratio of cells in each phase of the cell cycle relative to the total cell population analyzed 48 hrs following each treatment (RAD001 at a dose equivalent to respective GI_{50} for each cell line; RT at 4 Gy and a combination) and compared to untreated control cells. This cell distribution throughout the various phases of the cell cycle was determined in the KU7, UM-UC3, UM-UC6 and 253J-BV.

Results in Figure 11 show that when RAD001 was administered alone, it mainly induced an arrest in the G0/G1 phase across the four cell lines: in KU7, 62% (+/- 4%) of cells were detected in the G1 phase, compared to 54% (+/- 3%) in untreated control; For UM-UC3, UM-UC6 and 253J-BV, 71% (+/-6%), 77% (+/-3%) and 67% (+/- 4%) of cells were arrested in the G1 phase, compared to their untreated controls with 64% (+/-2%), 66% (+/-2%) and 55% (+/- 3%) respectively ($p<0.05$).

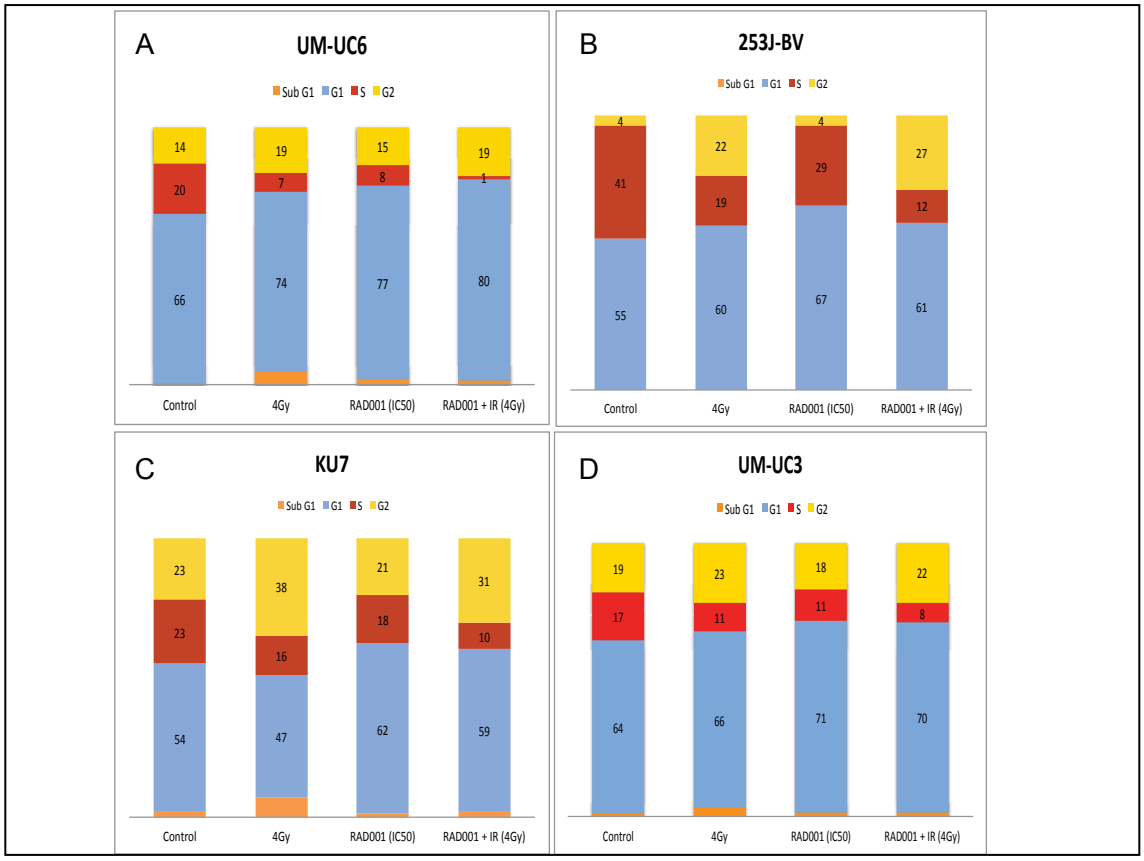

Figure 11: Effect of RAD001 and ionizing radiation on the cell cycle. UBC cell lines ((A) UM-UC6, (B) 253J-BV, (C) KU7, (D) UM-UC3) were treated with RAD001 alone, ionizing radiation alone and with the combination of RAD001 and radiation. For the latter series, samples were pre-treated with RAD001 for 6 hrs prior to radiation. Cells were fixed 48 hrs after treatment and stained for propidium iodide intake measured by flow cytometry. The proportion of cell populations in the different phases of the cell cycle is shown for each cell line by colored bars (G0/G1: Orange/Blue; S: Red and G2: Yellow).

When cells were subjected to 4 Gy of ionizing radiation alone, a clear arrest in the G2 phase was observed (Fig. 11), with significant increases ($p<0.05$) in the percentage of cells in this phase compared to the respective untreated controls, 38% (+/- 4%) vs. 23% (+/- 2%) of KU7 cells were detected in the G2 phase ($p<0.05$), 23% (+/- 4%) vs. 19% (+/- 3%) for UM-UC3 ($p<0.05$), 19% (+/- 4%) vs. 14% (+/- 3%) for UM-UC6 ($p<0.05$), and 22 % (+/- 3%) vs. 4% (+/- 2%) for 253J-BV ($p<0.05$).

The combination of RAD001 with ionizing radiation clearly resulted (Fig. 11) in an arrest of cell progression within the cell cycle characterized by both an arrest in G0/G1 and the G2 phases, which supports an additive inhibitory effect on cell cycle progression as compared to single treatments.

Interestingly, the observed increase in the G0/G1 and the G2 phases in the combined treatment occurred in parallel with a clear decrease in the percentages of cells in the S-phase compared to either the untreated control (20% → 1% for UM-UC6, 41% → 12% for 253J-BV, 23% → 10% for KU7 and 17% → 8% for UM-UC3, $p<0.05$) and to each treatment alone. This might be indicative of a shift in cell cycle distribution induced by RAD001, eventually sensitizing the cells to radiations and explaining the cytostatic effect that leads to the additive inhibition of cell cycle progression and subsequent growth as previously depicted.

In order to understand the molecular changes underlying the shifts in the cell cycle following combined treatment with RAD001 and ionizing radiation, we investigated the levels of expression of cell cycle regulators associated with the cycle checkpoints in the same set of UBC cell lines: UM-UC6, 253J-BV, KU7 and UM-UC3 and measuring by Western blots levels of the cyclin D1 protein, a positive regulator that mediates the G1/S transition, and the p27 and p21 proteins as inhibitors of cell cycle progression.

Results in Figure 12 show that for KU7 cells, which are relatively resistant to both RAD001 and ionizing radiation, the level of cyclin D1 was drastically decreased 24 hrs post-treatment with a dose of RAD001 equivalent to the GI_{50} alone, or with the RAD001 combination with ionizing radiation. Radiation alone also reduced cyclin D1 protein

levels but to a lesser extent (~30%; normalized to endogeneous actin) in comparison to almost non-detectable cyclin D1 with the two other treatment arms. Given the role of cyclin D1 as a positive regulator of the G1/S transition, the observed decrease in its expression levels in the two arms involving RAD001 is in line with the observed increase in cells arrested in the G1 phase along with the decrease in the percentage of cells in the S-phase.

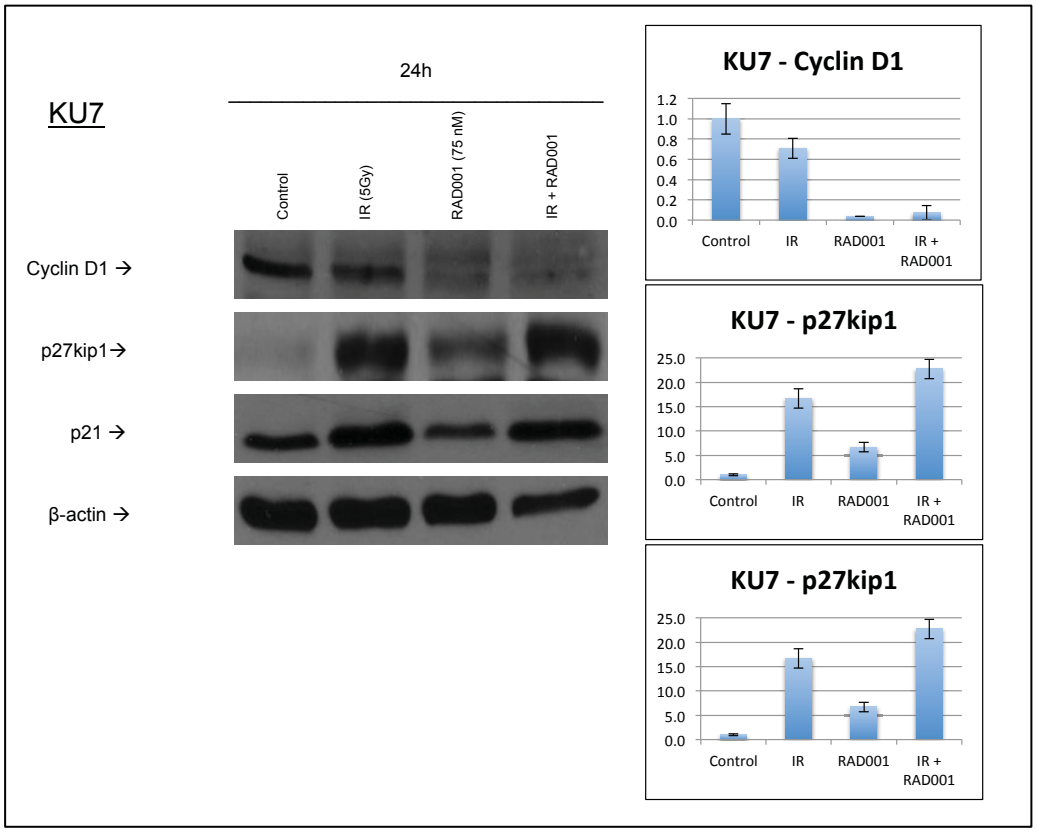

Figure 12: Expression of cell cycle regulatory proteins following RAD001 and IR treatments. Bladder cancer KU7 cells were treated with RAD001 or IR alone and the combined treatment. A pre-treatment period of 6 hrs with RAD001 preceded the treatment with ionizing radiation and proteins were extracted from lysed cells 24 hrs after treatment for Western blotting of cyclin D1, p27kip1 and p21 as described in Methods. Levels were determined by scanning each protein band and normalized in right panels (bar graph) as a function of actin levels measured in parallel in each cell lysate.

For p27, on the other hand, a 24-hr treatment with RAD001 alone, with ionizing radiation alone and with the combined treatment clearly led to a substantial increase in the expression levels of p27 (15- and 22-fold respectively), compared to the untreated control where p27 was barely detectable. RAD001 alone led to ~6-fold increase. These changes were significant and reproducible. Since p27 inhibits the G1/S transition, the increase in its expression level further supports the observed increase in the percentage of cells arrested in the G1 phase, and the decrease of the cells in the S-phase observed above.

The treatment of KU7 cells with ionizing radiation alone increased the levels of p21, a cell cycle progression inhibitor, by ~ 1.5 fold compared to the untreated control. The addition of RAD001 alone to KU7 cells induced a slight decrease, in the order of ~ 10% in p21 levels, overcome by the combination of RAD001 and ionizing radiation which led to highest p21 levels, ~ 2.2 fold compared to controls. Altogether these findings on cell cycle regulators being most affected in the combined treatment, with decrease of cyclin D1 attributed to RAD001 whereas increases in the p27 and p21 inhibitors reflect the IR response, speak in favor of additive effects, with RAD001 radio-sensitizing UBC and eliciting highest treatment response.

4.3 The role of p21 in RAD001-induced radiosensitization of bladder cancer: in vitro and in vivo

As stated in the introduction, the response to RAD001 or ionizing radiation may be due to the dual role that p21 plays in cell cycle regulation: a cell cycle checkpoint inhibitor acting at the G1 checkpoint, and an anti-apoptotic factor with radiation actually inducing cells to resist apoptosis.

In order to investigate the p21 role duality noted above in bladder cancer cells response to combined RAD001 and radiation, we determined baseline expression levels of p21 across the nine UBC cell lines and then tested if they correlate with treatments. Figure 13A shows Western blots of the p21 protein which was easily detected in UM-UC1, UM-UC6, 253-JP, 253J-BV, RT4 while in the other cell lines including KU7, p21 was minimally expressed and sometimes not detectable in comparison to most expressing cell lines. Interestingly, when p21 baseline levels were expressed relatively to endogeneous levels of actin, we observed a positive correlation with each cell line sensitivity to RAD001, with the exception of UM-UC5.

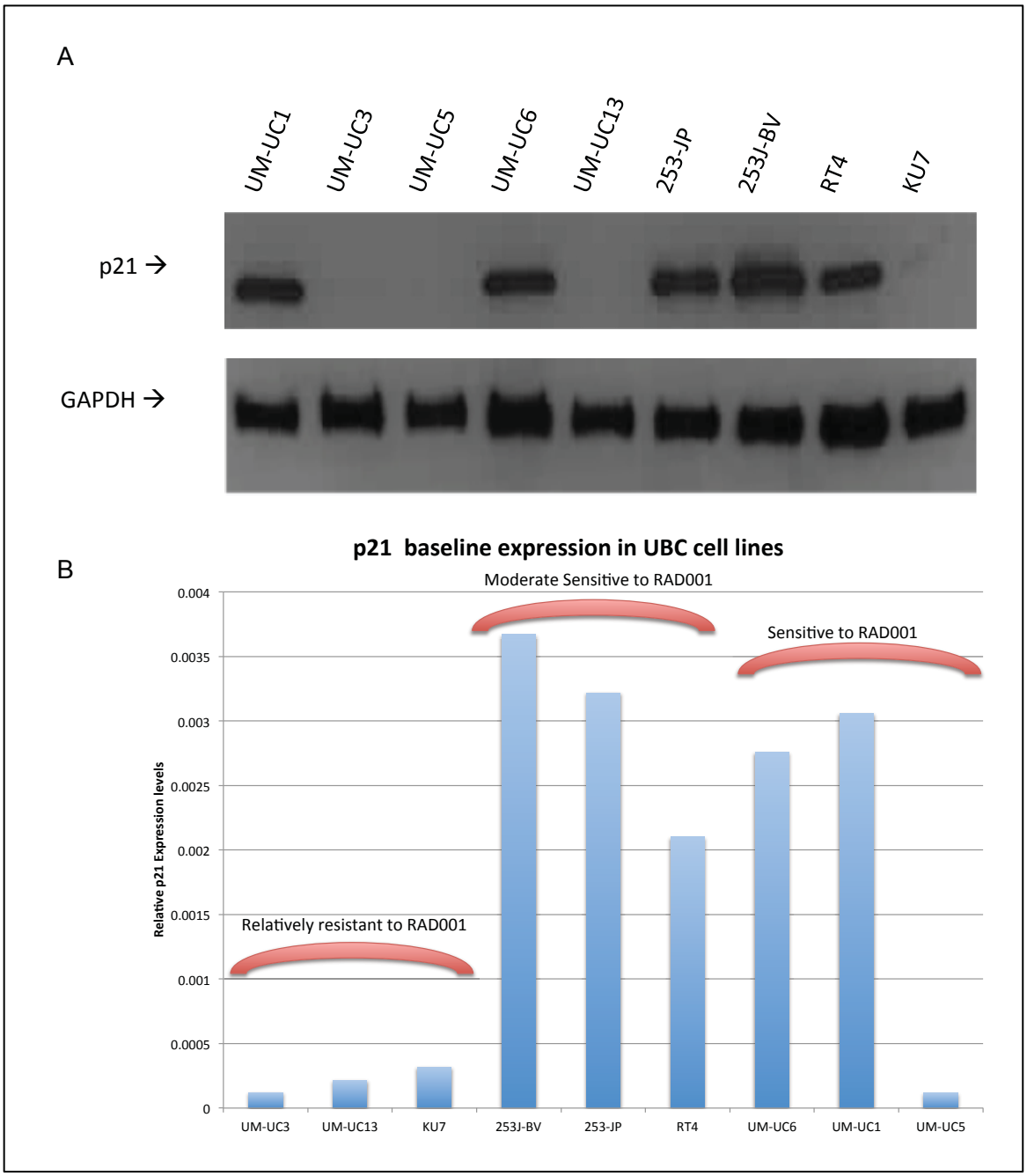

Figure 13: Expression of the p21 protein in the panel of bladder cancer cell lines and correlation with their RAD001 sensitivity. (A) Extracted proteins were submitted to electrophoresis and Western blotting with p21 antibody as described in Methods. Protein bands were scanned and normalized to levels of GAPDH, a 38kDa internal control protein band (loading control in lower panel) in all samples. **(B)** p21 expression was plotted in relation to RAD001 sensitivity in all cell lines tested, as obtained by MTT (Fig. 1) and represented by GI_{50} values. Cross-referencing points to a correlation between the sensitivity of the cell lines to RAD001 and the presence of the p21 protein endogenously, except for UM-UC5.

In a second step, p21 levels were re-assessed in four cell lines 24 hrs following treatment with RAD001 (at respective GI_{50}) and ionizing radiation (5Gy) alone, and combined. Figure 14 shows that p21 levels were higher in cell lines known to be sensitive to RAD001 (UM-UC6 and 253J-BV) compared to those highly resistant to RAD001 (UM-UC13) whereas p21 levels in untreated cells were considerably lower. KU7, however seems to be an exception, with relatively high levels of p21 expression, despite showing resistance to treatment with RAD001.

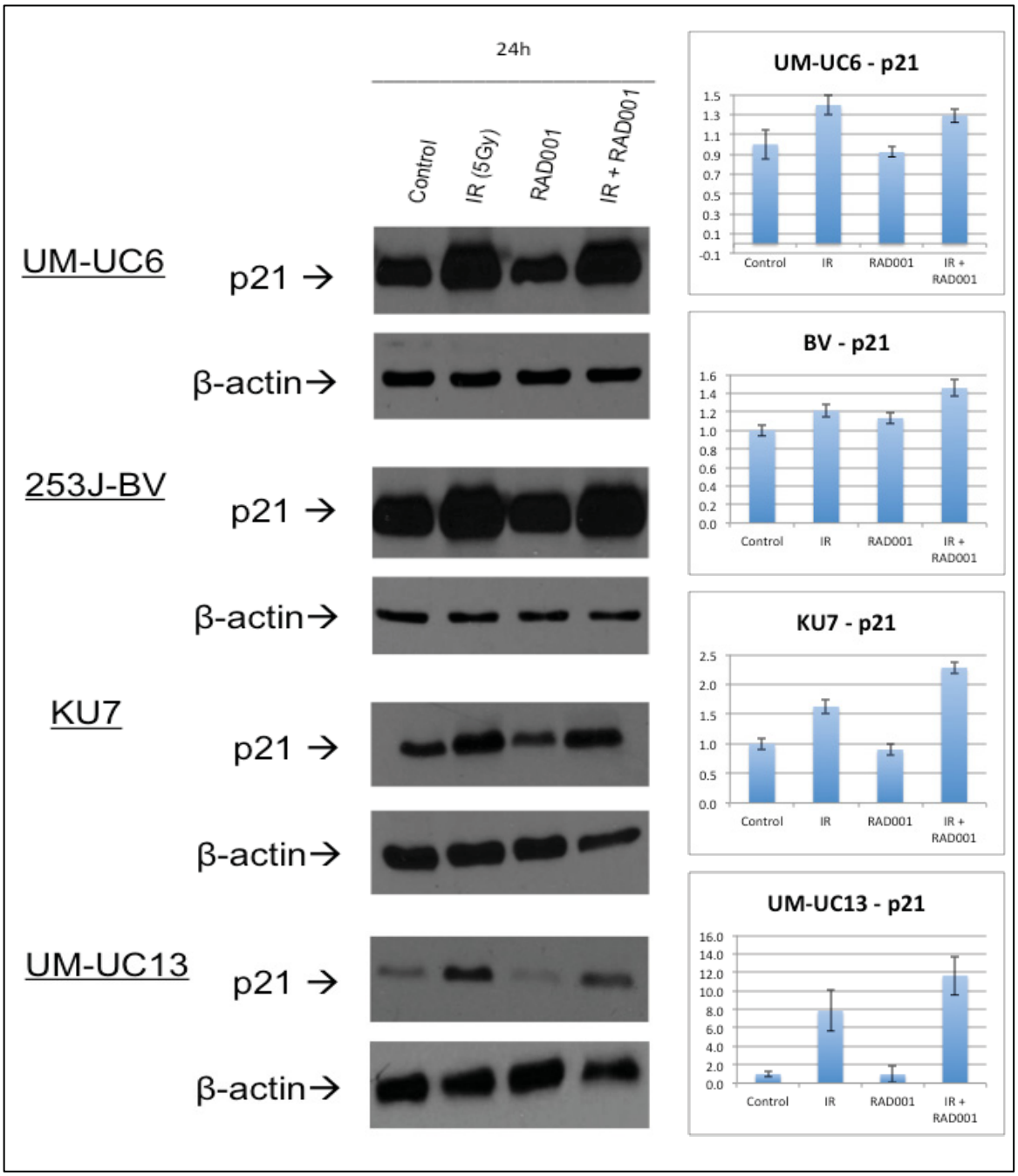

Figure 14: Expression of p21 protein following RAD001 and IR treatments alone and in combination. Bladder cancer cells (UM-UC6, 253J-BV, KU7 and UM-UC13) were treated with RAD001, IR or the combined treatment, as depicted above. After 24 hrs, proteins were extracted for Western blot analysis of p21 and β-actin in parallel. Bands were scanned and normalized to actin (right panels), ($p<0.05$).

We noticed, as shown in Figure 14, that as above treatment with the GI_{50} dose of RAD001 resulted in a slight decrease or no change in p21 levels (normalized to actin), while treatment with IR induced increases in p21 expression, at varying extent according to cell lines (~ 1.4-fold in UM-UC6, 1.2-fold in 253J-BV, 1.6-fold in KU7 and 7.5-fold in UM-UC13, $p<0.05$) The combined treatment resulted in a further enhancement of p21 expression in three of the four cell lines (~ 1.4-fold in 253J-BV, 2.2-fold in KU7 and 12-fold in UM-UC13, $p<0.05$) whereas in UM-UC6, p21 levels were at a level similar to radiation treatment alone.

Accordingly, we interrogated if p21 plays a functional role in mediating the growth response to these treatments. For that purpose, we specifically targeted p21 in KU7 cells using a short-pin RNA (shRNA). As Figure 15 shows the p21 shRNA was efficient in stably knocking down its expression, reducing levels by ~58%, while the scrambled control shRNA exhibited no significant effect as p21 expression remained similar to non-transfected cells.

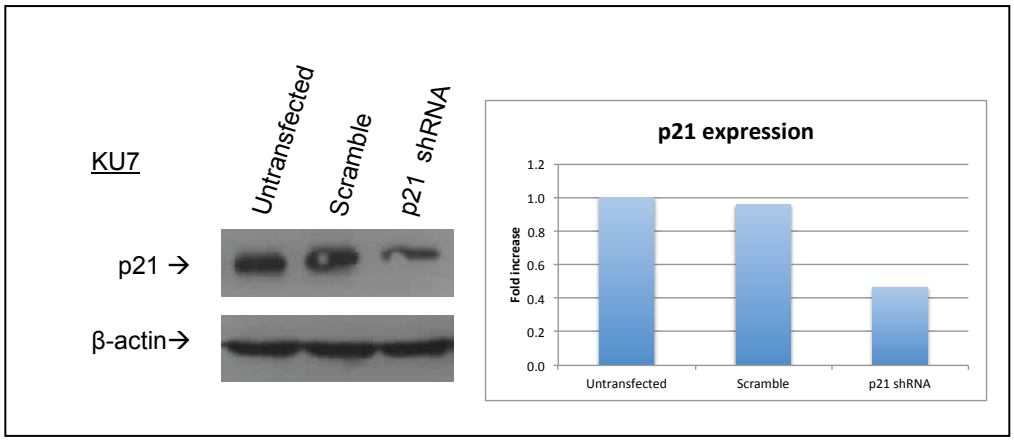

Figure 15: Targeting p21 with shRNA in KU7. Cells were treated with shRNA for p21 and scrambled, as described in Methods. The knockdown efficacy was measured by Western blot of p21 (top left panel), with levels expressed in the right panel relatively to actin, which was probed in parallel (lower left panel).

The stably transfected KU7 cells expressing lower p21 levels were morphologically similar to the parental control cell line (non transfected) or cells transfected with scrambled shRNA. However, KU7 expressing reduced p21 levels took a little longer time to detach during trypsinization, suggesting changes in cell-substratum (polystyrene, with deposition of extracellular proteins) or cell-cell interactions. In clonogenic assays performed on p21shRNA-KU7 cells exposed to increasing dose of ionizing radiation (0-8 Gy), with or without pre-treatment with RAD001 (at GI_{50}), we observed that p21 knockdown resulted in lower colony formation compared to non - transfected cells and cells transfected with scrambled shRNA (Fig.17).

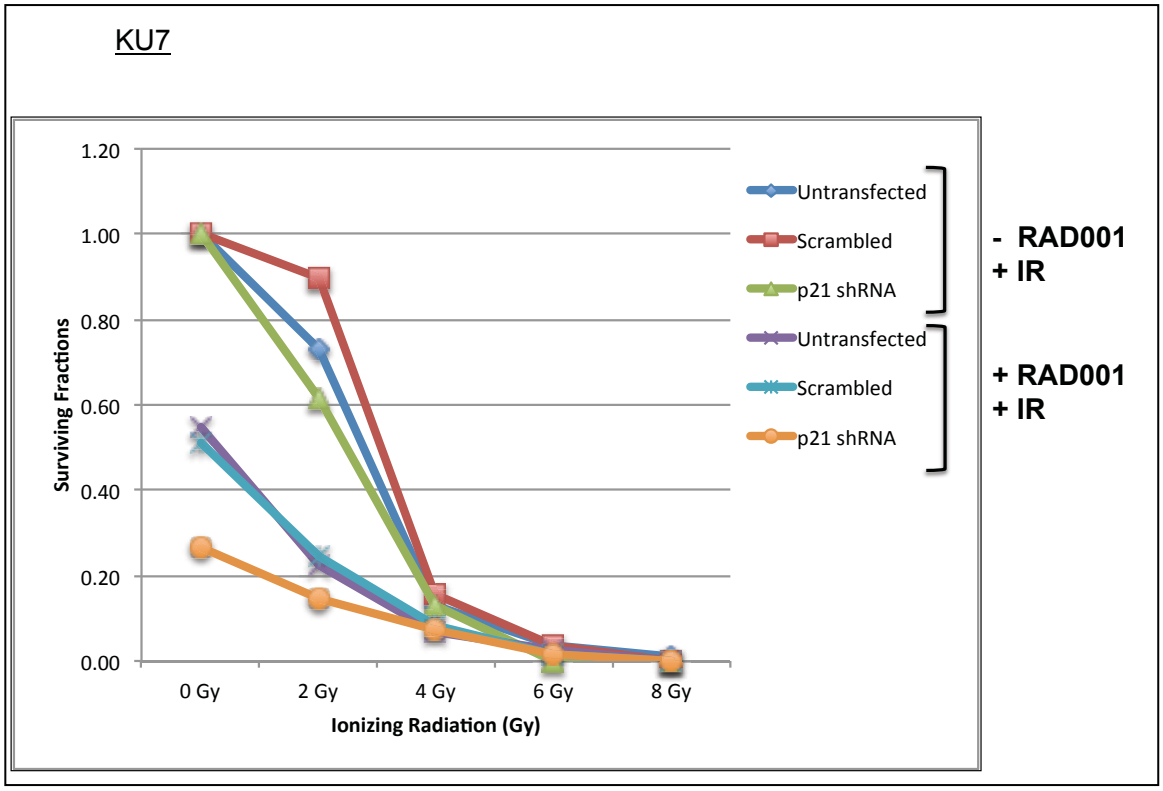

Figure 16: Role of p21 in RAD001-induced radiosensitization of bladder cancer cells. KU7-stably transfected with p21 shRNA, scramble shRNA and controls (parental) were treated with RAD001 for 12 hrs (or left untreated) before IR at varying doses (2-8Gy) and further grown to assess colony formation 10 days after treatment as indicated in Methods. Results were statistically significant ($p<0.05$) in the combined treatment compared to either treatment alone, as well as between the p21 shRNA transfected cells compared to the un-transfected/scramble shRNA-transfected cells.

This effect was evidenced with or without treatment with RAD001, implying a lower surviving fraction of cells expressing low levels of p21 in response to ionizing radiation. However the most significant inhibitory effect on colony formation ($p<0.05$) was observed when the mTOR inhibitor, RAD001, was coupled to the p21 knockdown compared to untreated (no RAD001) p21-knockdown cells. This effect was also seen but to a lesser extent with scrambled shRNA and un-transfected cells whereby the addition of RAD001 to ionizing radiation was enough to decrease KU7 cell colony formation, in agreement with earlier results, as illustrated in Figure 7. From these findings, we concluded that in addition to the role of RAD001 in providing additive effects to ionizing radiation, p21 plays a major role in mediating the UBC cell response to the mTOR inhibitor and also enhances their sensitivity to radiation.

The interplay between p21, RAD001 and ionizing radiation was further investigated in KU7-derived UBC tumors growing in nude mice by an assessment of p21 expression levels by IHC according to the treatment arms defined in Figure 9.

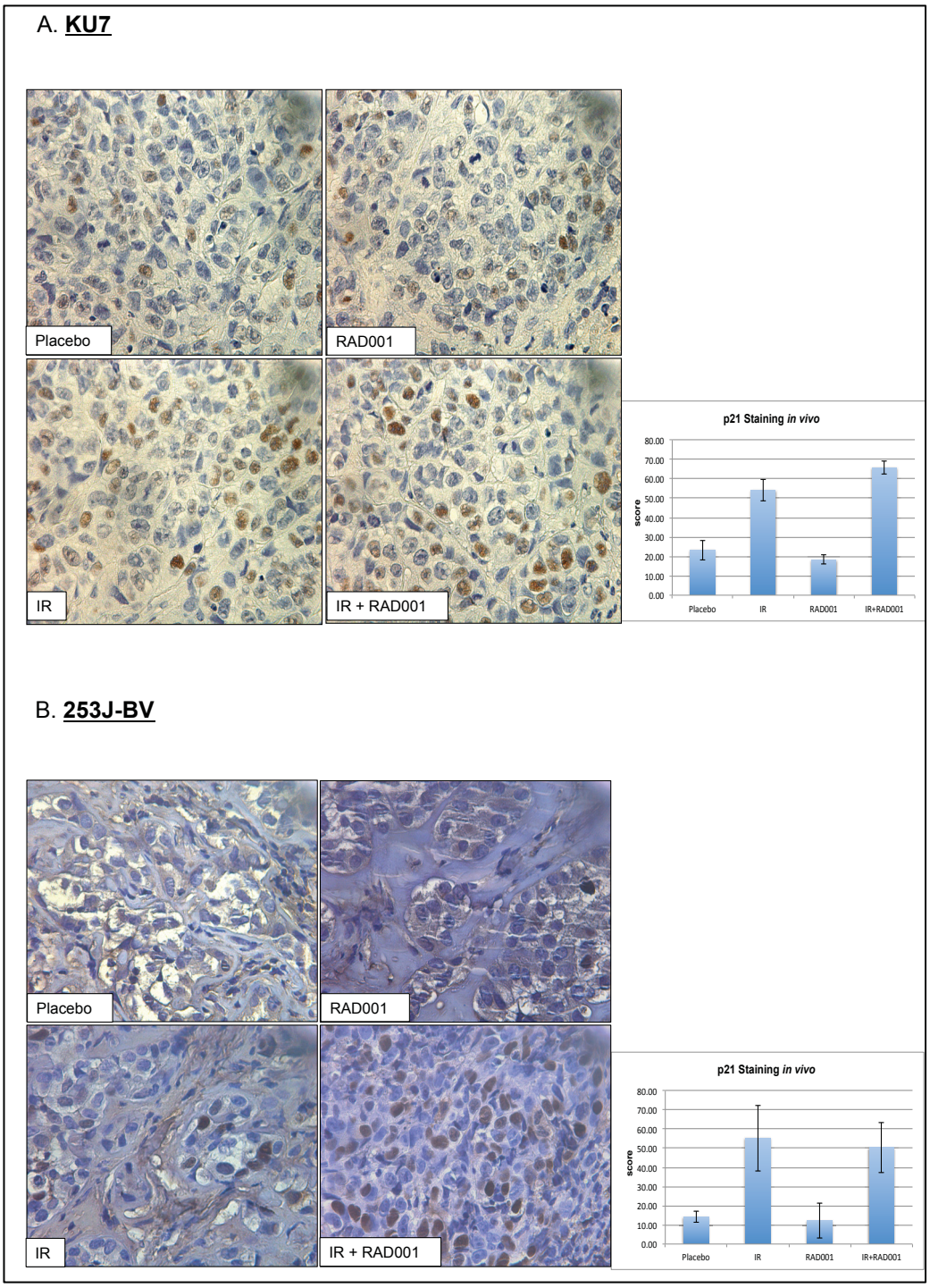

Figure 17: p21 expression in UBC tumors grown in mice treated with placebo, RAD001 or IR alone and in combination. (A) KU7 and **(B)** 253J-BV derived tumors. Paraffin-embedded tumors were processed for IHC with p21 antibodies and cells scored for their reactivity and the nuclear staining intensity to determine H scores as described in Methods.

As illustrated in Figure 17A (bar graph in right panel), radiations significantly enhanced the p21 nuclear protein level ($p<0.05$) compared the placebo, as quantified by H score, in otherwise morphologically similar KU7 tumors (microphotographs in left panels; 40X magnification). Levels were slightly lower in the RAD001 treatment arm ($p>0.05$) and highest with an H score of 65 in the combined RAD001 and ionizing radiation treatment arm ($p<0.05$).

Results observed in the 253J-BV xenograft model were relatively similar (Fig. 17B), with ionizing radiation significantly inducing nuclear p21 expression, while treatment with RAD001 had minimal marginal ($p>0.05$) effect compared to the placebo. Co-treatments with RAD001 and ionizing radiation yielded H scores similar to the radiation treatment arm alone (~50-53).

It is interesting to note that these *in vivo* observations mirror earlier *in vitro* findings on p21 levels measured in Western blots (Figs 12 and 14). However, while cells *in vitro* received their respective GI_{50} doses of RAD001, mice were administered RAD001 at clinical doses prior IR and continuously during follow up as described earlier. To further dissect out if the dose of RAD001 administered matters, KU7 cells were treated *in vitro* with increasing doses of RAD001 (100nM-10μM) for 24 hrs prior to analysis of p21 expression. Figure 18 clearly indicates that levels of p21 expression are inversely proportional to the concentration of RAD001 with higher doses (1-10 μM) inducing a more pronounced decrease in p21 expression (normalized to β-actin levels).

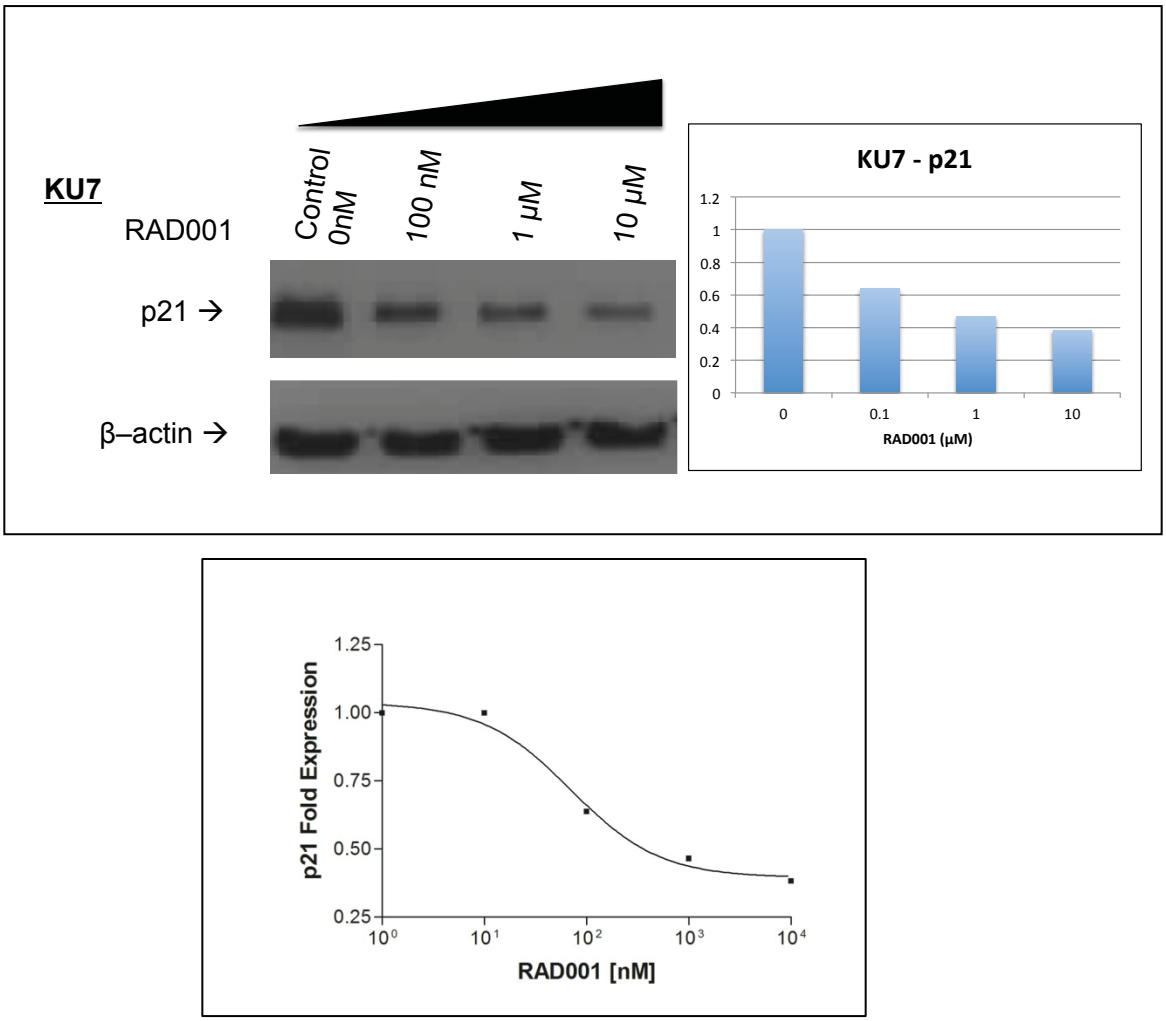

Figure 18: Dose-dependent RAD001 inhibition of p21 expression. KU7 cells were treated with various doses of RAD001 (ranging from 100 nM to 10 µM) and lysed 24 hrs after treatment for Western blot analysis of p21 (top left panel) and β-actin (lower left panel) in parallel, as described in Methods. The p21 protein bands were scanned and levels were normalized as a function of actin levels (bar graph in right panel). The relationship between p21 and doses of RAD001 is shown (lower middle panel).

4.4 Cell Death: Autophagy and Apoptosis

Given that p21 may be largely responsible for the radio-sensitizing effect of RAD001 leading to UBC cells kill, we wondered if p21 may play a role in cell autophagy and / apoptosis.

Currently autophagy is assessed by transmission electron microscopy in human tissues, and described as a punctate staining resulting from the accumulation of autophagy substrates such as SQSTM1.[335] However, this method has pitfalls associated with transmission electron microscopy on post-mortem tissues where vacuolization becomes a challenge. The induction of autophagy detected by immunofluorescence with the LC3I/II protein as a marker appears most reliable and easy to enable the monitoring of auto-phagosomes accumulating in cells, and detected as fluorescent dots. We thus choose the autophagic marker LC3 I/II which was measured by immunofluorescence in KU7 bladder cancer cells exposed to either treatments alone, RAD001 at 100 nM for 24 hrs or ionizing radiation (4 Gy), and to their combination and compared effects of the different treatments to untreated control cells, as described in Methods. Figure 19 shows a diffuse red LC3 signal in the cytoplasm of control KU7 cells, with no marked difference in cells irradiated with 4 Gy.

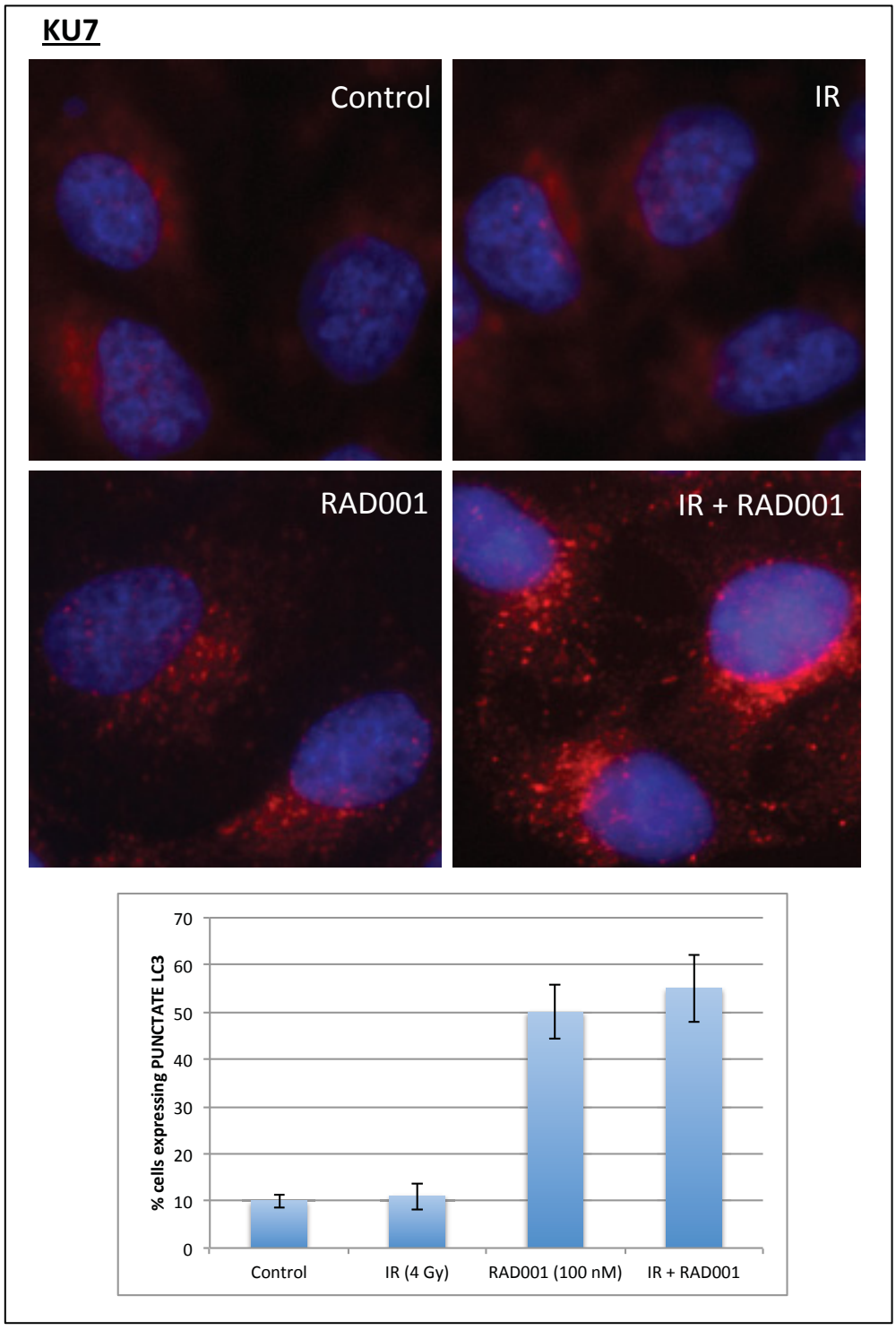

Figure 19: Autophagy following RAD001 treatment in KU7 blabber cancer cells. KU7 cells were treated with 4Gy of ionizing radiation and 100 nM of RAD001 alone and with a combination of the two treatments. Fluorescent LC3 levels ($p<0.05$) were measured by immunofluorescence as described in Methods. The presence of punctate LC3 is an indication of the onset of autophagy.

When cells were exposed to RAD001 however, there was a net increase in the punctate expression of the autophagic marker, and this increase was significant after quantification ($p<0.05$), as shown in the bar graph lower panel. Accordingly and as expected this punctate expression pattern was observed in cells submitted to the combined treatment of ionizing radiation and RAD001. In those experiments, Chloroquine was added to halt the rapid autophagic process in order to be able to assess LC3 levels. This is a standard procedure in the study of autophagy to prevent endosomal and lysosomal acidification; by doing so, it raises the pH in lysosomes, leading to inhibition of the fusion between the autophagosome with the lysosome, as well as inhibiting lysosomal protein degradation.[336] Altogether these findings demonstrate that the addition of 100 nM RAD001 to KU7 bladder cancer cells induced the punctate LC3 pattern characteristic of autophagy induction. Ionizing radiation alone was not able to induce this punctate pattern, but rather a diffuse LC3 I/II signal, similar to the untreated control cells.

As the addition of RAD001 induced a decrease in levels of p21 and also autophagy, we questioned whether p21 plays a functional role in the autophagic process. Towards this goal, we compared the levels of LC3 I/II protein expression by Western blots in control KU7 cells, in cells treated with ionizing radiation alone, RAD001 alone and a combined RAD001/ionizing radiation treatment. Levels of LC3 I/II were next assessed under these conditions, but when cells were transfected with p21 shRNA, scramble shRNA, or un-transfected controls.

Results in Figure 20 show that in the un-transfected KU7 cell series (the left side), the addition of RAD001 alone or in combination to ionizing radiation increased the levels of LC3 I/II expression. A slight increase was noticed in cells treated with ionizing radiation alone. These results recapitulate the findings observed in KU7 cells under the same conditions when monitoring the autophagic immunofluorescent signal of LC3.

Results observed with the scramble shRNA were similar with respect to RAD001 and IR and significantly increased LC3 II expression in the combination therapy ($p<0.05$), thus serving as an internal negative control. However no marked difference was noticed between levels of LC3 I/II when RAD001 was used alone vs. the control and the IR arm alone.

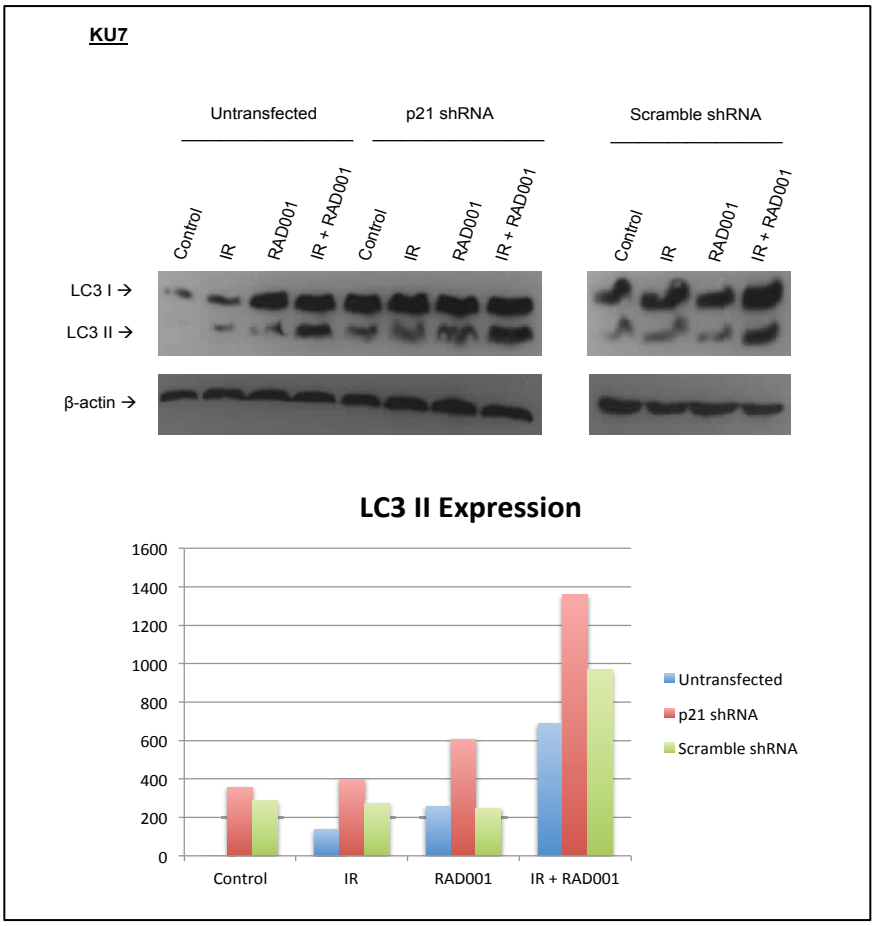

Figure 20: Interplay between RAD001, p21 and autophagy in bladder cancer cells. Levels of LC3 determined in three series of KU7 cells, un-transfected, transfected with scramble shRNA and p21 shRNA and treated with 100 nM RAD001 alone and for 12 hrs before exposure to ionizing radiation (4 Gy) and the combination therapy and compared to untreated controls in each series as described above. Chloroquine was added to stop the degradation of LC3 I yielding LC3 II next detected by Western blot. Accumulation of LC3 II served as an indication of autophagic onset. β-actin was probed in parallel for normalization and controlling for equal loading.

The knock-down of p21 by shRNA on the other hand induced an increase in LC3 I/II protein levels across all conditions: in fact, control cells (untreated) seem to show an increase in LC3 I/II levels upon p21 knock-down compared to un-transfected cells or corresponding control cells treated with scramble shRNA. The same observation held true for treatment with ionizing radiation alone, whereby p21 shRNA induced an increase in LC3 I/II levels. In line with our previous observations, addition of RAD001 alone, and more so when combined with ionizing radiation further increased the levels of LC3 I/II expression and of LC3 II when p21 was knocked down. Altogether, these results establish that, as demonstrated earlier, RAD001 induces autophagy, and more importantly, that this is mediated through p21 as demonstrated by increasing levels of LC3 I/II. This even occurred without RAD001 addition. Nevertheless, this increase in autophagy was more pronounced with the addition of the mTOR inhibitor, RAD001.

Given that RAD001 per se induces a slight decrease in levels of p21 in UBC cells (Figs. 11 and 14), and activates autophagy, and that p21 knockdown also induces autophagy, it is conceivable that RAD001 might be activating the autophagic process *via* the modulation of p21 expression. We thus evaluated whether the autophagic process serves cytoprotective or cytotoxic purposes.

Work from literature has established that ionizing radiation is cytotoxic and induces cell death whereas survival mechanisms may be activated in cells resisting to IR We also know that p21 plays a major role in controlling the cell cycle by inhibiting progression into the G1 phase to allow for DNA repair post radiation-induced damage.

To investigate whether the knockdown of p21, besides inducing autophagy, further contributes to apoptosis of bladder cancer cells, we monitored apoptosis by examining in KU7 cells levels of cleaved Caspase-3 compared to total Caspase-3 by Western blotting across the different conditions depicted above. Ionizing radiation was at 6 Gy and cells were cultured over a 72 hrs period prior analysis; the following series were compared: un-transfected, transfected with scramble shRNA, and transfected with p21 shRNA. Results are shown in Figure 21: in KU7 cells that received no radiation, Caspase-3 was intact, regardless of the transfection conditions. In fact, in the absence of ionizing radiation, knockdown of p21 in un-treated cells did not induce a change in Caspase-3 cleavage compared to un-transfected cells or cells transfected with a scramble shRNA.

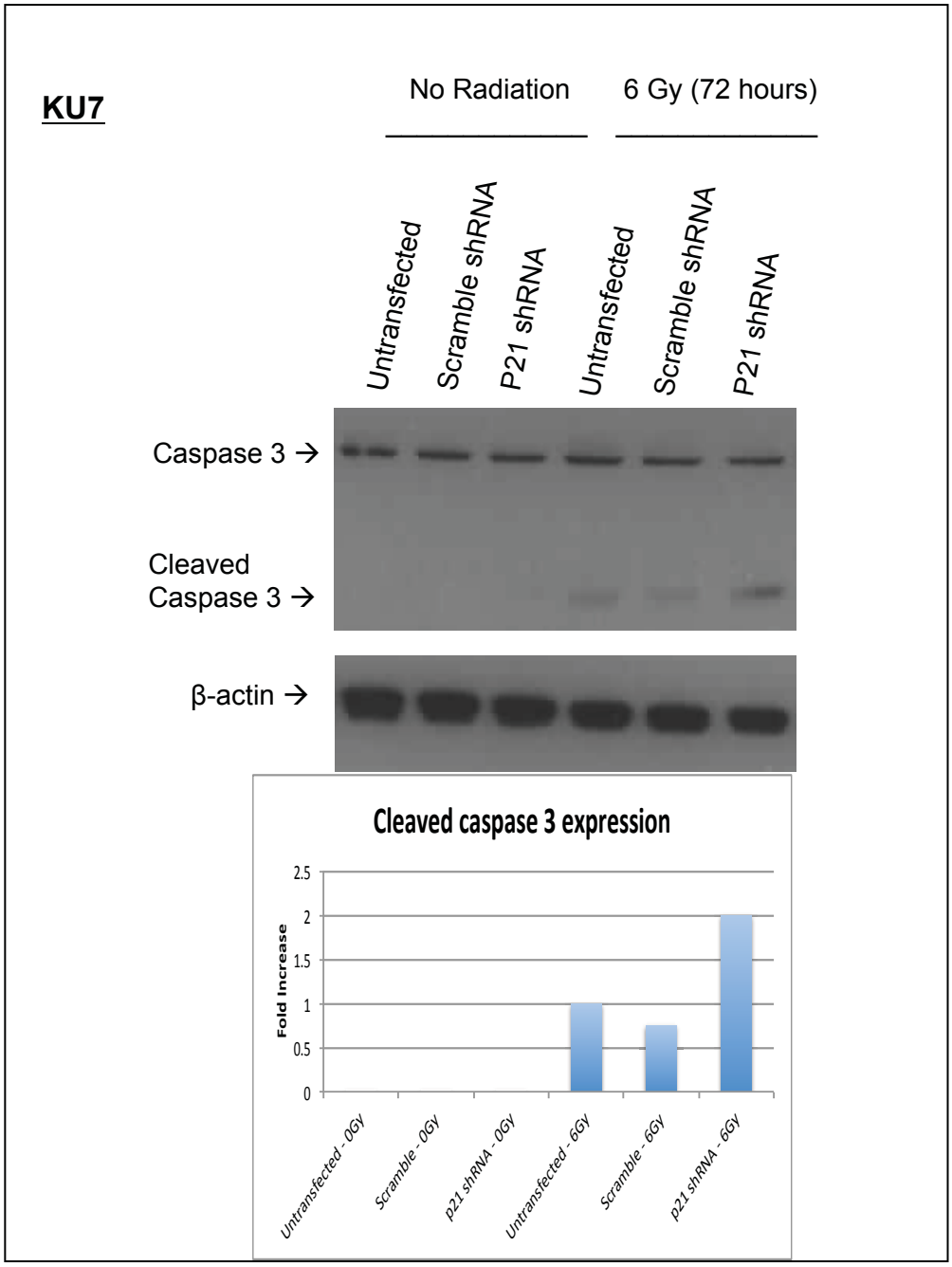

Figure 21: p21 knockdown and apoptosis following IR. The p21 knockdown was induced using shRNA as described in Methods, comparing scramble shRNA and un-transfected KU7 cells as controls. Cells were then treated with IR for 72 hrs. Caspase-3 levels, total and cleaved (indication of apoptotic death) were measured by Western blots (top row). Actin was blotted in parallel (lower row) to control for loading and normalize results (lower panel).

On the other hand, treatment with ionizing radiation induced the cleavage of Caspase-3 in un-transfected cells, in p21 shRNA-transfected cells and in cells transfected with scramble shRNA. One could thus conclude that, as expected, ionizing radiation acted on KU7 cells by inducing apoptosis. Moreover, apoptosis appeared more pronounced in irradiated cells when p21 was knocked down compared to un-transfected cells or scramble-shRNA transfected cells. Accordingly, the apoptotic, cytotoxic effects of ionizing radiation implicate p21 since inhibition of its expression with shRNA further enhanced apoptosis under radiation evidenced by cleaved Caspase-3. This observation, along with the observation that RAD001 induces autophagy potentially *via* modulation of p21 expression, invites to question the extent to which apoptosis and autophagy come into play, whether the two processes counter one another, balance one another or add up towards radio-sensitization when cells are co-treated with RAD001 and ionizing radiation.

CHAPTER V: DISCUSSION

Currently, ionizing radiation is used as a bladder-sparing treatment modality instead of cystectomy.[91] However, a major challenge is local control of the disease and IR-induced cytotoxicity that affects surrounding tissues. Therefore, the use of a radio-sensitizer that could ultimately enhance the final outcome of ionizing radiation, along with limiting the resulting side effects of IR is important in overcoming those challenges. In light of our results regarding the effects of RAD001 on radio-sensitization of human UBC cell lines *in vitro* and derived tumor xenografts *in vivo*, it is reasonable to envision the clinical use of RAD001 with its potential benefits and also challenges for the treatment of UBC with radiations.

Everolimus (RAD001) as a monotherapy

i. RAD001 is the second Rapamycin analog in the family of the first generation of mTOR inhibitors;
ii. Compared to other rapamycin analogs, RAD001 has the advantage of being a potent hydrophilic molecule, that is orally administered.
iii. Its safety profile revealed mild side effects such as hyperglycemia, fatigue and thrombocytopenia in patients with gastroentero-pancreatic neuroendocrine tumors.[337] Being also well tolerated in metastatic breast cancer patients, other common side effects beside hyperglycemia and fatigue are stomatitis, rash, hyperlipidemia and myelo-suppression.[338] More interestingly, and contrary to other drug cytotoxic effects, RAD001's adverse effects were resolved, thus reversible, through dose reductions or interruption of treatment.

iv. Since its FDA approval in 2011, RAD001 has been used alone for the treatment of advanced renal carcinoma following the failure of standard treatment.[339] More recently, RAD001 has been shown to significantly increase progression-free time in renal cancer as part of a phase III clinical trial (RECORD-1, *REnal Cell cancer treatment with Oral RAD001 given Daily*).[340]

v. RAD001 is currently an attractive molecule being evaluated in several clinical trials for the treatment of already mentioned solid tumors (metastatic breast cancer and pancreatic neuroendocrine tumors) as well as sub-ependymal giant cell astrocytoma resulting from tuberous sclerosis,[198, 339, 341-343], ovarian, endometrial, prostate, non-small-cell lung carcinomas and hematological malignancies.[344-349]

vi. The use of RAD001 has not been limited to oncological settings and has been shown to be effective in treatment of Tuberous Sclerosis, preventing surgical intervention, the only treatment that was available before RAD001.

Everolimus (RAD001) in combination with other drugs

i. RAD001 has shown great clinical efficacy in combination with drugs such as tamoxifen and letrozole for the treatment of estrogen receptor-positive (ER+) breast cancer.[350] Also, the use of RAD001 as a combination drug with other first-line therapies has shown the benefit of reversing resistance to these therapies. For example, RAD001 was assessed in ER+ breast cancer where the use of tamoxifen alone eventually leads to drug resistance and failure of the

anti-estrogen therapy. When RAD001 was added to tamoxifen in a randomized controlled trial, the combinatory treatment was successful at reversing tamoxifen resistance, thus prolonging the palliative use of anti-estrogen therapy and delaying the use of chemotherapy.[351, 352]

ii. In a phase II trial of RAD001 and octreotide, a somatostatin analog, evaluated for the treatment of neuroendocrine tumors, a dramatic improvement over RAD001 alone was shown (ORR of 20%).[353] The rationale is to block IGF production consequent to inhibition of PI3K activation.

iii. Combining mTOR inhibitors with erlotinib, an EGFR tyrosine kinase inhibitor is being investigated in a phase II clinical trial for non–small cell lung cancer.[354] The addition of LY294002, a PI3K inhibitor to tamoxifen and RAD001 in breast cancer cell lines (MCF-7 and BT474) improved the antitumor effect compared to tamoxifen alone or to tamoxifen and RAD001 combination, implying that combinatory regimen targeting several signaling molecules may be a promising avenue.[355] More importantly, the addition of LY294002 abrogated the AKT activation feedback loop, and inhibited the expression of HIF-1a, thus reducing angiogenesis *in vitro*. When tested in an MCF-7 xenograft model in mice, the combination of three agents showed the greatest efficacy in inhibiting tumor growth and angiogenesis.

However, it seems common across different cancers and non-cancerous diseases that the anti-proliferative efficacy of RAD001 was eventually compromised by the activation of feedback compensatory mechanisms via AKT activation, thus presenting a challenge to RAD001. In fact, and besides the fact that RAD001 only targets mTORC1,

the use of the drug alone induces an activation upstream of AKT as well as an activation of mTORC2 in a way that counteracts mTOR inhibition. Several potential mechanisms have been proposed to explain the feedback activation of the PI3K pathway. For example, a study has shown that when RAD001 inhibits mTORC1, the S6K1-dependent auto-inhibitory pathway that usually inhibits the PI3K signaling is interrupted, thus causing feedback activation of PI3K, and promoting resistance to the effects of rapamycin and its analogs.[356, 357] Another possibility is that the activation of mTORC1 leads to inhibition of mTORC2.[357] When mTORC1 is inhibited by RAD001, the inhibition of mTORC2 is relieved, thus mediating AKT activation. Furthermore, when mTORC1 is inhibited, MAPK gets activated via a feedback mechanism involving the S6K1/PI3K/Ras pathways.[358] Altogether, the promising cytostatic effects of RAD001 in cancer are limited by the activation of feedback mechanisms counter-acting the effects of mTOR inhibition on cell survival and growth.

In order to overcome these challenges, new research has been focusing on the use of dual inhibitors, along three different approaches: i) co-inhibition of mTORC1 and mTORC2, ii) dual inhibition of PI3K, upstream of AKT, and mTOR, and iii) dual inhibition of MEK and PI3K while administering RAD001. Since both mTORC1 and mTORC2 are responsible for AKT activation, as well as SGK1-mediated phosphorylation of p27, [359] the use of a dual mTORC1/2 inhibitors was proposed to restore the nuclear localization of p27, thus leading to effective cyclin-CDK2 inhibition.[354] Furthermore, this re-localization of p27 into the nucleus would prevent it from binding RhoA, thus abrogating actin stability and with that, tumor cell motility and metastasis.[360, 361] Although the dual inhibition of mTORC1 and mTORC2 is still relatively poorly investigated, a study by Chapuis *et.al*, examined NVP-BEZ235 (a PI3K inhibitor) in

Acute Myeloid Leukemia and showed that the co-inhibition of both mTORC proteins resulted in full inhibition of the 4E-BP1 phosphorylation, as well as protein translation.[362]

RAD001, mTOR inhibition and radiosensitization in bladder cancer

Our study tested for the first time the potency of RAD001-mediated mTOR inhibition as a growth inhibitor *in vitro*, on a panel of nine UBC cell lines, representing different origins, stages and grades of UBC, which translate into potentially promising clinical applications given that RAD001 could affect a wide spectrum of UBC stages and subtypes. Our results show that RAD001 potently inhibited the proliferation of these cell lines, to varying degrees: UM-UC5, UM-UC1 and UM-UC6 being the most sensitive and KU7, UM-UC13 and UM-UC3 relatively resistant. Furthermore, bladder cancer has been specifically associated with a specific deletion at the 9q34 loci, which encodes the TSC1 tumour suppressor that downregulates the mTOR pathway.[363] This observation adds promise to our proposal of a combination RAD001 and radiation therapy in bladder cancer.

We further tested the sensitivity of six UBC cell lines to ionizing radiation alone, without treatment with RAD001 through clonogenic assays. Of our initial cell line panel, RT4, 253-JP and UM-UC1 were excluded due their growth patterns, not proper or too slow for assessment *via* the assay of choice. Our findings reveal that the different cell lines exhibited different levels of sensitivities to ionizing radiation, which might mirror the different responses to IR observed in a clinical setting. Furthermore, the UBC cell

sensitivity *vs*. resistance to IR did not strictly correlate with their RAD001 sensitivity. UM-UC5 for example was shown to be very sensitive to RAD001 but most resistant to IR. This observation actually reflects the differential effects of IR and RAD001, which we clearly established with the two interventions targeting different signaling molecules. This, as proven, supports combining RAD001 with IR to achieve a complementary additive effect with regards to UBC treatment.

Interestingly, results of combined RAD001 and IR treatment investigated *in vivo* in two UBC xenograft models echoed observations made through clonogenic assays *in vitro*. To our knowledge, this represents the first observation of a radio-sensitizing effect of RAD001 on bladder cancer cell growth *in vivo*. These novel findings of efficiently sensitizing UBC cells to radiation compare to reported data in other cancer types, including prostate, breast, non-small cell lung, and head and neck squamous cell carcinomas.[178, 364-366]

We were able to clearly show that, similarly to *in vitro* observations, the combined IR and RAD001 therapy resulted in tumors of a significantly slower growth rate, reflected through smaller volumes and weights of tumors in comparison to each individual treatment which also significantly inhibited growth compared untreated controls but to a lesser extent than combined therapy. The rapid tumor growth of controls determined the experimental end points at 5 weeks following implantation. It is worth mentioning that our findings were consistent in both the KU7 and 253J-BV xenograft models. Given the phenotypes of these cell lines and their differential sensitivity to IR (KU7 is moderately resistant while 253J-BV is sensitive), our results hold clinical significance and are promising in terms of covering different tumor behaviors observed in the clinic.

Another aspect to consider is the very potent radio-sensitizing effect of RAD001 in bladder cancer observed *in vivo,* with a pronounced reduction in tumor weight compared to the *in vitro* results. This can be attributed to differences in the pre-treatment protocol to administer RAD001, notably at their respective GI_{50} doses (76 nmol/L for KU7 and 5.3 nmol/L for 253J-BV) and at 6-12 hrs prior to treatment with ionizing radiation *in vitro* whereas in the *in vivo* model, RAD001 was introduced 24 hrs prior to exposure to IR and afterwards at a daily dose of 1.5 mg/kg for 4 weeks, similarly to the arm of RAD001administered alone. This difference in RAD001 pre-treatment time might be sufficient to better sensitize bladder cancer cells to IR *in vivo* compared to *in vitro*. In fact, the half-life of RAD001 in blood (~21-22 hrs) is comparable to its half-life once in tumors.[367] More importantly, and in addition to longer exposure, a higher RAD001 dose may prove to be more successful at inhibiting growth of bladder tumors (as we showed *in vitro*), emphasizing further the potency of RAD001 radio-sensitization effects.

In a clinical context ionizing radiation *per se* might be explored; while bladder cancer cells were subjected to 4 Gy of continuous radiation *in vitro*, the bladder tumor xenografts were exposed to fractionated radiations over a longer period of time. The importance of dose fractionation, whereby the total dose is spread over a period of time and administered in different, smaller dose "fractions", is well documented.[356] Enhanced potency is attributed to: i) Allowing cells in the S-phase that are normally more resistant to radiation to cycle into the more sensitive G2 phase prior to the new fraction; ii) Allowing re-oxygenation of the tumor between fractions, and thus avoiding a hypoxic stage that renders the cells more radio-resistant and iii) Allowing the recovery of normal cells while tumor cells, usually deficient in proper repair mechanisms are less efficient in

repairing between fractions. Currently, radiation therapy for bladder cancer is often administered in fractions over 4-5 weeks;[91] thus, the *in vivo* model presented in this thesis is better at mirroring the clinical setting, adding value to our findings for future clinical applications

Molecular basis of the combination therapy

The PI3K/AKT pathway, also known as the survival pathway plays a major role in cancer cell survival and has been shown to be over-activated in different cancer cells (reviewed in[333]). In order to understand the pathways underlying resistance to IR, and the targets that potentially mediate radiosensitization by RAD001, we investigated the levels of AKT activation as a survival pathway when KU7 and 253J-BV cells were subjected to IR. The choice of cell lines was meant to reflect in KU7 a moderately resistant model to IR/relatively resistant to RAD001 phenotype, whereas 253J-BV represented a sensitive model to IR/moderately sensitive to RAD001 phenotype. Results clearly showed that indeed IR rapidly induced an activation of AKT phosphorylation, in a time-dependent manner. Our results confirmed the rapid response observed within 5 min to the stress imposed by IR, which may then be explained by post-translational changes or degradation rather than their de novo synthesis or expression of several components of this pathway.

Several genomic modifications related to the AKT pathway were reported in cancer and may then affect cell response to therapy. Besides the amplification of the gene encoding AKT itself, several partners and downstream targets of AKT are altered; for example, *PTEN (10q23)*, which encodes a phosphatase and acts as a negative regulator of

AKT is often deleted,[261, 297, 298, 334, 364] while *PDPK1 (16p13.3), which encodes an AKT-activating kinase,* is amplified.[357, 358, 368] Furthermore, the activation of the survival pathway eventually translates into an increase in mTOR activity, downstream of AKT. In UBC, the characterization of this unique molecular landscape involving the PI3K/AKT/mTOR pathway provide important evidence and opportunities to use molecular targets within the pathway in the treatment of the disease as highlighted by the TCGA study in 2014.[270] As a whole, IR contributes to an unfavorable increase in pro-survival pathway that may eventually confer resistance to treatment through the PI3K/AKT/mTOR. These findings substantiate the need to combine a radio-sensitizer with IR to minimize, counteract or prevent a pro-survival reaction due to IR alone in cancer cells that resist treatment.

As mTOR activates the S6 kinase *via* phosphorylation,[369] we obtained further insights in this radio-sensitization effect of RAD001 on the AKT survival signaling by showing that activation of S6, a direct target of mTOR, was indeed inhibited. This was achieved in KU7, 253-BV, as well as UM-UC3 and UM-UC6 cell lines, chosen for their resistance and sensitivity to RAD001, respectively. We determined that RAD001 potently inhibited the activation of S6 *via* its phosphorylation across the four cell lines.

The clear decrease in p-S6 expression relative to stable S6, reflects an effective inhibition of mTOR by RAD001 in bladder cancer cells, which has not been reported before. Of interest, S6 inhibition was most pronounced and rapid in UM-UC6 and 253J-BV in comparison with KU7 and UM-UC3. The differences among cell lines are thus in line with their growth response and sensitivities to RAD001, thereby proving that the sensitivity to RAD001 correlates positively with the inhibition of S6 activity and growth.

It is thus conceivable, that RAD001 in bladder cancer affects cell growth *via* the inhibition of the mTOR activity, as shown *via* a clear decrease in S6 activity following treatment with the drug. Given that the PI3K/AKT/mTOR pathway is activated by IR in bladder cancer while RAD001 effectively reduces UBC cell growth *via* mTOR inhibition, we can confidently propose that the combination of RAD001 to IR as a promising avenue to increase the efficiency of radiation therapy.

In that perspective, and as we had shown earlier, IR alone did not affect the levels of S6 activation but consistently increasedAKT activation in the cell lines tested. Intriguingly, it seems from our data, that RAD001 alone could also contribute to the activation of AKT despite still being effective at inhibiting S6 activation. This can be explained by the fact that RAD001 targets the mTORC1 protein, thus inhibiting one negative feedback loop that signals to AKT, but it does not inhibit the mTORC2 protein which plays a role in the central negative feedback loop, signaling to increase AKT phosphorylation at Ser473 residue.[273, 370, 371] Furthermore, a study performed in both cell lines and human prostate and breast cancer tissues showed evidence that mTOR inhibition by RAD001 induced the expression of the insulin receptor substrate-1 expression, and thus abrogated the feedback inhibition of the pathway, resulting in AKT activation.[372] The exact mechanism by which RAD001 enhanced AKT activation effects remains to be elucidated, as it might open the way to investigate modalities to block this rebound activation, and thus help in decreasing radio-resistance.

RAD001 decreases bladder cancer cell growth following IR; a first step towards combating radio-resistance

Whether this observed activation of AKT when RAD001 is combined with IR has consequences on the actual behavior of cancer cells towards combined therapy was not known. In order to answer this question this fact, we assessed the effect of a combined therapy on UBC cell colony formation. Across six UBC cell lines (253J-BV, UM-UC6, KU7, UM-UC3, UM-UC13 and UM-UC5), we observed a clear reduction in cell proliferation or colony formation potentials when IR and RAD001 were combined, compared to IR alone. This combination therapy led to an additive, but not synergistic, effect thus supporting a role for RAD001 in radio-sensitizing bladder cancer cells to therapy. Until further dissection of the pathway, one could speculate that blocking any AKT activation, following the inhibition of mTOR, could eventually enhance the radio-sensitizing effect of RAD001 in bladder cancer cells.

The cytostatic effects of the combined therapy on the cell cycle

mTOR is a member of the PI3K-kinase related kinases superfamily (PIKK) along with ATM, ATR and DNA-PK (reviewed in [373]). Several studies have investigated the role of mTOR and its inhibitors in cell cycle progression: early studies in yeast models have pointed to the role of *TOR2* in regulating the cell cycle whereby deletion of the gene was reported to be lethal due to arrested proliferation at all points of the cell cycle.[374] In the same model, deletion of a *TOR2* analogue was attributed to an arrest at the G1 phase,[375] similar to our observations inhuman UBC models.

In mammalian systems, mTOR was shown to modulate cell cycle progression and shares the function of cell cycle checkpoint control. Inhibition of mTOR affects the G1-to-S-phase transition [376] across different cell systems, which is further in line with our observations of an increase of cells in the G1 phase, and a decrease in the cells entering the S-phase. This G1 arrest is often attributed to the regulation of S6K levels of activation, [377] as we observed.

As far as ionizing radiation is concerned, studies have clearly shown a delay in G2 in response to irradiation in virtually all eukaryotic cells, [218, 220, 226] similar to what we demonstrated in the UBC model.

Our results indicate that RAD001 or ionizing radiation contributed to cell cycle arrest, each at a different phase of the cycle: while RAD001 induced a G0/G1 arrest, ionizing radiation was responsible of stopping cells at the G2 phase. More interestingly, these selective effects reflected in an additive effect when the two treatments were administered together and in arresting the cell cycle progression at two levels: G1 and G2.

These observations of a dual inhibition of the cell cycle imply that the mTOR inhibitor and ionizing radiation affect cell cycle progression in a complementary fashion, thereby explaining the inhibition of UBC cell growth and supporting higher potency when sequentially applied to target different stages of the cell cycle. It thus seems that the addition of RAD001 to the conventional ionizing radiation treatment would enhance tumor growth inhibition by interfering at different checkpoints.

Cell cycle checkpoints are tightly regulated by a large set of cell cycle regulators.[177, 179] In this work we closely investigated the levels of cyclin D1, p27 and p21 expression. Cyclin D1 is a positive cell cycle regulator that mediates the G1/S transition, while p27 and p21 are negative regulators that inhibit the cell cycle progression.

In line with the cell cycle, we showed that pre-treatment with RAD001 alone or in combination with ionizing radiation induced a significant decrease in levels of cyclin D1, and an increase in the levels of p27. The combined effect of cyclin D1 inhibition and p27 overexpression leads to cell cycle inhibition. More interestingly, the patterns of p21 deregulation in response to RAD001 alone or with ionizing radiation was different than that observed for p27, although both proteins play inhibitory effects on cell cycle progression. In fact, addition of RAD001 alone induced a slight decrease in the levels of p21 unlike what one would expect given the reported inhibitory function of the protein. Combination of RAD001 and ionizing radiation however restored p21 to levels similar to what is observed with ionizing radiation alone, which significantly increased p21 levels.

The understanding of p21 function has evolved over time: initially, the role of p21 was limited to mediating cell cycle arrest at G1 in a p53-dependent fashion. [228, 230, 231] Later on, p21 was described as having other p53-independent activities related to regulating different transcriptional factors,[233-235] and more interestingly a cytostatic role whereby it confers anti-apoptotic functions.[239, 240] This functional dichotomy could explain the effect of RAD001 having no effect or slightly decreasing levels of p21 expression rather than its increase, whereby the drug might be specifically targeting the "oncogenic", anti-apoptotic p21, and not the cell cycle inhibitor role. The mechanism of this specific targeting and its role in radio-sensitizing bladder cancer remains poorly understood, and warrants further investigation.

The mechanism by which RAD001 radio-sensitizes UBC cells may be attributed the selective arrest leading to a higher number of cells in the G1 and G2 phases, and less in the S-phase. In line with our observations, it appears that different cancer cells are most resistant to ionizing radiation at the S-phase, and least in the growth phases.[378] It is

thus conceivable that the cell cycle arrest at G1 and G2 could be one mechanism by which RAD001 radio-sensitizes bladder cancer cells, whereby arresting the cells in certain stages renders them more sensitive to radiation. These results, if prospectively applied in a clinical setting, invite to additional investigations to examine whether the effects of the RAD001 and ionizing radiation treatments when administered in fractionated doses rather than a single dose, would further increase the efficacy of RAD001 in addition to ionizing radiation in bladder cancer cells.

Of note, cell cycle analysis revealed no significant cell death whether the cells are treated with ionizing radiation alone, RAD001 alone or a combination of both. Furthermore, the fact that both treatments, administered separately or in combination lead to cell cycle arrest, and to a decrease in cell proliferation rather than cell death raises the question of a cytostatic effect of RAD001 and ionizing radiation rather than a cytotoxic one. However, whether a longer time of treatment will induce cell death would need to be explored.

The role of p21 in modulating the radio-sensitization of UBC cells by RAD001

Previous studies has heavily investigated the interaction between p53 and p21.[228] The *TP53* gene which encodes the p53 protein plays a major role in cell cycle regulation, especially in response the DNA damage, *via* transcriptional regulation of several proteins including p21.[157] p53 is a positive regulator of p21: once p53 is active in response to DNA damage, it stimulates the transcription of p21, thus inducing cell cycle arrest and preventing the replication of the damaged DNA. In two independent studies by Bunz *et.al* and Waldman *et.al* it was shown that the interaction between p21

and p53 is essential for cell cycle arrest, and that p21 is required for p53 to fulfill its role as a "guardian of the genome" in response to DNA damage.[379, 380] In fact, in the HCT116 colon cancer cell line, for example, which harbors a wild-type p53 protein, cell cycle arrest in response to 6Gy of ionizing radiation was successful only in p21-positive cells, while p21-deficient cells had their G1 arrest abrogated.[379] Similar studies were carried out in prostate cancer where co-transfection with p53 and p21 cDNA was shown as a potential gene therapy modality. [381] In breast cancer, the co-expression of p21 and p53 was indicative of a better prognosis.[382] In hepatocellular carcinoma cell lines (Hep 3B and HepG2), it was shown that stress caused by exposure to nitric oxide resulted in an up-regulation of both p21 and p53.[383] Altogether, these results indicate the need for p21 as an essential mediator of p53 activity in response to DNA-induced damages. In light of that, our results regarding the baseline expression of p21 across the various bladder cancer cell lines is in concordance with the literature. In fact, p21 expression was not or minimally detected in UM-UC3 and UM-UC13, two cell lines that harbor mutations in *TP53* rendering p53 nonfunctional.[384] In one of our studies Kassouf *et.al*, unpublished work), we observed the UM-UC5 cell line to be p53-mutant, whereas the cell lines UM-UC6, UM-UC1, 253-JP, 253J-BV and RT4 on the other hand, are all p53-wild-type and express a functional p53 protein. Here we showed that they also express p21. Given the known interaction between p53 and p21, these differences in baseline levels of p21 expression could be in part attributed to the p53 status: in cells expressing wild type p53, p21 is detectable, while those that are p53-deficient seem to lack or display low p21. KU7 however, seem to be an exception, with no reported p53 mutation, and low p21 levels. One possible explanation refers to the p53 functional status: while the gene *TP53* is not mutated, the protein itself is still subjected to post-translational modifications such

as phosphorylation, acetylation, and proteosomal degradation which modulate its stability and its relatively short half-life. [385]

Of note, the role of p21 in DNA damage repair has been shown to go beyond mediating cell cycle arrest and p53 –an arrest that allows damage repair- to direct involvement in the DNA repair mechanism.[386] A better understanding of this aspect of p21 functions in our model is warranted.

Across the different cell lines tested, and in another interesting finding, we have observed that the levels of p21 expression correlated well with the cell line's sensitivity to treatment with RAD001 as defined early in this work. With the exception of UM-UC5 which is sensitive to RAD001 but shows non detectable p21 expression, levels of p21 expression in other cell lines seems to correlate with sensitivity to RAD001, with those highly expressing p21 being sensitive to the drug, and those with no, or low p21 expression being resistant. This observation allows us to conclude that mTOR plays an important role in the p53-p21 interaction in response to radiation-induced DNA-damages. Cell lines that are *TP53*-mutant (UM-UC13 and UM-UC3) have low levels of p21 expression, and are resistant to RAD001 treatment. KU7, for which no mutations in the *TP53* are reported, has low levels of p21 and is resistant to treatment with RAD001. On the other hand, the other cell lines are *TP53*- wild type, express high levels of p21 and are sensitive to RAD001.[387]

Previous work has reported that ionizing radiation affects mTOR-mediated protein synthesis in a bimodal fashion,[283] depending on the dose of radiation administered: low doses that are less than 2 Gy, trigger a radiation-induced activation of multiple growth factor signaling pathway that leads to the activation of mTOR with subsequent increase in eIF4 levels, and thus the cap-initiation of translation. This cellular response provides the

cell with increased protein synthesis associated with the production of DNA damage response protein. At higher doses of radiation (higher than 20 Gy), mTOR is inhibited in a p53-dependent fashion once DNA damage is detected. Our experiments were carried at relatively low doses of ionizing radiation (4-6 Gy). In fact, when cell lines were treated with ionizing radiation alone, RAD001 alone and with both RAD001 and radiation, p21 levels were expressed at a much higher levels in RAD001-sensitive, p53-wild type cell lines. Of note, and across all the cell lines tested, the response to ionizing radiation alone was common, showing an increase in p21 expression regardless of the p53 status.

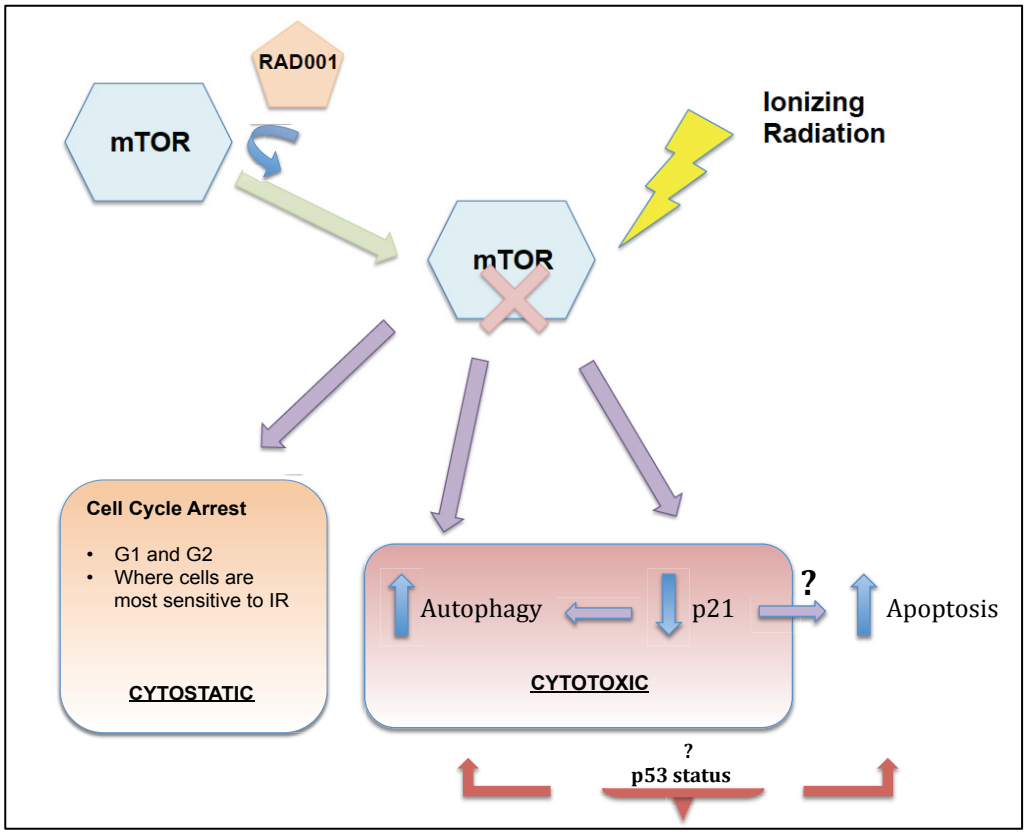

Figure H: Proposed model for the RAD001-induced radiosensitization in bladder cancer

In light of these results, we propose a model (Figure H) through which RAD001 provides radio-sensitization to UBC cells: in the absence of mTOR inhibition (no RAD001 treatment), ionizing radiation works at two levels: first, by triggering several survival pathways that lead to mTOR activation, and thus the translation of proteins needed for DNA damage response; and second by triggering a DNA damage response mediated *via* the p53 pathway, and for which the increased mTOR activity provides the proteins necessary for the response. Once p53 is activated, p21 transcription is induced, and results in higher expression of p21 (observed in our results in response to ionizing radiation alone), and thus cell cycle arrest. In light of this hypothesis, one could expect that cells that are *TP53* mutant, and thus with non-functional p53, are unable to trigger a proper response *via* p21, thus explaining the low levels of p21 expression in UM-UC3, UM-UC13 and UM-UC5. Furthermore, in these cells and still in the absence of RAD001, ionizing radiation might be increasing the activity of mTOR to higher levels in order to compensate for the lack of functional p53-p21 axis acting *via* the synthesis of other proteins needed for DNA repair. As a consequence, this could explain how these cell lines are resistant to RAD001 treatment. In fact, given the absence of a functional p53, and the potential higher translational activity of mTOR caused by ionizing radiation, RAD001 might be needed at much higher doses in order to be effective. Cell lines that have a wild type p53 will be able to trigger the p21-mediated DNA damage response on one hand which reflects in high levels of p21 expression in our results; on the other hand, the existence of the p53-p21 mediated response might keep the levels of mTOR activation within levels controllable with smaller doses of RAD001, hence the reported sensitivity. Thus, in these cell lines, the existence of the p53-p21 response, along with the sensitivity

of the cells of RAD001 explains the higher efficiency of the RAD001/ionizing radiation co-treatment in inhibiting cellular proliferation.

In order to challenge this model and better understand the role of p21 in RAD001 mediated radio-sensitization, we knocked-down p21 using an shRNA strategy to next assess KU7 cell growth. The cells were subjected to RAD001 treatment and an increasing dose of ionizing radiation. Results clearly demonstrated that reduced p21 levels hampered their abilities to form colonies. Furthermore, the addition of RAD001 to the p21 knockdown had more drastic inhibitory effect than with radiation alone. These findings pinpoint to a functional role played by p21 in mediating the RAD001 radio-sensitization, probably through interacting with p53 and mTOR as we suggested in our model. Our findings add to those obtained with breast cancer cells,[388] glioblastoma cells,[389] colorectal tumour cells [390] and melanoma cells [391], that have provided evidence of the radio-sensitizing effect of p21 and suggested it could be the result of a p53-p21 interaction.

When levels of p21 were evaluated in UBC *in vivo* tumors, treatment with RAD001 induced a slight decrease in p21 levels while ionizing radiation induced an increase in p21 levels. Interestingly, the combination treatment restored the levels of p21 that remained high, similar to the *in vitro* observations. Although one would expect p21 levels to remain low when cells are treated with RAD001, some possible explanations might account for our observations. *In vitro*, cells were treated according to the GI_{50} values determined earlier, while *in vivo*, mice were treated at higher doses of RAD001. By comparing the two models, it is clear that the response to higher doses of RAD001 was more pronounced *in vivo* compared to *in vitro*.

As this invites questions on the ideal dose to observe an effect, we assessed the levels of p21 in cells exposed to increasing doses of RAD001. Significant reductions in the levels of p21 were indeed achieved but at a much higher dose (10 to 100 fold) than the GI_{50}. Another aspect to consider is the localization of p21 in the cell besides translocating to the nucleus upon p53 activation to inhibit cell cycle, p21 also performs cytoplasmic activities, mainly anti-apoptotic and oncogenic ones. In fact, cytoplasmic p21 inhibits pro-apoptoticproteins such as pro-caspase 3, caspase 8 and caspase 10 *via* positive feedback, ultimately leading to the amplification of survival signaling.[392] In our *in vivo* model, p21 was limited to the nucleus, which likely reflects transcriptional-related functions, while the effects of RAD001 and ionizing radiation on the cytoplasmic processes remain unknown. It would thus be interesting to investigate the variation of p21 levels in the cytoplasm, and with that, other processes that are involved in cellular response to ionizing radiation, and RAD001.

Whether the consequences of the p53-p21 interaction in response to ionizing radiation is only limited to proliferation and growth arrest is worth considering in the scope of better understanding the mechanism by which RAD001 sensitizes the bladder cancer cells to radiation, and also to decipher whether the effect is cytostatic as we show, or could be cytotoxic under different conditions.

The introduction of a new drug treatment to radiation therapy aims at reducing radiation dose while preventing growth and ultimately killing UBC cells

We show that RAD001 induced autophagy of UBC cells. To our best knowledge, no previous work has clearly demonstrated such a role for RAD001 in UBC cells.

However, previously published articles have closely studied the role of this mTOR inhibitor in several cancer cell lines. The implication of RAD001 in inducing autophagy is an emerging topic of investigation.

Our findings as presented in this thesis and reported in 2009 by Mansure *et.al* [304] indicated that RAD001 was effective at inhibiting UBC cell growth *in vitro* and to treat UBC tumors in nude mice. Similar to our results, a 2011 study by Chiong *et.al* [306] reported on RAD001 effects on cell cycle progression and proliferation in a panel of UBC cell lines. Interestingly, we did not detect cell death *in vitro* while analyzing the cell cycle. The Chiong *et.al* 2011 study reports no effect of RAD001 on apoptosis and propose autophagy might be a potential mechanism behind the observed cell death. [306]

In this work, we obtained convincing evidence supporting the role of RAD001 in inducing autophagy. However, and as mentioned above, the role of RAD001 as an autophagy-inducer remains a relatively recent, emerging topic, as much as the understanding of the role of autophagy in cancer is.

Given that the role of mTOR as an inhibitor of autophagy has been established and shown to be conserved from yeasts to mammals, whereby inactivation of mTOR leads to autophagy, and its activation inhibits autophagy, it is not surprising that RAD001, an inhibitor of mTOR produces relatively similar results across different cancers. In fact, several reports reported a link between RAD001 and induction of autophagy. In hepatocellular carcinoma,[393] acute lymphoblastic leukemia,[394] gliomas,[395] *PTEN*-null prostate cancer,[364] papillary thyroid cancer,[252] lung cancer, [396] and breast cancer, [397] addition of RAD001 to cells *in vitro*, or administering the drug *in vivo* to mice models, induced autophagy.

Of note, in nasopharyngeal carcinoma, RAD001 was shown to induce apoptosis and autophagy in two nasopharyngeal carcinoma cell lines with different sensitivities to the drug.[398] When the cells were co-treated with RAD001 and 3-methyladenine, an autophagy inhibitor, cellular growth was significantly inhibited. However, when the cell lines were co-treated with RAD001 and an apoptotic inhibitor (z-VAD-fmk), the effect on cellular growth was not as pronounced and the percentages of apoptotic cells significantly decreased. Based on this evidence, the authors concluded that the main mechanism by which RAD001 induces cell death is apoptosis and not autophagy. Although these results somehow contradict other findings regarding the role of RAD001 in inducing autophagy, we think that the involvement of the mTOR inhibitor in autophagy is not exclusive nor does it prevent a role in controlling apoptosis. In fact, and as our results show, RAD001 was also effective at inducing apoptosis, possibly *via* the modulation of p21 levels.

This duality in RAD001 inducing autophagy and apoptosis has been discussed in earlier work,[364, 399] as a venue to sensitize cells to certain treatments such as chemotherapeutic agents or ionizing radiation. However, the mechanism by which RAD001 acts and its implication in cellular signaling related to either autophagy and/or apoptosis remains poorly understood. We have shown that RAD001 at its GI_{50} induces a slight decrease in levels of p21 expression, which became clear when increasing the dose. Furthermore, knockdown of p21 by shRNA induced autophagy regardless of other treatments. These novel observations undoubtedly point to a functional role for p21 in mTOR-mediated the autophagic process.

Previous work, in different cancer models has also shown an involvement of p21 in the regulation of autophagy in response to treatment. In a mantle cell lymphoma model, the mTOR inhibitor temsirolimus was successful at inhibiting proliferative activity in

three different cell lines, at inducing cell cycle arrest in the G0/G1 phase, as well as inducing autophagy without affecting apoptosis. When the mechanism of action was further investigated, it was shown that Temsirolimus caused a decrease in levels of p21 expression without altering p27 or cyclin D1 levels, simultaneously to increasing the number of acidic vesicular organelles and of microtubule-associated protein 1 light-chain 3 processing, all characteristic of autophagy. Thus, similar to our work, an association between the inhibition of mTOR and autophagy was clearly demonstrated with the decrease of p21 levels being the link between the two observations.

The mechanism *via* which p21 could potentially be contributing to the induction of autophagy was described by Yang *et.al* using atorvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, and not an mTOR inhibitor, on hepatocellular and colorectal carcinoma models.[400] In fact, in hepatocellular carcinoma, there was a positive correlation between the decrease in levels of p21 expression and the expression of the autophagic marker beclin-1. Furthermore, it was shown that in colorectal carcinoma, the alteration of the p21 signaling led to autophagy *via* the induction of endoplasmic reticulum stress response. In a similar study by Liu *et.al,*[401] the induction of autophagy in hepatocellular carcinoma cells *via* the administration of histone deacetylase inhibitors was shown to mainly occur *via* the downregulation of mTOR, thus echoing the results in our own work, whereby inhibition of mTOR by RAD001 induced autophagy. The Liu *et.al* study further dissected the role of p21 in inducing autophagy: although p21 was not essential in mediating the effect of the HDAC inhibitors on autophagy *per se*, the study discussed the negative effects of p21 on autophagy induction, whereby the silencing of p21 *via* siRNA increased autophagy.

Taken together, these different results from the literature are in line with our findings showing that on the one hand, inhibition of mTOR induced autophagy in a p21-mediated fashion, and that on the other hand, the role played by p21 is that of negative regulation known to relate to stress to the endoplasmic reticulum. In light of that, further dissection of the interaction between p21 and induction of autophagy induction is warranted in UBC cells.

In this thesis, we also showed that the administration of ionizing radiation induced apoptosis, and that p21 knockdown in cells receiving no treatment resulted in no cell death. Interestingly, the combination of ionizing radiation and the p21 knockdown resulted in apoptosis, at levels higher than those observed when cells were subjected to radiation alone. These results point to the fact that the apoptotic, cytotoxic effects of ionizing radiation is carried through p21 as inhibition of p21 expression further enhanced apoptosis under radiation. As mentioned previously, p21 is a negative regulator of the cell cycle progression inhibiting progress into the G1 phase to allow for DNA repair post radiation-induced damages. The combination of ionizing radiation, along with the knock-down of p21 is thus resulting in pushing the cell through the G1 phase without permitting repair of the DNA damage induced by radiation, ultimately leading to apoptotic cell death.

Taken all together, it seems that p21 is playing a central role in mediating both autophagy and apoptosis in UBC cell models thus inviting to question the extent to which apoptosis and autophagy come into play, in what context, and whether the two processes counter one another, balance one another or have some additive effect towards radio-sensitization when cells are co-treated with RAD001 and ionizing radiation. Our findings on the sequence and duration of treatments, the administration of RAD001 at a proper

dose together with higher efficacy of fractionated radiations also open new research avenues and deserve further investigations for rapid translation into the clinical setting.

The dual role for p21 is to act first as, a growth inhibitor, tumor suppressor role; and second, an anti-apoptotic, oncogenic role which was reported to occur through p21 selective inhibition of pro-apoptotic genes such as pro-caspase 3, caspase 8 and caspase 10.[239, 240] The dichotomy in p21 functions depends on its cellular sub-localization: while p21 is a nuclear regulator of cell growth and proliferation, it also accumulates in the cytoplasm where it mainly carries anti-apoptotic, oncogenic functions. This dichotomy of p21 functions has been addressed in the literature, whereas while some studies showed that p21 acts to impair autophagic activity, other reports pointed to enforced expression of p21 inducing autophagy.[388, 402, 403] This could be explained by the fact that regulatory effects of p21 on autophagy may depend on its subcellular localization.

Our results, along with other reports thus raise the question regarding the balance between apoptosis and autophagy, when cells resort to one mechanism and not the other, and the cues involved in the choice of mechanism. Furthermore, whether the reported autophagic activity serves cyto-protective or cyto-toxic functions is also worth further investigation. Fujiwara *et.al* studied the machinery that determines the type of cell death, whether *via* apoptosis or autophagy, and interestingly focused on the role of p21 in this process.[402] In $p21^{+/+}$ mouse embryonic fibroblasts, treatment with C2-ceramide induced the cleavage of caspase-3 and the degradation of autophagy-related Beclin 1 and Atg5 proteins, indicative of active apoptosis and inhibition of autophagy. On the other hand when $p21^{-/-}$ cells were treated with C2-ceramide, no cleavage of caspase-3 was observed, and Beclin-1 and Atg5 protein levels remained stable, culminating in autophagy. Furthermore, inhibition of p21 by siRNA in the $p21^{+/+}$ cells, induced autophagy rather

than apoptosis, while p21 exogenous expression in p21-/- cells increased apoptosis and decreased autophagy. Of interest, C2-ceramide is used for studies of apoptosis, and is endogenously generated by cells under ionizing radiation. This information along with our results could thus explain how, under irradiation, and when levels of p21 are reduced, cells are geared towards autophagy when mTOR is inhibited by RAD001. In the absence of RAD001, levels of p21 are not decreased, and autophagy is inhibited by the active mTOR, thus favoring apoptosis. One could thus speculate that p21 acts to balance and direct the effect of ionizing radiation: in the presence of RAD001, p21 levels are decreased and autophagy is activated thus inducing autophagic activity; on the other hand, in the absence of RAD001, levels of p21 are close to normal, autophagic activity inhibited, and the ionizing radiation then leads the cell towards apoptotic death.

Based on these novel promising findings, we would propose a multi-targeted therapeutic regimen in the clinical setting whereby mTOR and p21 are co-targeted along with exposure to ionizing radiation, leading to clinical advantages.

CHAPTER VI: CONCLUSIONS

To our best knowledge, no previous work has examined the effect of RAD001 on radio-sensitization of UBC cells. The novelty brought up in this thesis presents several pieces of evidence in support of a potential translation of our findings into clinical applications for the treatment of bladder cancer.

First, our data clearly shows a role for RAD001, an mTOR inhibitor, in inhibiting cell proliferation, and that, across a wide array of UBC cell lines of different stages and grades that reflect the heterogeneity of bladder cancer as encountered in patients. When we combined RAD001 with ionizing radiation, these different cell lines, which exhibit different levels of sensitivities to ionizing radiation alone, showed significantly lower proliferation rates; using *in vivo* UBC xenograft models, we further demonstrated the radio-sensitive effect of RAD001 on bladder cancer cell growth. Further investigation into the molecular pathways underlying the observed effect of RAD001/IR combination treatment revealed the involvement of the PI3K/AKT/mTOR pathway, whereby RAD001 counter-acts the ionizing radiation-induced activation of the AKT survival pathway, thus indicating that a combination of RAD001 and IR might increase the efficiency of radiation therapy. As shown in cell proliferation and colony formation assays, the combination of IR and RAD001 led to an additive, but not synergistic cytostatic effect. No cell death was observed per se, although apoptosis occurred when modulating p21 expression.

We have also presented evidence showing that RAD001 and ionizing radiation contributed to cell cycle arrest by acting at difference phases of the cycle progression:

while RAD001 induced a G0/G1 arrest, ionizing radiation was responsible of stopping cells at the G2 phase, potentially explaining the increased efficiency upon combination of RAD001 and IR compared to each treatment alone.

Another novel aspect presented in this work pertains to the role of RAD001 in inducing autophagy, a relatively new process whereby cells engage in a catabolic, auto-degradation process through lysosomes. Although several articles have studies the role of RAD001 in inducing autophagy in different cell lines, no previous work has clearly demonstrated such a role in bladder cancer cells. Furthermore, we have shown throughout this work that RAD001 seems to be playing a role in balancing between apoptosis and autophagy since RAD001 was able to control both of these processes, which could present a promising way to sensitize cells to treatment. Through knockdown experiments our results present evidence in support of a functional role for p21 in mediating the autophagic process, thus promoting p21 as a potential player that can be modulated in the treatment of UBC.

CHAPTER VII: FUTURE DIRECTIONS AND CLINICAL RELEVANCE

From the bench…

Despite novel and promising results, several avenues are yet to be explored in order to better understand the molecular pathways behind our observations. First, it would be interesting to investigate the effects of varying the administered doses of RAD001; in fact, and through our experiments, a differential response was noticed between low doses of RAD001 as applied to the cell lines *in vitro*, and higher doses with continuous administration of the drug *in vivo*, with mice. Thus, one could wonder whether a higher dose of RAD001 can achieve a higher efficiency, or whether there could be a certain toxicity-related limit. Within the same line of thoughts, it is also interesting to investigate the effects of administrating ionizing radiation in fractions, as currently done in a clinical setting. Could such a mode allow a deviation from our observations towards a higher efficacy?

In addition to varying the dose of RAD001 and/or the mode of irradiation, this work invites to further investigating the DNA repair mechanisms involved in this process. Although our work has shown a G0-G1 cell cycle arrest, we have not had the possibility to further investigate the mechanism involved in DNA repair; in fact, targeting these mechanisms is one potential way that could increase the kill efficiency of bladder cancer cells. Furthermore, the role that p21 plays in the DNA repair mechanisms would warrant close consideration in the context of combination therapy.

Our experiments have shown an involvement of apoptosis in the cellular response to RAD001-induced radio-sensitization. Further and deeper investigation of the apoptotic

pathway, in our models is warranted. Similarly, exploring novel inhibitors that could be tested along with RAD001 and ionizing radiation is also an interesting prospect emanating from this study. Of the most obvious potential targets are members of the PI3K/AKT pathway. As shown, in the course of this work, RAD001 seems to counter-act the effect of ionizing radiation in activating the AKT. It is thus very conceivable that targeting activated members of this pathway, along with administration of RAD001 could help achieve an even higher therapeutic efficiency in bladder cancer cells.

… to the bedside

Currently, ionizing radiation is used as a bladder-sparing treatment modality instead of cystectomy with proper local control of radiations, and optimal management of the cytotoxicity remaining major challenges. The significance of our results stems from the fact that the use of RAD001 as a radio-sensitizer could ultimately enhance the final outcome of ionizing radiation along with reducing the side effects. Furthermore, and as far as the patient's quality of life is concerned, the implementation of a radio-sensitizer as part of the ionizing radiation treatment would hopefully save the urinary and sexual functions of patients, and with that allow him to overcome a major psychological challenge.

In addition, the introduction of a radio-sensitizer in the radiation of the bladder cancer could allow the treating physician a certain "marge-de-manoeuvre", as far as radiation doses are concerned. In fact, the additive effect of RAD001 on cell growth presents an opportunity to achieve similar efficacy as ionizing radiation alone, but with lower doses, thus sparing the patient the known short and long-term side effects of

radiations. Furthermore, being able to achieve the same efficacy with smaller doses would allow an increase in dose fractionation, thus permitting the normal surrounding cells to recover, the radio-resistant cells to enter sensitive phases of the cycle before the next fraction, as well as the re-oxygenation of tumor cells; these different aspects would allow, not only better recoveries and minimization of side-effects, but also a lowering in the chance of recurrence following ionizing radiation treatment.

Given that RAD001 is an orally administered drug, with mild side-effects, introducing it as a combination with ionizing radiation could potentially reduce the need for hospital admission, normally required for cystectomy, delay the surgery or even spare the bladder altogether; such combination treatment could thus be provided at specialized care centers, thus presenting economic advantages to healthcare provision.

On a different level, a better understanding of the molecular mechanism, and more specifically of the role of p21 in mediating the RAD001 radio-sensitization is warranted. In fact, one could easily envision that patient profiling or signature based on their p21 and/or p53 status could open the gate to personalized, targeted therapy, thus sparing some patients the burden of inefficient, unnecessary over-treatment.

It is worth noting that results from this work have provided the basic evidence for a clinical trial that was opened at the McGill University Health Center, assessing the advantage of introducing RAD001 to ionizing radiation as one treatment arm, compared to ionizing radiation alone. Preliminary results from the trial are positive, and seem promising in generating evidence in support of RAD001 use as a radio-sensitizing agent in urinary bladder cancer treatment.

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THE END

Roland S. Nassim

ANNEXES

ANNEX A: Journal article (2008)

Mansure, J.J., **Nassim, R.**, Chevalier, S., Rocha, J., Scarlata, E., and Kassouf, W., *Inhibition of mammalian target of rapamycin as a therapeutic strategy in the management of bladder cancer.* Cancer Biol Ther, 2009. **8**(24): p. 2339-47.

RESEARCH PAPER

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Inhibition of mammalian target of rapamycin as a therapeutic strategy in the management of bladder cancer

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Key words: mTOR, bladder cancer, experimental therapeutics, everolimus

We examined whether mTOR inhibition by RAD001 (Everolimus) could be therapeutically efficacious in the treatment of bladder cancer. RAD001 markedly inhibited proliferation of nine human urothelial carcinoma cell lines in dose- and sensitivity-dependent manners in vitro. FACS analysis showed that treatment with RAD001 for 48 h induced a cell cycle arrest in the G_0/G_1 phase in all cell lines, without eliciting apoptosis. Additionally, RAD001 significantly inhibited the phosphorylation of S6 downstream of mTOR and VEGF production in all cell lines. We also found tumor weights from nude mice bearing human KU-7 subcutaneous xenografts treated with RAD001 were significantly reduced as compared to placebo-treated mice. This tumor growth inhibition was associated with significant decrease in cell proliferation rate and angiogenesis without changes in cell death. In conclusion inhibition of mTOR signaling in bladder cancer models demonstrated remarkable antitumor activity both in vitro and in vivo. This is the first study showing that RAD001 could be exploited as a potential therapeutic strategy in bladder cancer.

Introduction

The incidence of bladder cancer has increased gradually since 1973. In North America, it is now the fourth most frequently diagnosed cancer in men and significantly increasing among women as smoking becomes more prevalent in this group. Radical cystectomy remains the main form of treatment for invasive bladder cancer and patients with clinically organ-confined disease have a 5-year overall survival rate of only 60% despite standard of care therapy. Furthermore, once distantly metastatic, virtually all bladder cancer patients will succumb to the disease within 18 mo. While some newer chemotherapeutic regimens are less toxic, there is yet no compelling evidence of improved patient survival. Hence, there is a desperate need not only to improve efficacy of organ-sparing therapies for clinically localized disease, but also to improve treatment for metastatic bladder cancer. As understanding of the biology of urothelial carcinoma increases, novel therapeutic approaches need to be investigated.

In recent years, discoveries in the field of signal transduction have led to identification of key molecules involved in the pathways that regulate cell survival, apoptosis, proliferation and tumor-associated processes such as angiogenesis and metastasis. One signaling molecule that is extremely attractive and has recently drawn much attention for targeted therapy is the mammalian target of rapamycin (mTOR). mTOR is a downstream serine/threonine-specific protein kinase of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which plays a critical role in oncogenesis.[1] Dysregulation of the PI3K/mTOR pathway generates a favorable oncogenic environment and has been documented in a variety of human transformed cells and tumors.[2-4] The mTOR kinase integrates extracellular and intracellular signals of growth factors and nutrients, through PI3K/AKT pathways.[5-7] AKT modulates mTOR activity by direct phosphorylation of a complex known as tuberous sclerosis (TSC1/TSC2), which has been established as the major upstream inhibitory regulator of mTOR.[8] Moreover, mTOR regulates translation by controlling key molecules of protein synthesis, notably Ribosomal S6 kinase (S6K)1 and eukaryote initiation factor 4E binding protein 1 (4EBP1), the most extensively studied substrates of mTOR.

Promising preclinical data have led to rapid translation of mTOR inhibitors as anticancer therapy into the clinical setting.[9] The first generation of mTOR inhibitors that has entered the medical oncology includes three rapamycin derivatives, Torisel (CCI-779), AP23573 and Everolimus (RAD001), which are in development for various solid tumors.[10,11] Recently, the first successful Phase III clinical trial involving a mTOR inhibitor occurred in patients with advanced renal cell carcinoma.[12,13] Patients who received monotherapy with Torisel showed 50% increase in overall survival relative to patients who received standard of care with interferon. Based on these results, Torisel was approved by the Food and Drug Administration for the treatment of patients with advanced renal cancer in May 2007. Lately, preliminary studies in vitro have reported rapamycin to inhibit proliferation of human bladder cancer cell lines.[14,15] To our knowledge, the mechanism and effect of mTOR inhibition in bladder cancer in vivo has never been studied. In the present study, we

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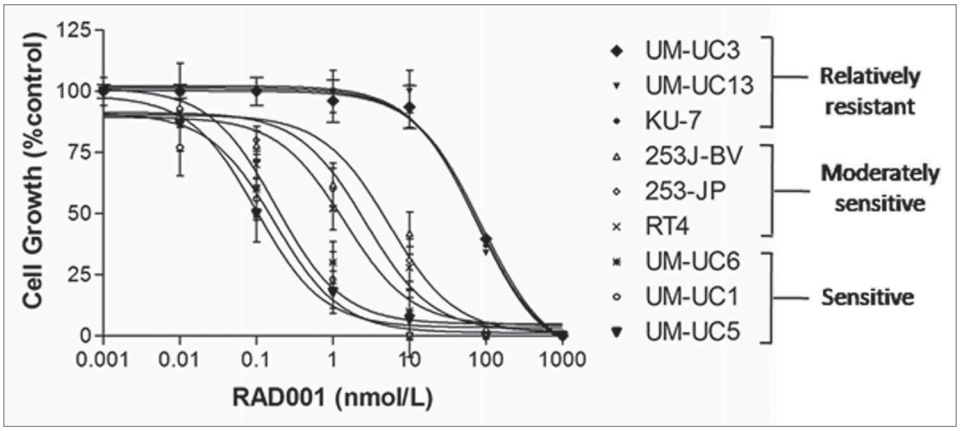

Figure 1. Dose-response of bladder cancer cell lines to the mTOR inhibitor, RAD001. Proliferation assays were performed after treatment with RAD001 at concentrations ranging from 0.001–1,000 nmol/L for 72 h. The GI_{50} value was defined as the mean concentration of drug that generated 50% of growth inhibition. RAD001 sensitivity was referred to as high at a GI_{50} <0.5 nmol/L in UM-UC5 (0.1 nmol/L), UM-UC1 (0.17 nmol/L), UM-UC6 (0.2 nmol), moderate at 0.5 nmol/L < GI_{50} < 50 nmol/L in RT4 (1.4 nmol/L), 253-JP (2.7 nmol/L), 253J-BV (5.3 nmol/L) and relatively resistant at GI_{50} >50 nmol/L in KU-7 (76 nmol/L), UM-UC13 (77 nmol/L) and UM-UC3 (86 nmol/L).

report for the first time that RAD001 potently exhibits antitumorigenic activity on bladder cancer cells in vitro and bladder tumors in vivo. Our findings suggest that inhibition of mTOR signaling holds great potential for bladder cancer therapy.

Results

Effects of RAD001 on cell proliferation. In order to determine the effect of RAD001 on bladder cancer cell growth in vitro, we screened by performing MTT assays on a panel of nine urothelial carcinoma cell lines to establish sensitivity by dose-response curves for RAD001. Briefly, these cell lines represent different stages and grades of bladder cancer. UM-UC3, UM-UC5 and UM-UC6 originated from invasive urothelial carcinoma, whereas UM-UC1 and UM-UC13 were isolated from lymphatic metastases of bladder cancer.[16] Similarly, the 253-JP and 253J-BV cell lines were derived from an invasive urothelial carcinoma.[17] Unlike UM-UC cell lines, the RT4 cell line was derived from a well-differentiated superficial bladder tumor.[18] Cells were treated with RAD001 at concentrations ranging from 0.001 nmol/L to 1,000 nmol/L in the presence of 10% FBS for 72 h (**Fig. 1**). RAD001 was a very potent drug for bladder cancer cells and did inhibit the proliferation of all cell lines in a dose-dependent manner but cells exhibited different sensitivities to RAD001. Of the nine cell lines tested, three lines (UM-UC5, UM-UC1, UM-UC6) presented a GI_{50} <0.5 nmol/L and were considered most sensitive to the anti-proliferative effect of RAD001; whereas 3 lines (RT4, 253-JP, 253J-BV) showed a GI_{50} between 0.5 nmol/L and 50 nmol/L, and were classified as moderately sensitive. The other three lines (KU-7, UM-UC13, UM-UC3) had a GI_{50} >50 nmol/L and were then considered as relatively resistant to RAD001.

RAD001 significantly increases the proportion of cells in G_0/G_1 arrest. The anti-proliferative activity of mTOR inhibitors is assumed to be primarily due to G_0/G_1-phase arrest.[19] To ascertain the potential effect of RAD001 on the cell cycle in bladder cancer, we investigated the distribution of cells in different phases of the cell cycle after RAD001 treatment. When cells were treated with different concentrations of RAD001, ranging from 0.5 to 100 nM (based on GI_{50}) for 48 h, a significant increase in the percentage of cells arrested in the G_0-G_1 phase was observed for all cell lines. This was accompanied by a corresponding decrease in the fraction of cells in the S-phase but no significant changes in the sub-G_1 fraction (**Fig. 2**). According to cell line's sensitivities to RAD001, 88% ± 3.2% of the most sensitive lines (UM-UC5; UM-UC1; UM-UC6) were arrested in G_0-G_1 phase as compared to 66% ± 1.4% in the corresponding untreated controls. Values for cells arrested in G_0-G_1 phase under RAD001 treatment in the moderately sensitive lines (RT4; 253-JP; 253J-BV) were 85% ± 2.9% as compared to 69% ± 0.6% in the untreated controls. For RAD001 relatively resistant cell lines (KU-7; UM-UC13; UM-UC3), 72% ± 0.9% were found in G_0-G_1 as compared to 61% ± 0.9% for the controls. At a 95% confidence interval, there was no overlap between RAD001 (sensitive: 73–100%; moderate: 72–97%; resistant: 68–71%) and control (sensitive: 59–71%; moderate: 66–71%; resistant: 56–65%) series. These data provide strong evidence for a cell cycle arrest induced by RAD001 and, in turn, growth inhibition of all cell lines, supporting that RAD001 has a cytostatic effect in bladder cancer.

Expression levels of mTOR and its associated upstream/downstream signaling partners and effects of RAD001 on activated S6. To better evaluate the effect of RAD001 in this set of human urothelial carcinoma cell lines, we characterized the baseline expression levels of mTOR and its close upstream and downstream signaling partners by western blotting. **Figure 3A** reveals that key signaling molecules of the pathway, PTEN, AKT, mTOR and S6 were expressed at significant levels in all bladder cancer cell lines. However, when levels of each of PTEN, AKT, mTOR and S6 were reported relatively to β-actin, there was neither an association with the aggressive features of the cell lines, nor with their relative RAD001 sensitivity (GI_{50} values). Nonetheless, the activated

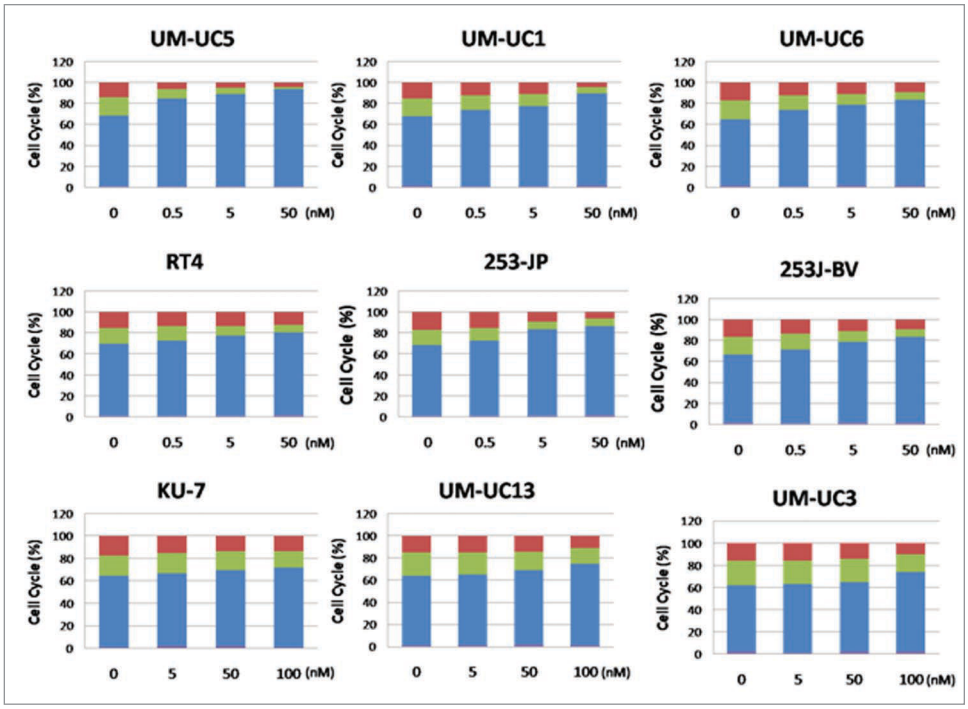

Figure 2. Effect of RAD001 on the cell cycle. FACS analysis of the cell cycle in all bladder cancer cell lines treated for 48 h with different concentrations of RAD001, as indicated. Phases of cell cycle: Sub-G_1 (purple); G_1 (blue); S (green) and G_2 (red).

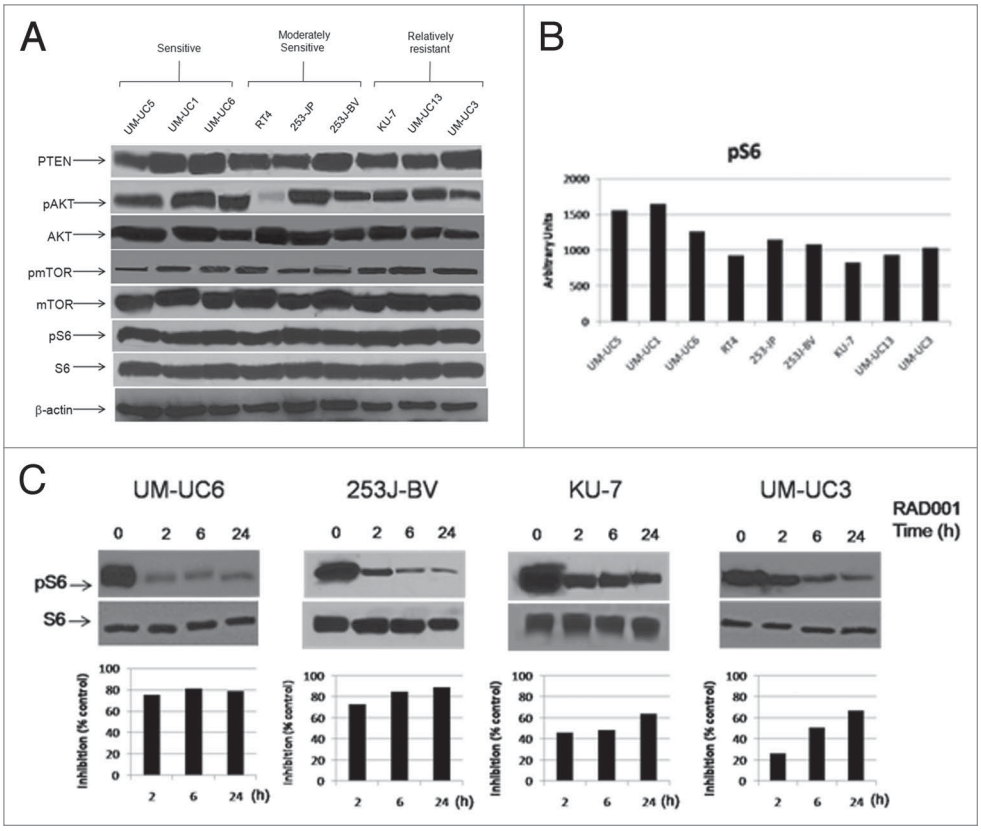

Figure 3. For figure legend, see page 2342.

Figure 3. Expression of upstream and downstream targets of mTOR and effects of RAD001 on S6 activation. (A) Total protein levels of PTEN (lane 1), AKT (lane 3), mTOR (lane 5), S6 (lane 7) and phosphorylated (p) AKT (lane 2); pmTOR (lane 4) and pS6 (lane 6). β-actin was used as a loading reference. (B) Levels of phosphorylated S6 (Ser240/244) reported over total S6 and presented according to their relative RAD001 sensitivity. (C) Time-course effects of RAD001 on pS6 at the GI_{50} concentration, as determined in sensitive (UM-UC6 at 0.5 nmol/L), moderately sensitive (253J-BV at 5 nmol/L) and relatively resistant (KU-7 and UM-UC3 at 75 nmol/L) bladder cancer cell lines (upper blots). Total S6 levels were also determined (lower blots) and used as above to calculate the extent of S6 activation (% of controls).

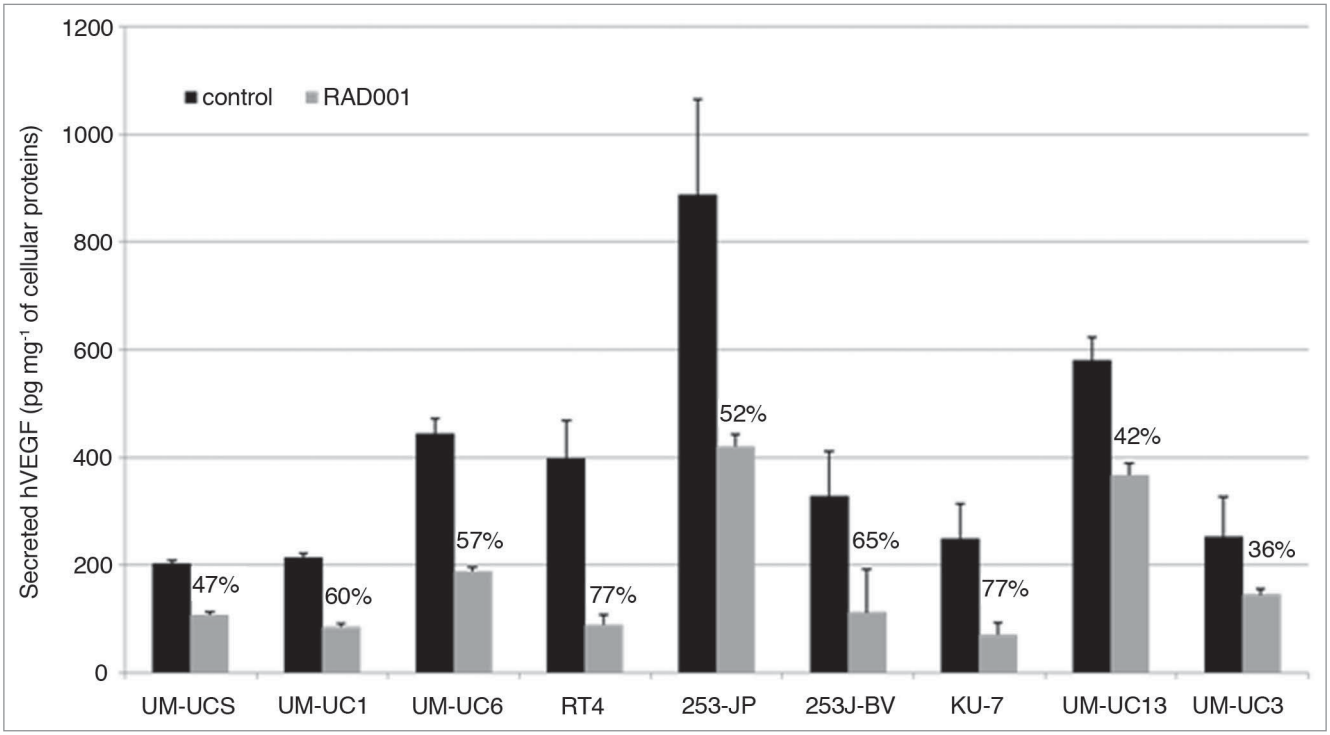

Figure 4. RAD001 inhibits the secretion of VEGF in bladder cancer cell lines. ELISA assays were used to determine hVEGF levels in the conditioned media of bladder cancer cells, treated at a GI_{50} concentration of RAD001 for 48 h. The secreted VEGF (pg per mL) for each cell line was expressed relative to their respective cellular protein content. Percentages of inhibition are shown above each grey bar.

or phosphorylated (p) forms of AKT (Ser473), mTOR and pS6 (Ser240/244) were detected in all cell lines (**Fig. 3A**), implying that the pathway is likely operational. In addition, there was no correlation between activated AKT and mTOR with aggressive features of the cell lines and RAD001 sensitivity. Interestingly, this was not the case for activated S6 whose levels appeared higher in most sensitive compared to relatively resistant cell lines. This is illustrated in **Figure 3B** showing pS6 levels for each cell line in the order of their RAD001 sensitivity.

Based on these findings, S6 activation was chosen as the signaling readout of RAD001 inhibitory effect on mTOR. This was investigated in the highly sensitive, UM-UC6, moderately sensitive, 253J-BV, and two relatively resistant, KU-7 and UM-UC3, cell lines. For this purpose, cells were exposed to RAD001 (at their GI_{50} concentration) as above, and for time-periods varying from 0, 2, 6 and 24 h (**Fig. 3C**). As expected, levels of the S6 protein were not affected by RAD001 and thus remained relatively constant over time. On the other hand, RAD001 markedly reduced the activation of pS6 in comparison to untreated cells. Notably, RAD001 decreased the phosphorylation levels of S6 more rapidly and at a greater extent in the sensitive, UM-UC6, and moderately resistant, 253J-BV, cell lines (**Fig. 3C**). Indeed in UM-UC6 and 253J-BV cells, RAD001 inhibitory activity reached 70% at 2 h, which was almost the maximal level detected at 24 h (80%) in these cell lines. In contrast, in the relatively resistant KU7 and UM-UC13 cell lines, the inhibition was in the order of 20–40% at 2 h, and increased with time but to attain only 60% at 24 h. Altogether, these data demonstrate the potency of RAD001 to efficiently target the mTOR pathway in these bladder cancer cell lines.

RAD001 reduces the production of VEGF in bladder cancer cell lines. It has been reported that mTOR has the ability to inhibit the production of VEGF in adenocarcinoma cells.[20] Therefore, to test whether this may also apply in bladder cancer, we evaluated effect of RAD001 on VEGF production among the nine bladder cancer cell lines. Significant reduction of human VEGF (hVEGF) production was observed in all cell lines treated with RAD001 for 48 h in serum-free medium at their GI_{50} concentration, compared to untreated controls ($p < 0.03$) (**Fig. 4**). Effects ranged from 22–63% of inhibition and, in the conditions used, were not correlated with the sensitivity/resistance of the cell lines. These findings indicate that inhibition of mTOR signaling by RAD001 also translates in diminished VEGF secretion.

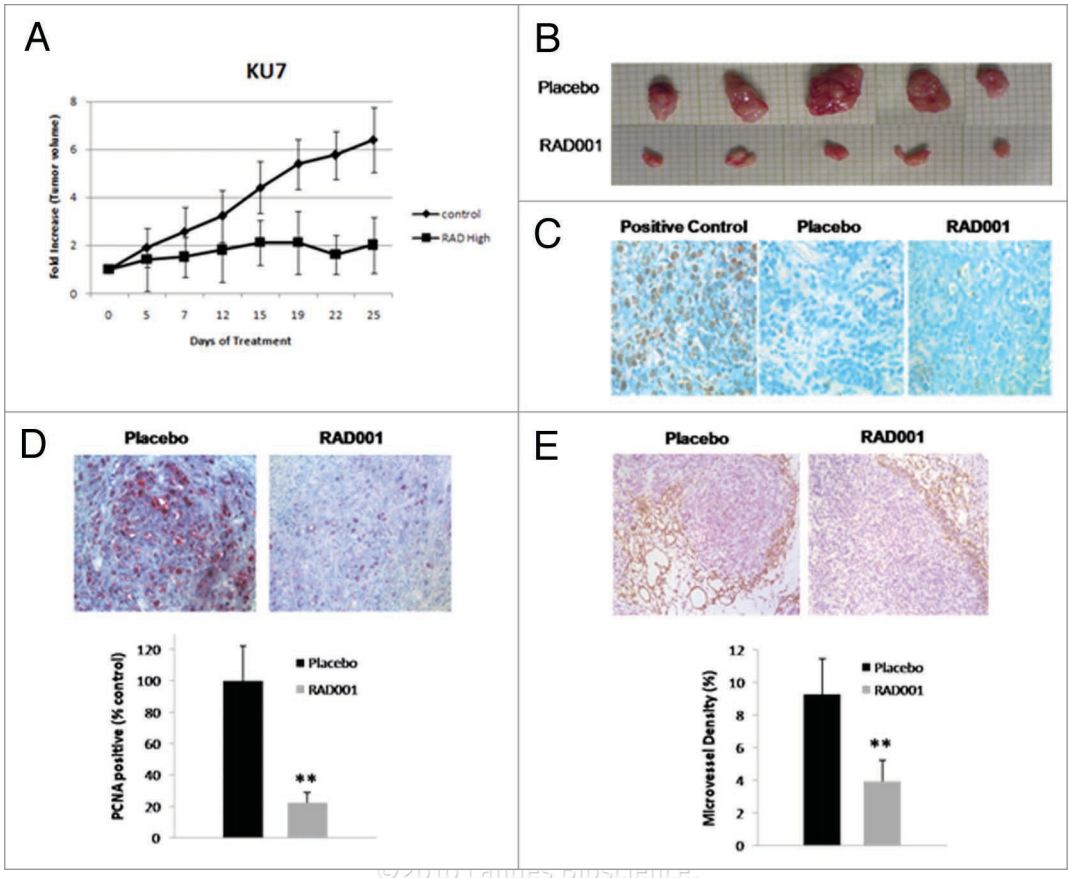

Figure 5. RAD001 inhibitory effects on bladder tumors in vivo. Tumor growth was assessed in control (placebo group) and RAD001-treated nude mice bearing s.c. KU-7 xenografts, as described in Methods. (A) Tumor volume is expressed as the mean fold increase over time. (B) Appearance of KU-7 tumors at the end of the 4 w treatment period. (C) Cell death was investigated in situ by TUNEL assays, as described in Methods. A positive control (created by treating any tissue sample with DNase I) is included in the left panel. (D) Tumor cell proliferation was assessed by PCNA staining, as described in Methods. The mean number of $PCNA^+$ cells per 100 cells is expressed graphically in tumor xenografts treated with RAD001 vs. control (**$p < 0.01$). (E) Microvessel density revealed by CD31/PECAM-1 staining in tumor xenografts from mice treated with placebo and vehicle RAD001, as described in Methods and shown at a x20 magnification. Histograms in lower panels represent average number of microvessels in the placebo and treated arms. **$p < 0.01$ vs. control.

RAD001 inhibits tumor growth and angiogenesis in human bladder cancer xenograft models. To determine if inhibitory effects of RAD001 on bladder cancer cells in vitro would result in bladder cancer cell growth inhibition in vivo, we employed nude mice bearing subcutaneous human bladder cancer xenografts of the relatively resistant KU-7 cell line. Almost all mice developed bladder tumors (n = 26/28). The drug was well tolerated, and no evident treatment toxicity was observed after 4 w. As illustrated in **Figure 5A**, the monitoring of tumor volume revealed that RAD001 treatment delayed KU-7 tumor progression. Moreover, this was associated with a markedly significant reduced mean tumor weight in RAD001-treated mice as compared to the tumor weight of the placebo-treated mice (KU-7; 87 mg vs. 340 mg, $p < 0.02$), implying that treatment with RAD001 has clear antitumor growth activity (**Fig. 5B**).

Consistently with these findings and because tumor growth reflects a delicate balance between cell death and proliferation, tumors were next submitted to TUNEL assays to detect apoptotic cells and, in parallel, proliferating cells immunostained with PCNA antibody. TUNEL assays revealed no positive cells in both situations; whereas DNase I treated samples were extensively labelled (**Fig. 5C**). However, PCNA positive cells were significantly less abundant upon treatment with RAD001 (**Fig. 5D**; **$p < 0.01$**). This implies that the extent of cell death was negligible whereas tumors among RAD001-treated grew at a slower rate than in placebo-treated mice. These findings are in line with the above in vitro data, and indicate that RAD001 inhibits tumorigenesis in this bladder cancer model mainly by inhibiting cell proliferation rather than by inducing apoptosis.

Tumor progression, including bladder, largely depends on optimal nourishment of malignant cells, a process achieved by the tumor cell's ability to provide factors such as VEGF, which in turn promotes the formation of blood vessels.[21] Since RAD001 inhibited VEGF production in all tested bladder cancer cells in vitro, we evaluated the effect of RAD001 on tumor angiogenesis in vivo. The endothelial marker CD31 was examined by IHC to account for microvessel density in the same tumors. As shown by CD31-positive vessels in KU-7 tumors from RAD001-treated mice were relatively smaller and fewer than in KU-7 tumors from placebo-treated mice, where larger vessels were observed

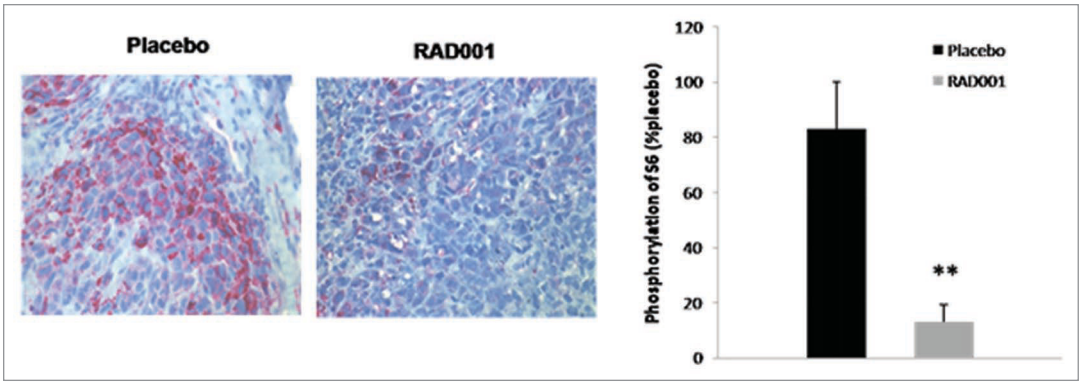

Figure 6. RAD001 inhibits S6 activation in vivo. Serial sections of tumor xenografts from mice treated with placebo and RAD001 were stained with pS6 antibody, as described in Methods and quantified in the right panel relatively to values in controls (**p < 0.01).

(**Fig. 5E**; **p < 0.01**). These results indicate that the antitumor effect of RAD001 was associated, at least in part, with the inhibition of tumor angiogenesis.

RAD001 attenuates S6 phosphorylation in vivo. To further dissect out the underlying mechanisms and in line with above in vitro findings on signaling, IHC was carried out to verify if RAD001 also inhibits S6 activation in tumors. As shown in **Figure 6**, RAD001 treatment significantly reduced the phosphorylation of S6 in KU-7 tumors of RAD001-treated mice as compared to the placebo group (p < 0.01). These results confirm that the RAD001 inhibition of tumor growth via mTOR signaling leads to a decrease activation of S6 in vivo.

Discussion

Current efforts in anticancer drug development are based on inhibiting the activity of key targets required for the maintenance of the transformed state, such as those implicated in the AKT pathway. Among AKT numerous substrates, mTOR is thought to be one of the major targets of relevance to cancer therapy.[22-24] Since the original demonstration of rapamycin tumor suppressing properties, rapamycin and its analogs have been tested for their effects on a number of tumor-derived cell lines and mouse xenograft tumor models.[25] Moreover, all mTOR inhibitors under clinical development have shown safety and efficacy activity; temsirolimus has been recently granted FDA approval for the treatment of metastatic renal cancer. Lately, in the largest Phase III clinical trial (RECORD-1, *REnal Cell cancer treatment with Oral RAD001 given Daily*) investigating the effects of RAD001 in patients who have failed standard therapies, RAD001 has shown to significantly extend the time without tumour growth from 1.9–4 mon and to reduce the risk of cancer progression by 70%.[26] While these findings in kidney cancer are highly promising, the likelihood of applications for other types of cancer sites largely depends on particular changes in the AKT/mTOR signaling axis in tumor cells. Indeed although mutations of mTOR itself have not been reported, bladder cancer has been associated with a specific deletion on chromosome 9. Interestingly, chromosome 9 harbors the TSC1 tumor suppressor gene, at the 9q34 loci, whose product downregulates the mTOR pathway.[27] Since this deletion is commonly found in all stages of bladder cancer progression, RAD001 has potential therapeutic properties to be exploited in bladder cancer therapy.

In the present study, we have shown that RAD001 potently inhibits the proliferation of several cell lines representing different stages of bladder tumorigenesis in vitro and also significantly inhibits human bladder KU-7 tumor xenografts growing in nude mice. We characterized the sensitivity of a panel of urothelial cell lines to mTOR inhibition, and while RAD001 markedly inhibited the proliferation of all bladder cancer cell lines tested in vitro, remarkable differences in dose response were observed among these cell lines. Interestingly, our findings indicate that even relatively resistant cells, in vitro, demonstrated sensitivity in vivo. Whether the dose used was already optimal to reproduce in vitro conditions or else that additional mechanisms of resistance to rapamycin analogues apply remains to be investigated. For instance, several factors intrinsic to bladder cancer cells such as the diversity of molecules in the mTOR signaling network may enter into play. This may imply altered levels of expression and/or activation of mTOR upstream targets such as hyperactive AKT signaling, which is associated with elevated mTOR signaling in some cancers.[28] Similarly, the loss of PTEN leading to activation of AKT was reported to correlate with increased sensitivity to mTOR inhibitors in prostate and breast cancer cell lines.[29] The two distinctive complexes of mTOR, named mTORC1 and mTORC2, may also exert different physiological functions and bind their own sets of partners and regulators.[30-32] Furthermore, the responsivenes of tumor cells to rapamycin and its analogs may also include an inability to regulate the CDK inhibitors p21 and p27 levels,[33,34] activity of cyclin D1,[35] as well as potential mutations in S6 or amplification of S6K1.[36] In the latter context, our studies provide evidence that mTOR inhibition does not seem to be profoundly affected by expression and phosphorylation levels of some upstream and downstream targets, since no apparent correlation with RAD001 sensitivity or cell aggressive features was observed among bladder cell lines with the exception of pS6. The finding of lower levels of S6 phosphorylation in more resistant cell lines is intriguing and worthy

of further characterization since the degree of inhibition at which RAD001 affects S6 activation was related to the RAD001 cell sensitivity. Indeed, the reduction in phosphorylation levels of S6 was more rapid and greater than in relatively resistant cell lines. Additionally, longer exposure was needed in more resistant cell lines to yield the same magnitude of inhibition. Such a correlation appears to exist in vivo as well since RAD001 decreased levels of S6 phosphorylation in KU-7 bladder tumors, and this most likely contributed to RAD001 antitumor activity. These findings support that RAD001 potently inhibits mTOR signaling at the level of S6 activation, which is determinant for protein synthesis. They also suggest that S6 inhibition reflects cell sensitivity to the drug. Nonetheless, it has been reported that different rapamycin sensitive signals others than S6K inhibition are required for its anti proliferative effects.[37] Clearly, the mechanism of bladder cancer may particularly be sensitive to mTOR inhibitors need to be further elucidated. We are currently developing bladder cancer cell lines stably transfected with a RAD001 resistant S6K to address whether such construct can rescue the antiproliferative effect of mTOR inhibitors.

We demonstrated that the anti-tumor activity of RAD001 was attributed to the regulation of cell cycle progression, particularly at the G_1-to-S transition. This may occur by blocking the signal of the downstream messengers, S6K1 and 4E-BP1, reported to prevent translation of key mRNAs required for cell cycle progression.[38,39] Consistently, our studies in bladder cancer cell lines revealed a cytostatic effect with no significant change in the sub-G_1 fraction and no cell loss in MTT assays. Along the same line, there was no concomitant observation of apoptotic tumor cells in xenografts. Hence beside the induction of cell cycle arrest, RAD001 was quite potent in exerting antitumor activity by inhibiting angiogenesis. This was evidenced by the decrease in VEGF production in vitro, and significant reduction of microvessels in tumors from RAD001-treated mice compared to control tumors in the placebo treated mice. These findings are consistent with the literature on RAD001 affecting angiogenesis.[40,41] Another possible mechanism by which RAD001 may inhibit tumor growth is by inducing autophagy,[42] a mode of cell death currently under investigation in human urothelial cell lines.

Taken together, this is the first study reporting that RAD001 has remarkable antitumor activity in bladder cancer both in vitro and in vivo. Notably, treatment was well tolerated. Moreover we also observed tumor growth inhibition in vivo in another relatively resistant bladder cancer cell line, UM-UC13 (unpublished), supporting applicability in a larger spectrum of tumors. Furthermore, these preclinical data provide a rationale and guidance for future clinical trials with RAD001 in bladder cancer patients.

Materials and Methods

Cell lines, antibodies and reagents. The bladder cancer cell lines (UM-UC1, UM-UC3, UM-UC5, UM-UC6, UM-UC13, RT4, 253JP, 253J-BV, KU-7) were kindly provided by Dr. Colin P.N. Dinney from M.D. Anderson Cancer Center, Houston, Texas. All cell lines were maintained as monolayers in modified Eagle's MEM supplemented with 10% fetal bovine serum (FBS), vitamins, sodium pyruvate, L-glutamine, penicillin, streptomycin, and nonessential amino acids at 37°C in the presence of 5% CO_2.

Drugs. RAD001 (Everolimus) was supplied by Novartis (Basel, Switzerland) as dry powder and microemulsion for oral use. For in vitro studies, the RAD001 was reconstituted to 10 mmol/L in DMSO (Sigma-Aldrich, Canada) and stored at -20°C. The stock solution was diluted in the culture medium supplemented with 10% FBS just before use so that DMSO concentration never exceeded 0.1%.

Cell survival and proliferation assay. For anti-proliferative effects of RAD001 and GI_{50} determination, bladder cancer cells were treated with different concentrations of RAD001 (0.001 nmol/L to 1,000 nmol/L) in medium supplemented with 10% serum for 72 h. Cell viability was evaluated using MTT assays 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, Canada) as described by manufacturer. Each experimental data point represented average values obtained from six replicates, and each experiment was done at least twice. The percentage of growth inhibition was calculated as: [(T - Ct24)/(C - Ct24)] * 100, where Ct24 is the baseline of cells seeded. The GI_{50} value was defined as the mean concentration of drug that generated 50% of growth inhibition. Dose-response curves were constructed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA).

Cell cycle analyses. After 24 h seeding, cells were treated with RAD001 at different concentrations (0.5 nmol/L to 100 nmol/L) for 48 h. Cells were washed once with PBS but keeping floating cells to analyse jointly with attached cells harvested by trypsinization. After two washes with cold PBS, cells were fixed in absolute ethanol, and stained with 50 μg/mL of propidium iodide in PBS. Samples were analyzed in a fluorescence-activated cell sorter, FACS Calibur (Becton Dickinson, Franklin Lakes, NJ USA), collecting 10,000 events. Cell cycle distribution was analyzed, with calculations done using Cell Quest© software. FL2 was used as threshold and subG_1 peak was applied to detect cumulative apoptosis.

Western blot analysis. Cells lysates and western blot were prepared as described.[43] Western blot analysis were done with 1:1,000 diluted primary monoclonal antibodies [mTOR, phospho-mTOR (Ser2448), PTEN, S6, phospho-S6 (Ser240/244), AKT, phospho-AKT (Ser473), β-actin (all from Cell Signaling Technology, New England, MA)]. Density histograms were created using NIH ImageJ software.

VEGF secretion. For in vitro measurements of VEGF production, bladder cancer cell lines were cultured in six-well plates for 24 h. The medium was replaced by fresh serum-free medium or medium supplemented with different concentrations of RAD001, according to GI_{50} values for each cell line. They were further cultured for 48 h and the cell supernatants were quantified (in triplicate assays) using the human VEGF Quantikine Immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The concentration of secreted VEGF was expressed relatively to the respective cellular protein content of each monolayer.

Bladder tumor xenografts. Female nude mice, 4–6 w old, were purchased from Charles Rivers Laboratories, Inc., (Wilmington, MA). The research protocol was approved by the Animal Care Committee of McGill University Health Center, in accordance with institutional and governmental guidelines and Declaration of Helsinki. Mice were injected subcutaneously with the urothelial carcinoma KU-7 cells (10^6 cells per injection) resuspended in 200 µL of Matrigel (BD Biosciences Co., Franklin, NJ). Treatment started on day seven after implantation. By this time, tumors reached 4–5 mm in diameter. Animals of each series (14 mice per group) were randomised and assigned to treatment and placebo arms. RAD001 microemulsion (2% RAD001) was diluted in water and administered at 5 mg/kg daily by oral gavage. Tumor growth was monitored at least twice weekly by Vernier calliper. Tumor volume was calculated as $V = [(\text{Length} \times \text{Width}^2) \times (\pi/6)]$.[44] Body weight was recorded weekly. Treatment was continued for 4 w when all mice were euthanized to harvest tumors. Half of each tumor was formalin-fixed and paraffin-embedded for immunohistochemistry and routine H&E staining, which confirmed the presence of tumor(s) in each specimen. The other half was snap-frozen in liquid nitrogen and stored at -80°C. Student's t test analysis using unpaired two tailed between the two samples was employed to assess differences in mean of fold increase in tumour volume among the treatments across time, adjusting for baseline. Natural logarithmic transformation was used to normalize the distribution of tumor volumes. Mean and median differences of tumor weight were obtained at the end point. Statistical significance was considered to be present at levels >95% ($p < 0.05$).

Immunohistochemistry for PCNA. Quantification of PCNA was determined as described.[45] Sections were incubated overnight at 4°C, with primary specific antibodies against PCNA (rabbit polyclonal Proliferation Marker, Abcam, MA; 1:200 dilution). Goat polyclonal anti-rabbit IgG secondary antibody, conjugated with HRP was added and incubated for 1 h at room temperature. Color development was performed with DAB substrate (Sigma Aldrich, Canada), according to manufacturer's instructions.

Slides were viewed under an Olympus IX81 inverted microscope system (Olympus, Inc.), equipped with CoolSnap HP digital camera (Hewlett Packard, USA). Images were acquired using Image Pro+ advanced software (version 5.0.1.; Media Cybernetics). The proliferative index of tumors was calculated from the average of five foci showing cells with highest PCNA density within a single 40 x field. Values were compared using unpaired Student's t test.

TUNEL assay. Cell death was detected in situ by enzymatic labelling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (Chemicon International Inc., Germany), according to the manufacturer's instructions. Formalin-fixed paraffin-embedded sections were processed as for PCNA staining. Control was created to positively identify apoptotic cells containing labelled DNA strand breaks in each tumor sample by treating sections with DNase I to a final concentration of 0.5 µg/mL (specific activity 5,000 U/mL). TdT was omitted from negative control slides, which were included in each run. After washing, reactions were revealed by incubating sections with DAB as above and viewed by light microscopy.

Microvessel density. Frozen sections (4 µm thick) were stained with CD31 (PECAM-1) antibodies from BD Pharmingen (San Diego, CA), following manufacturer's instructions and analyzed as described above for PCNA. Any distinct area of positive staining for CD31 was counted as a single vessel. A total of ten random high power fields at x20 magnifications were examined from tumors of each treatment groups and vessel density were then examined under higher magnification (x40) to count microvessels. Values were subjected to unpaired Student's t test analysis.

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Annex B: Journal article (2013)

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Combining mTOR Inhibition with Radiation Improves Antitumor Activity in Bladder Cancer Cells *In Vitro* and *In Vivo*: A Novel Strategy for Treatment

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Abstract

Purpose: Radiation therapy for invasive bladder cancer allows for organ preservation but toxicity and local control remain problematic. As such, improving efficacy of treatment requires radiosensitization of tumor cells. The aim of study is to investigate if the mammalian Target of Rapamycin (mTOR), a downstream kinase of the phosphatidylinositol 3-kinase (PI3K)/AKT survival pathway, may be a target for radiation sensitization.

Experimental Design: Clonogenic assays were performed using 6 bladder cancer cell lines (UM-UC3, UM-UC5, UM-UC6, KU7, 253J-BV, and 253-JP) in order to examine the effects of ionizing radiation (IR) alone and in combination with RAD001, an mTOR inhibitor. Cell cycle analysis was performed using flow cytometry. *In vivo*, athymic mice were subcutaneously injected with 2 bladder cancer cell lines. Treatment response with RAD001 (1.5 mg/kg, daily), fractionated IR (total 9Gy = 3Gy×3), and combination of RAD001 and IR was followed over 4 weeks. Tumor weight was measured at experimental endpoint.

Results: Clonogenic assays revealed that in all bladder cell lines tested, an additive effect was observed in the combined treatment when compared to either treatment alone. Our data indicates that this effect is due to arrest in both G1 and G2 phases of cell cycle when treatments are combined. Furthermore, our data show that this arrest is primarily regulated by changes in levels of cyclin D1, p27 and p21 following treatments. *In vivo*, a significant decrease in tumor weight was observed in the combined treatment compared to either treatment alone or control.

Conclusions: Altering cell cycle by inhibiting the mTOR signaling pathway in combination with radiation have favorable outcomes and is a promising therapeutic modality for bladder cancer.

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Competing Interests: The mTOR inhibitor RAD001 was kindly provided by its manufacturer, Novartis. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Bladder cancer is a very prevalent disease in North America. In 2012, 55,000 men and 18,000 women were diagnosed with bladder cancer; 1 in 5 men and 1 in 4 women will die from their disease [1]. . Radical cystectomy which consists of the complete removal of the bladder, remains the "gold standard" treatment for invasive bladder cancer [2]. Radiation therapy is an attractive alternative as it preserves the bladder and allows for normal urinary and sexual functions [3]. However, the lack of local control of the disease as well as the significant toxicity that is associated with radiation therapy remains problematic [4–6]. To improve efficacy, several clinical trials on organ-sparing management were carried out to test the effects of combined chemotherapy and radiation [7,8]. However despite numerous efforts, chemoradiation studies remain associated with suboptimal local control of disease and decrease survival compared with radical surgery. As such, there is an imperative need to increase radiosensitization of bladder cancer to increase efficacy by improving local control of disease and allowing for dose reduction to decrease toxicity of radiation therapy.

A signaling molecule that is extremely attractive and has recently drawn much attention for targeted therapy is the mammalian target of rapamycin (mTOR). More specifically, mTOR is a downstream serine/threonine protein kinase of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway which plays a critical role in oncogenesis [9,10]. Deregulation of the PI3K/AKT/mTOR pathway generates a favourable oncogenic environment and has been documented in a variety of human tumours including bladder cancer [11]. mTOR inhibition became an active

area of research to develop and test small inhibitory molecules such as rapamycin analogues -notably RAD001 (Everolimus, Novartis) and CCI-779 (Torisol, Wyeth) to treat diverse diseases, including cancer. Recently, the first successful Phase III clinical trial involving an mTOR inhibitor was realized in patients with advanced renal cell carcinoma, who experienced an improvement in overall survival [12]. We recently published the first report demonstrating significant antitumor activity *via* inhibiting mTOR with RAD001 in bladder cancer models *in vitro* and *in vivo* [13]. Interestingly, remarkable differences in sensitivity to mTOR inhibition were noted among nine human bladder cancer cell lines. Moreover, there was no correlation between activated AKT and mTOR levels with cell aggressive features. However, this was not the case for activated S6 whose levels appeared higher in RAD001 sensitive compared to relatively resistant cell lines. Of interest, some studies have reported that mTOR inhibition may sensitize tumors of the prostate, breast, and brain to ionizing radiation [14–16]. Since radiation was shown to activate the PI3K/Akt survival/growth pathway which may be responsible for the cell death escape and radioresistance [17,18], concurrent mTOR inhibition may potentially overcome resistance to radiation in bladder cancer. To follow up on this hypothesis, the present study examined the effects of combining RAD001 and ionizing radiation, *in vitro* and *in vivo*, on cell survival and growth in an array of bladder cancer cell lines. In addition, we attempted to shed light on the mechanism by which this combination of treatments might inhibit tumor growth.

Materials and Methods

Ethics Statement

All ethical standards associated with the use of our animal xenograft model were fully followed and respected. The McGill University Health Center's Facility Animal Care Committee approved our animal protocols (protocol #5428) before the beginning of the study. Furthermore, the animals were maintained and kept in state-of-the-art facilities that follow the stringent procedures for conducting animal research, which includes constant monitoring and inspection of the animals and the users.

Cell culture

The UM-UC3, UM-UC5, UM-UC6, and KU7 cell lines were characterized and provided by the Specimen Core of the Genitourinary Specialized Programs of Research Excellence in bladder cancer at M. D. Anderson Cancer Center [19]. The 253-JP and 253J-BV were kindly provided by Dr Colin P.N. Dinney from M.D. Anderson Cancer Center, Houston, Texas [20]. The cell lines were routinely cultured at 37°C in a 5% CO_2 incubator, maintained in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) (Wisent, Saint-Jean-Baptiste QC) and passaged when reaching 80% confluence. The mTOR inhibitor RAD001 was kindly provided by its manufacturer, Novartis.

Clonogenic assay

Cells were seeded in a 6-well plate at a density of 200 cells per well and maintained in the growth medium. Once attached, they were treated with RAD001 at doses equivalent to the GI50 for each cell line, as previously described [13]: UM-UC3 (75 nM), KU7 (50 nM), 253J-BV (8 nM), 253-JP (8 nM), UM-UC5 (0.5 nM) and UM-UC6 (0.5 nM) and maintained at 37°C in a 5% CO_2 incubator for 12 hours. This was followed by radiation treatment at different dosages, with and without RAD001. Controls included untreated cells along with cells treated with each of radiation and RAD001 treatment alone. Cells were further cultured at 37°C and allowed to form colonies for 10–14 days. An approximate cutoff of 50 viable cells/colony was chosen. The cells were washed with phosphate balanced salt solution (PBS) and fixed for 15 min using 3.7% formaldehyde in PBS. After a second PBS wash, cells were stained with crystal violet (0.4% w/v in PBS; Fisher Scientific, Waltham, MA) and left to air dry before counting of colonies. Each treatment consisted of duplicate wells of a 6-well plate and the experiment was performed twice. The surviving fraction was calculated as (the mean colony count at the end of the experiment)/(cells inoculated at the beginning)×(plating efficiency). The plating efficiency was defined as (mean colony count)/(cells plated in the non-radiated control). The non-irradiated cells were used as a control.

Flow cytometry

Cells were seeded in culture plates and allowed to attach. RAD001 was added to the appropriate samples 12 hours before radiation at a dose equivalent to the GI50 of each cell line. This was followed by a dose of 4Gy of ionizing radiation (based on previously determined sensitivity experiments) and the cells were further cultured for 48 hours. Cells were then trypsinized, washed once with PBS, and fixed with 100% cold ethanol for 60 minutes at 4°C. After centrifugation, cell pellets were resuspended in a solution of propidium iodide (PI) (50 g/ml, Invitrogen, Carlsbad, CA) in PBS, supplemented with RNase (100 g/ml; Invitrogen, Carlsbad, CA) then transferred to fluorescence-activated cell sorting (FACS) tubes and incubated in the dark for 30 min at 40°C to allow propidium iodide intake in the nucleus. PI intake was then assessed using a Coulter Flow Cytometer (BD Biosciences, Franklin, NJ).

Western blot

Cells were grown and treated as per the regimen described above (RAD001, ionizing radiation, and both in combination), with untreated cells serving as controls. Following treatments, cells were scraped on ice and re-suspended for 30 minutes at 4°C in cold RIPA (lysis) buffer containing a cocktail of phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, IN). Cell suspensions were then centrifuged to collect clear lysates in the supernatant. The protein concentration was measured by the bicinchoninic acid (BCA) assay (Pierce Scientific-Thermo Fisher Scientific, Rockford, IL). Protein samples (40 μg–60 μg) were submitted to polyacrylamide gel electrophoresis, as previously described [13]. Proteins in gels were transferred onto membranes, blocked with a 5% non-fat milk and/or 5% bovine serum albumin solution, and immuno-blotted with the following monoclonal primary antibodies (all rabbit): phospho-AKT, total AKT, phospho S6, total S6, p21, p27kip1, and cyclin D1 (Cell Signaling Technology, Beverly, MA) at concentrations recommended by the manufacturer. The membranes were then incubated with the appropriate anti-rabbit secondary antibodies and an ECL chemiluminescence detection system (Amersham-GE Healthcare, Piscataway, NJ) was used to reveal protein bands of interests on X-ray film. The films were scanned and protein levels were normalized against actin (Cell Signaling Technology, Beverly, MA), a control 42 kDa housekeeping protein present in all samples and served as our loading control.

In vivo—Xenograft model

All protocol approvals were obtained prior to the onset of the study from the Animal Care Committee of the McGill University Health Center. Female athymic mice (Nu/Nu strain, 4–6 weeks old; Charles River Laboratories, Wilmington, MA) were used for

our xenograft bladder cancer model, as previously reported [13]. Briefly, mice were subcutaneously injected with KU7 (10^6 cells per injection). Another experiment with the same methodology was performed using the 253J-BV bladder cancer cell lines. To facilitate adhesion, cells were suspended in 200 µl Matrigel (BD Biosciences, Franklin, NJ) prior to injection. Tumors were allowed to implant and grow for one week prior randomization into 4 groups corresponding to the different treatment arms, with each group consisting of 14 mice. The 1st group was treated with a placebo (5% glucose solution in water). The 2nd group received RAD001 orally (microemulsion diluted in 5% glucose solution) at a dose of 1.5 mg/kg daily. In the 3rd group, tumors were exposed to ionizing radiation at a fractionated dosage totaling 9 Gy (3×3Gy) every second day during the first week of treatment. In the 4th group, mice were given RAD001 at the above-mentioned dosage 1 day before the start of the tumor radiation treatment. Mice were followed for 4 weeks from the onset of treatments. Body weight and animal behavior were monitored throughout the experiment. Tumors were measured (length and width) twice a week using a Vernier caliper in order to calculate volumes ($V = [(\text{length} \times \text{width}^2) \times (\pi/6)]$ as previously reported [13]. Mice were euthanized in a CO_2 chamber at the end of treatment. Tumors were harvested, immediately weighed, and conserved fixed or frozen for future studies.

Immunohistochemistry

Tumor sections were obtained from mice treated with placebo, radiation, RAD001 and the combination regimen. The paraffin embedded tumors sections were mounted on glass slide for staining. Following de-paraffinization and hydration, antigen retrieval was performed in heating the samples with 5% citrate buffer solution (pH 7.0). The sections were incubated overnight at 4°C with a p21 specific antibody (dilution 1:25). HRP-conjugated goat polyclonal anti-rabbit IgG secondary antibody was added and incubated at room temperature for 1 hour. 3,3′-Diaminobenzidine (DAB) substrate (Sigma Aldrich, St. Louis, MO) was used for color development according to manufacturer's instructions. Slides were viewed under a Leica Diaplan inverted microscope equipped with a Leica DFC300FX Camera (Leica, Wetzlar, Germany). Pictures were captured using a Leica Application Suite. Analysis was based on an average of 5 foci, at 40× magnification, showing viable cells, and a computed H-score was calculated by summing the products of the percentage cells stained at a given staining intensity (0–100) and the staining intensity (0 for negative staining, 1 for low and 2 for high staining).

Statistical analysis

Student's T-test (unpaired, two-tailed) was used in all statistical analysis. Significance was set at $p<0.05$.

Results

Relative sensitivity of a panel of bladder cancer cell lines to RAD001 and ionizing radiation

We recently demonstrated that a panel of nine bladder cancer cell lines exhibits relative differences in their RAD001 sensitivity and accordingly, RAD001 treatment resulted in relative differences in mTOR inhibition and growth arrest, as monitored by MTT assays. With this data, we were able to divide our cells lines into 3 groups based on their RAD001 sensitivity [13] as follows: relatively resistant (UM-UC3, UM-UC13, KU7 (GI50≥50 nmol/L)), moderately sensitive (253J-P, 253J-BV, RT4 (GI50<50 nmol/L)) and finally highly sensitive (UM-UC1, UM-UC5, UM-UC6 (GI50≤0.5 nmol/L). In this study looking at the effects of combined treatments (RAD001 and radiation), clonogenic assays was used to classify the six cell lines tested according to their relative sensitivities to IR to various doses of radiation (Fig. 1A). Based on these relative sensitivities to radiation, cell lines were divided into three groups, resistant, moderately resistant, and sensitive. The resistant group includes UM-UC5 with the highest surviving fraction, the moderately resistant included UM-UC13, KU7, UM-UC3, UM-UC6 whereas 253J-BV had a lower surviving fraction and was therefore defined as a radiation-sensitive cell line. We compared the response of these six cell lines to each of RAD001 and ionizing radiation. Based on the data in Figure 1B and Table 1, we concluded that there is no correlation between the sensitivity to RAD001 and the sensitivity to ionizing radiation.

Ionizing radiation activates AKT while RAD001 inhibits S6 phosphorylation

It has been reported that ionizing radiation activates AKT in the surviving cell fraction [17,21]. As this may be associated with resistance to treatment, cell death escape and survival, we sought to determine if radiation exposure of bladder cancer cells would lead to AKT activation. For this purpose, a relatively resistant cell line, KU7, was exposed to ionizing radiation over time (0 to 60 min) and lysed to analyze pAKT by direct Western blotting using phospho-specific AKT antibodies directed against the S473 phosphorylation site. Results in Figure 2A show that indeed AKT was rapidly activated following 15 min of radiation treatment and this activation persisted at 30 and 60 min. These results thus imply that KU7 undergo an activation of the pro-oncogenic survival pathway following exposure to ionizing radiation. In all experiments, the levels of pAkt increased following the treatment with ionizing radiation to a maximum and decreased afterwards. Similarly, as KU7 cells are also relatively RAD001 resistant, they were treated with RAD001 to ascertain that its target mTOR was inhibited. For this purpose, levels of phosphorylated S6 were determined using an antibody specific to serine residues 240/244 in the S6 protein. As expected, RAD001 was potent in decreasing phosphorylation levels on the mTOR downstream signaling molecule and target S6, as shown at 30 minutes post-treatment (Fig. 2B) and this inhibition is sustained at 24 h post-treatment (data not shown). Furthermore, similar results were obtained with other bladder cancer cells lines (253J-BV, UM-UC3, and UM-UC6) treated with radiation and RAD001 (data not shown).

Combining RAD001 with ionizing radiation significantly reduces colony formation

To provide insight on effects of combining RAD001 with ionizing radiation on bladder cancer cell lines, we monitored the fraction of surviving cells over time using clonogenic assays. Following treatment, plated cells were monitored over time and the number of colonies was counted. The RAD001 dose was maintained at the GI50 for each cell line, while the radiation dose was varied. In all cell lines tested (253J-BV, UM-UC6, KU7, UM-UC3, UM-UC13, and UM-UC5), a significant decrease in the number of colonies was observed for cells treated with the combination therapy compared to either ionizing radiation alone, or the untreated control (Fig. 3). Interestingly, while this decrease in the surviving fraction was seen in all cell lines tested, the most dramatic relative decrease when both treatments were combined was seen with two most sensitive cell lines to RAD001 (UM-UC5 and UM-UC6). In all tested cell lines, our results point to an additive effect on growth when combining RAD001 with ionizing radiation. It is worth noting that a lower inhibition of colonic

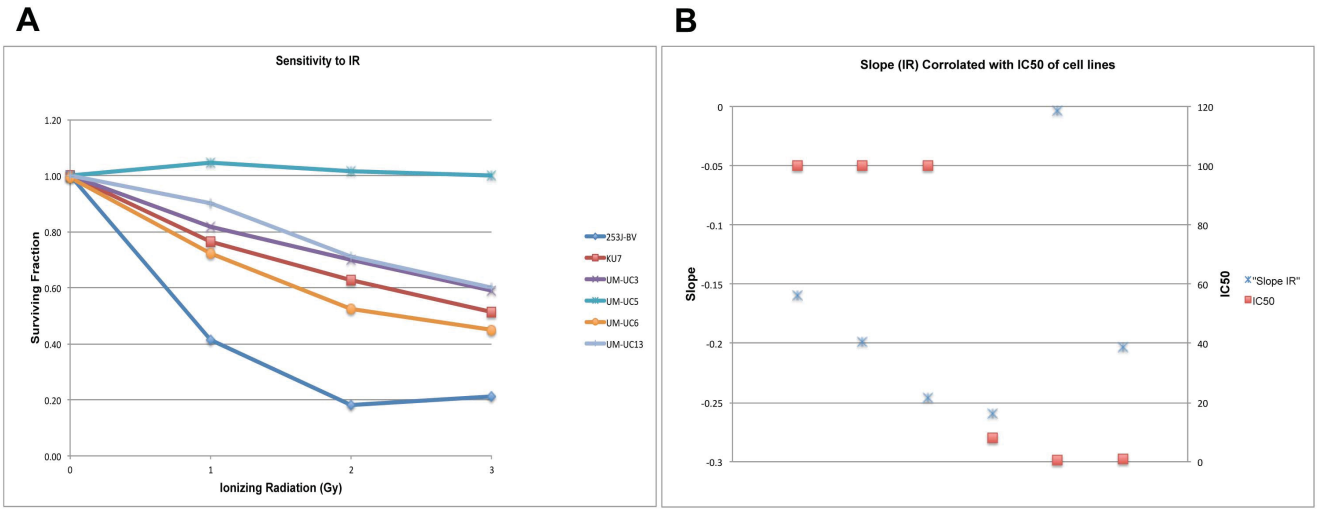

Figure 1. Response of a panel of bladder cancer cell lines to ionizing radiation. Plated cells were exposed to ionizing radiation to measure the effects on growth by clonogenic assay, as described in Methods. (**A**) Based on the gathered results, we were able to classify these cell lines as radiation–sensitive, moderately sensitive and -relatively resistant. (**B**) The RAD001 IC50 was plotted against the slope of the curve for each cell line in the clonogenic assay when treated with IR.
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formation was observed in the two cells lines (UM-UC5 and UM-UC6) that were characterized originally by our laboratory to being the most sensitive to RAD001. This lies primarily with the colonogenic assay itself where colonic formation (as determined by a universally set colony size) whereas the sensitivity to RAD001 was done with an enzymatic assay (MTT). This discrepancy in sensitivities of the assays, length of assay, combined with the quick doubling time, could explain the clonogenic results for these two cells lines.

The treatment with RAD001 and ionizing radiation induces both an increase of the percentage of cells in the G0/G1 and the G2 phases of the cell cycle

To get insights into the mechanism underlying the observed growth inhibition, cell cycle analysis was performed by flow cytometry to study the distribution of cells throughout the various phases of the cell cycle 48 hours following each treatment alone and in combination. The cells were treated with a dose of RAD001 equivalent to their GI50 (ranging from 0.5 to 75 nmol/L) as well as 4Gy of ionizing radiation. Results are shown in Figure 4. RAD001 induced a G0/G1 arrest in all the bladder cancer cell lines tested: KU7 62%±4%, UM-UC3 71%±6%, UM-UC6 77%±3% and 253J-BV 67%±4% compared to their untreated controls, 54%±3%, 64%±2%, 66%±2% and 55%±3%, respectively. Percentages represent the ratio of cells in each phase relative to the total number of cells. As expected, ionizing radiation led primarily to a G2 arrest, illustrated by a significant increase in the percentage of cells in this phase following treatment with ionizing radiation: KU7 38%±4%, UM-UC3 23%±4%, UM-UC6 19%±4% and 253J-BV 22%±3% compared to their respective untreated controls: 23%±2%, 19%±3%, 14%±3% and 4%±2%, respectively. In the combined arm with RAD001 and ionizing radiation, we observed both an increase in the percentage of cells in G0/G1 and G2 phases (Fig. 4). More specifically, a decrease in the percentage of cells in the S-phase was observed compared to either treatment alone or to the control (no treatment) and this was paralleled with an increase of the percentage of cells in the G0/G1 and the G2 phases. Taken together, we concluded that the cytostatic effect of RAD001 combined with ionizing radiation exhibits an inhibitory additive effect on the progression of cells through their cycle.

Table 1. Classification of bladder cancer cell lines based on their relative response to RAD001 and ionizing radiation.

	Ionizing Radiation			RAD001		
Cell Line	**Sensitive**	**Moderately Resistant**	**Relatively Resistant**	**Sensitive**	**Moderately Sensitive**	**Relatively Resistant**
253J-BV	x				x	
KU7		x				x
UM-UC3		x				x
UM-UC5			x	x		
UM-UC6		x		x		
UM-UC13		x				x

No correlation was noted when the RAD001 response, as reported [13], was compared to the response to ionizing radiation.
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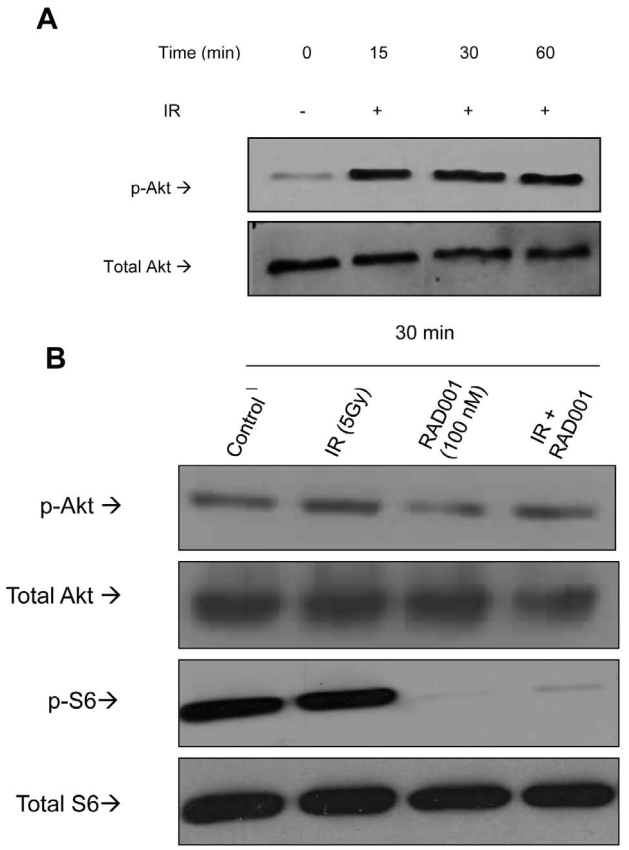

Figure 2. Ionizing radiation activates AKT by phosphorylation and RAD001 inhibits S6 phosphorylation. (**A**) KU7 cells were treated with 4 Gy of ionizing radiation. Cells were lysed 15, 30 and 60 minutes following treatment to analyze AKT activation (p-AKT; upper row) by Western blot. Total levels of AKT are shown in the lower row. (**B**) KU7 cells were treated with 5 Gy of radiation, 100 nM of RAD001 or both, and lysed. Levels of Akt and S6 phosphorylation were analyzed by Western blot. Total levels of Akt and S6 expression are shown. Similar results were obtained when 253J-BV, UM-UC3, and UM-UC6 were treated with radiation and RAD001 alone and in combination (data not shown).
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RAD001 and ionizing radiation alter the levels of the cell cycle checkpoints cyclin D1, p27kip1 and p21

Based on the above cell cycle analysis data and to further understand the mechanism by which RAD001 and ionizing radiation act together to inhibit cell growth, we tested the likelihood of changes in expression levels of diverse regulators of the cell cycle, particularly associated with checkpoints such as cyclin D1, p27, and p21. Results in Figure 5 illustrate the case of KU7, used as a representative of the group of cell lines found to be relatively resistant to both ionizing radiation and RAD001. That being said, a dose of RAD001 equivalent to the GI50 of that cell line was used to ensure a response to the RAD001 treatment. Our results show that the level of cyclin D1, which is a protein required for the G1/S transition through the cell cycle, was decreased following 24 hours of treatment with RAD001 (Fig. 5), a finding that supports our observations on cell cycle changes. In contrast, levels of p27, which is a protein also associated with the G1/S transition, changed in an inverse correlation (increased) following 24 hours of treatment with RAD001, ionizing radiation, and the combined treatment compared to the control (Fig. 5). Interestingly, levels of p21, which is also associated with inhibiting cell cycle progression, were decreased in cells treated with RAD001 alone compared to control, or radiation (Fig. 5). The levels of p21 increased in response to the treatment to radiation compared to the control, and the levels of p21 are at their highest when cells are treated with both RAD001 and ionizing radiation. It is noteworthy that similar results were obtained for cyclin D1, p27, and p21, in all tested cell lines: UM-UC3, UM-UC6 and 253J-BV.

Combining RAD001 treatment with ionizing radiation significantly inhibits tumor growth in human bladder cancer xenograft model, compared to either treatment alone

To ascertain significance and to verify if *in vitro* data with regards to effects of combining RAD001 and ionizing radiation on growth of bladder cancer cell lines can be transposed *in vivo*, we used the KU7 bladder cancer cell line to grow as subcutaneous tumor xenografts in athymic mice. In all the mice, tumors were evidenced within the first 10 days after implantation. There was no body weight loss or any significant toxicity directly associated with RAD001 and ionizing radiation treatments during the entire duration of the study (a total of 5 weeks). In mice treated with combined RAD001 and ionizing radiation, there was a maximal inhibitory effect on bladder cancer progression, as indicated by the significant decrease in tumor weight compared to either treatment alone or placebo group (average tumor weight 31 mg for combination arm vs. 117 mg with RAD001 alone, 80 mg with IR, or 340 mg for placebo, $P<0.05$) (Fig. 6). Similar findings were also obtained using the 253J-BV cell lines where combined therapy achieved maximal inhibitory effect on bladder cancer progression after 4 weeks of treatment compared to control. The same results were demonstrated using tumor growth kinetics when tumor volume was measured throughout the treatment duration (data not shown). Our p21 immunohistochemistry staining on the xenograft sections confirmed our findings from the western blot analysis for p21 levels *in vitro* (Fig. 7). The levels of p21 significantly ($p<0.05$) increased following the treatment with radiation and the combination regimen when compared to the placebo. Furthermore our data indicated a slight decrease, although statistically non-significant, of the p21 levels in the RAD001-treated group alone.

Discussion

Radiation therapy is a key element of many cancer treatment regimens hence its widespread use. However, ionizing radiation appears to contribute to an unfavorable increase in signaling through the PI3K/AKT/mTOR pro-survival pathway. In the present study, we observed differences in the sensitivity of a panel of six bladder cell lines to ionizing radiation, with some being were more resistant than others. We also demonstrated the activation of AKT following exposure to ionizing radiation. Several factors may potentially be determinant in the activation mechanisms of the PI3K/AKT pathway following ionizing radiation and then help cancer cells in the establishment of resistance [22]. Among others, the enhanced activity of key enzymes such as telomerase activity [23] as well as the involvement of signaling molecules such as the epidermal growth factor receptor (EGFR) and RAS [24,25] may explain why some tumors do not respond to radiation as effectively as others. Notably, EGFR signaling through the PI3K/AKT was reported to regulate the DNA-dependent protein kinase catalytic subunits, which are part of the DNA repair machinery turned on following radiation [26]. While these observations emphasize the important role that the activation of PI3K/AKT plays in the cancer radioresistance, we demonstrate that blocking the PI3K/

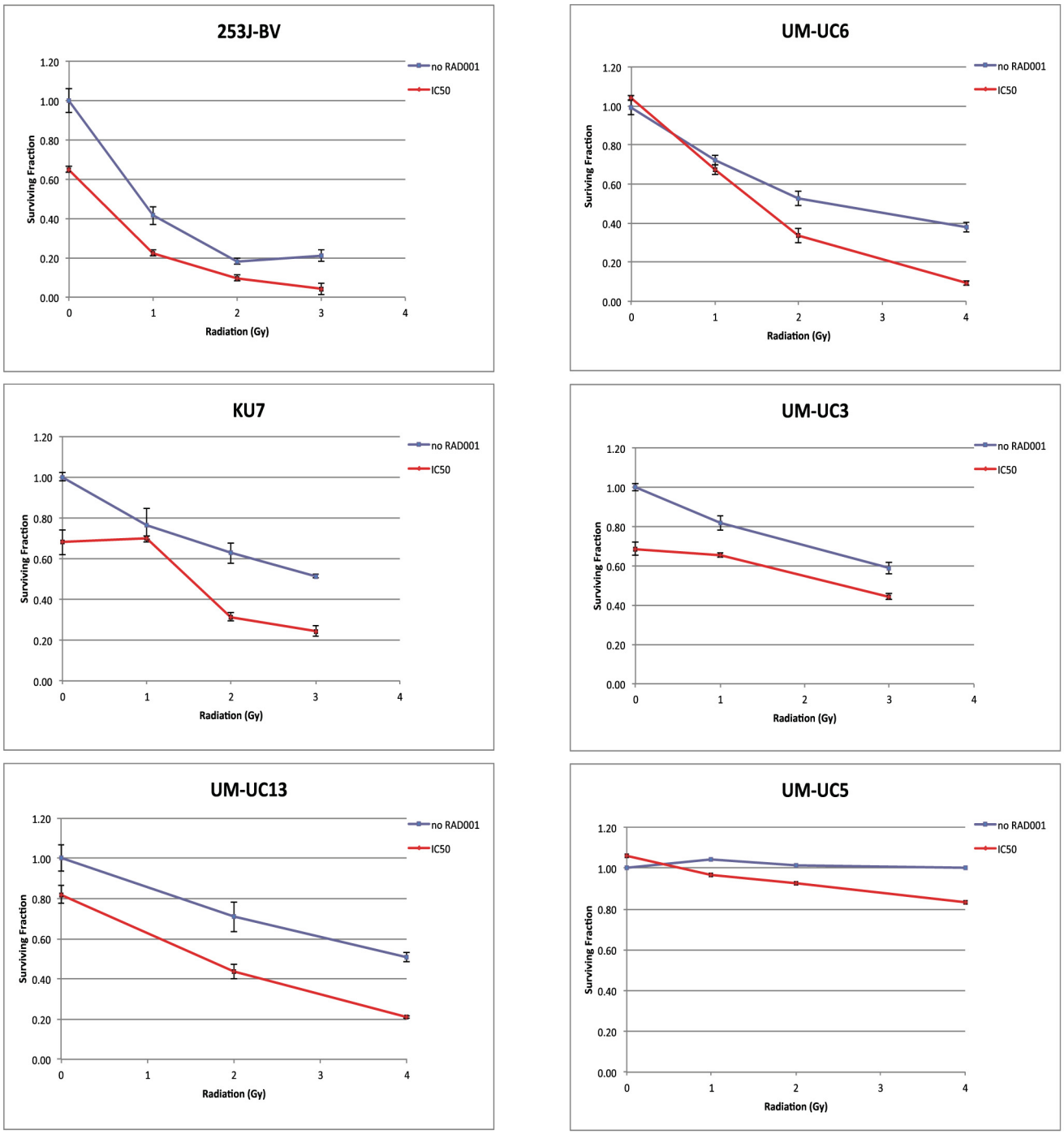

Figure 3. Effect of RAD001 and ionizing radiation on colony formation. Six cell lines were treated with RAD001 for 12 hours before exposure to ionizing radiation and further grown as indicated in Methods. Colony formation was measured after cell fixation and staining with crystal violet, 10–14 days after treatment depending on cell lines. Results were statistically significant ($p<0.05$) in the combined treatment compared to either treatment alone in all tested cell lines.
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AKT/mTOR pathway with RAD001 appears as a valuable mean to enhance the efficacy of radiation treatment in bladder cancer cells. The mechanism by which RAD001 exhibits this enhanced effect still needs further evaluation. It could simply be that blocking the rebound activation of the pathway following radiation is sufficient to decrease radioresistance; a plausible mechanism as the two treatments do share common targets in the cell such as the hypoxia inducible transcription factor (HIF-1), a molecule downstream of mTOR [27].

In addition to cellular signaling, the efficacy of the treatments may lie on their effects on the cell cycle. Our analyses show that RAD001 induces a G0/G1 arrest in the cells while ionizing radiation induces an S/G2 arrest. In the combined therapy, we observe both a G0/G1 as well as a G2 arrest. These changes in the

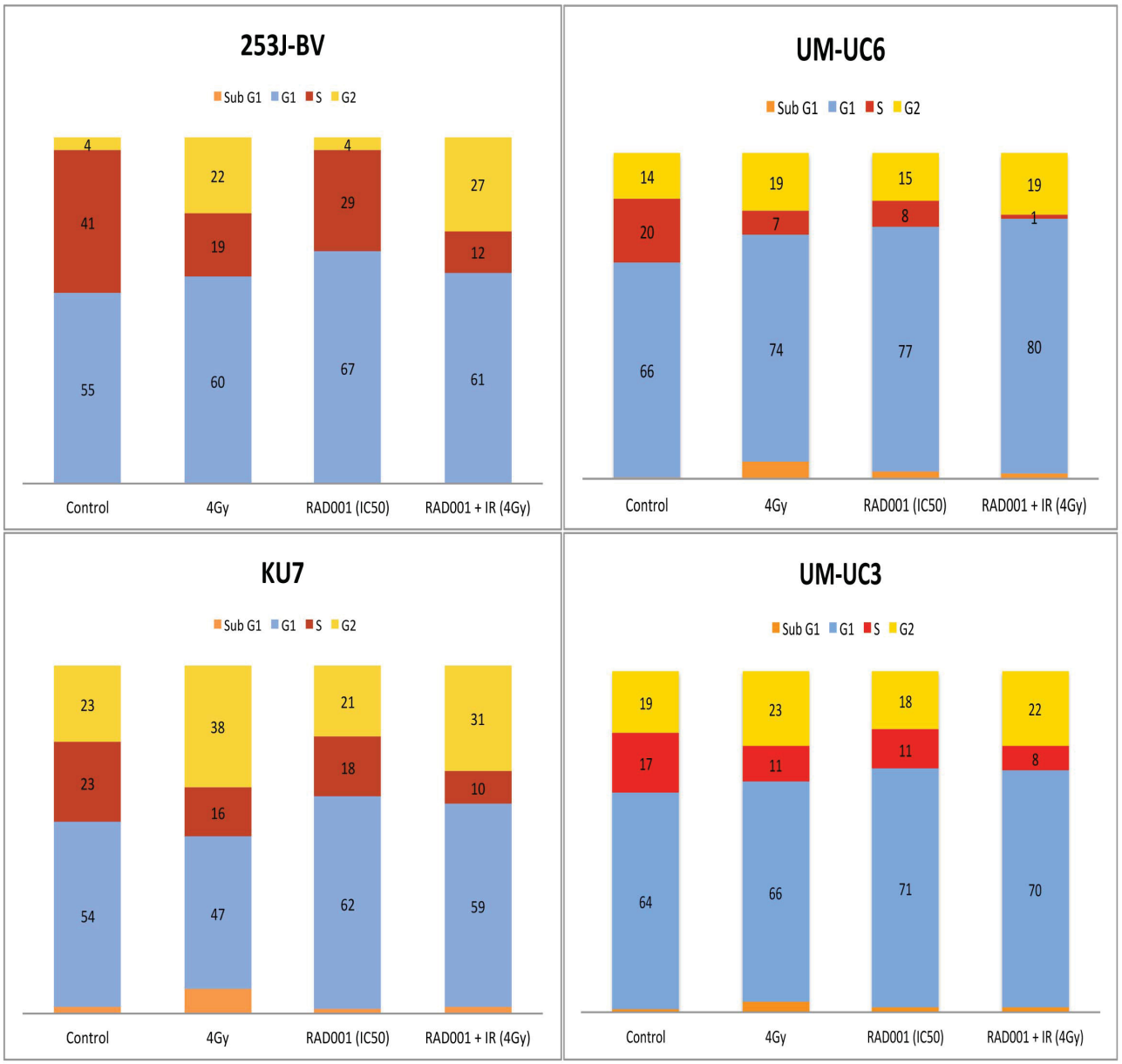

Figure 4. Effect of RAD001 and ionizing radiation on the cell cycle. The cell lines were cultured and treated with RAD001 alone, ionizing radiation alone and with the combination of RAD001 and radiation. For the latter series, samples were pre-treated with RAD001 for 6 hours prior to radiation. Cells were fixed and stained for propidium iodide intake at 48 hrs after treatment, and then measurements were performed by flow cytometry. The proportion of cell populations in the different phases of the cell cycle is shown for each cell line by colored bars (G0/G1: Orange/Blue; S: Red and G2: Yellow).
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cellular population within each part of the cycle were compensated by a decrease of cells in the S-phase. Since the early 1960s, scientists have confirmed that the sensitivity to radiation is dependent upon the phase of the cell cycle whereas cells are most sensitive to radiation in the late G1 and the G2/M phase, and are least sensitive in the S Phase [28–30]. These responses include chromosome aberrations, delay in division, alterations in DNA division and survival [31]. Our findings indicate that shifting cells within the phases of the cell cycle, following the treatment with RAD001, and arresting them in specific phases will alter their sensitivity to ionizing radiation. Furthermore, our preliminary data indicate that when the cells are examined at 12 h post-radiation treatment, a more dramatic shift to the G2 phase occur in the combination treatment as opposed to radiation alone, potentially rendering them more sensitive to IR. In our experiments, cells were arrested at the G1 phase with decreased proportion of cells in the S-phase following the pre-treatment with RAD001, and this arrest is rendering the cells more sensitive to ionizing radiation. It would be interesting to examine whether the effects of the combination in the regimen consisting of fractionated doses rather than a single dose will further increase the efficacy of RAD001 in addition to radiation in bladder cancer cells.

The cycle arrest induced following the treatment alone or in combination is underlined by changes in the levels of various proteins that control passage through the phases and the progression of the cycle. Here, we report a decrease in cyclin

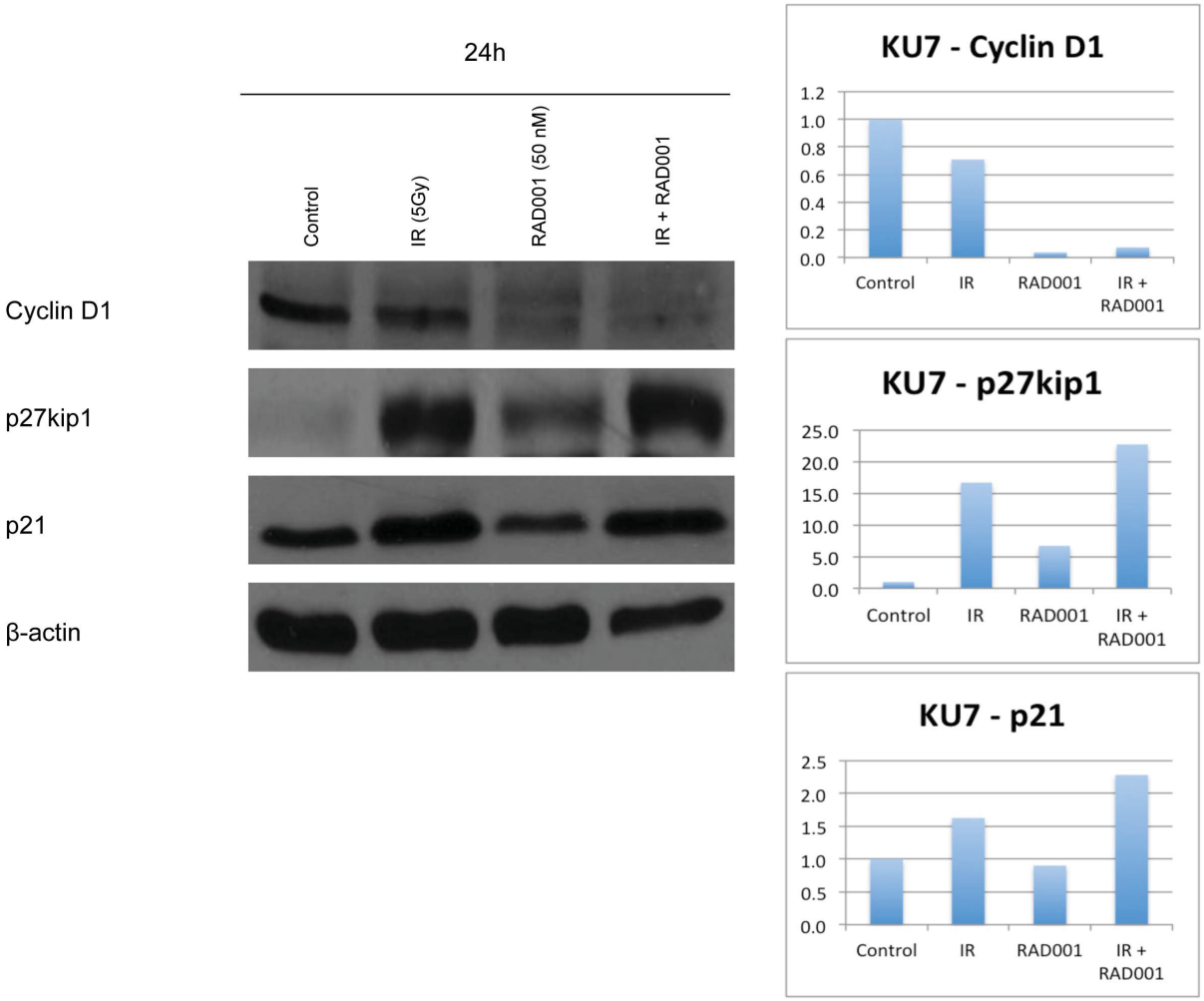

Figure 5. Expression of cell cycle regulatory proteins following RAD001 and IR treatment. Bladder cancer cells were treated with RAD001, ionizing radiation (IR) or the combined treatment. They were lysed 24 hours after treatment as described in Methods. Western blot analysis for Cyclin D1, p27kip1 and p21 in KU7 cells and normalized in lower panels as a function of the actin level measured in parallel. Similar results were obtained for all cell lines tested, UM-UC3, UM-UC6 and 253J-BV (not shown).
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D1 levels following treatment with RAD001 alone and when combined with radiation. A decrease in cyclin D1 results in the lack of cyclin D1/cdk complex formation required for transition past the G1 phase. Furthermore, our report points towards an increase in p27kip1 when treated with RAD001 and a maximal effect is observed in the combined treatments. This increase in the p27kip1 expression levels, which is an inhibitor of cyclin D1/cdk4 complex, support involvement of inhibitors of the cell cycle in the mechanism by which these treatments alter cellular proliferation [32–35].

Although the entire mechanism for the inhibitory role that RAD001 and ionizing radiation exhibit on the cell cycle remains unclear, one important protein that needs to be studied closely with that regards is the cyclin dependant kinase inhibitor 1, p21. It has been reported that the rapamycin-induced disruption of the cdk2 interaction with PCNA was due to the down regulation of p21, which affects the interaction between cdk2 and cyclin D1, leading to the malformation of the complex required to move the cells past the G1 phase [34]. Aside from being a G1/S regulatory molecule, p21 is also involved in DNA damage repair following exposure to ionizing radiation. Our results indicate that levels of p21 decrease when treated with RAD001 alone and increase following the treatment with ionizing radiation. *In vitro* and *in vivo*, p21 levels were maximally elevated in the combination arm, pointing towards the involvement of p21 in the increased cell cycle arrest observed previously by flow cytometry. It has been shown that p21 interacts with PCNA [36], the proliferating cell nuclear antigen. This p21/PCNA interaction has an inhibitory effect on DNA synthesis, a major process in DNA damage repair, and subsequently leading to an arrest in the G2/M phase [37,38]. Hence, pretreatment with RAD001 can enhance the effects of radiation through alteration in p21 levels that affects DNA damage repair leading cells to further arrest in G2. This may seem contradictory to the inhibitory role of p21, but studies have shown that p21 may exhibit a cell proliferation role [39]. p21 may exist at an optimal level in the cells and that a certain fluctuation from the basal level can lead to its inhibitory effect. Of note, p21 activity can also be influenced by its state of phosphorylation and location within the cell [40].

Another key element that might be playing an important role in determining the response of the cells to radiation and the effects on cell cycle is the tumor suppressor p53. P53 is an important DNA

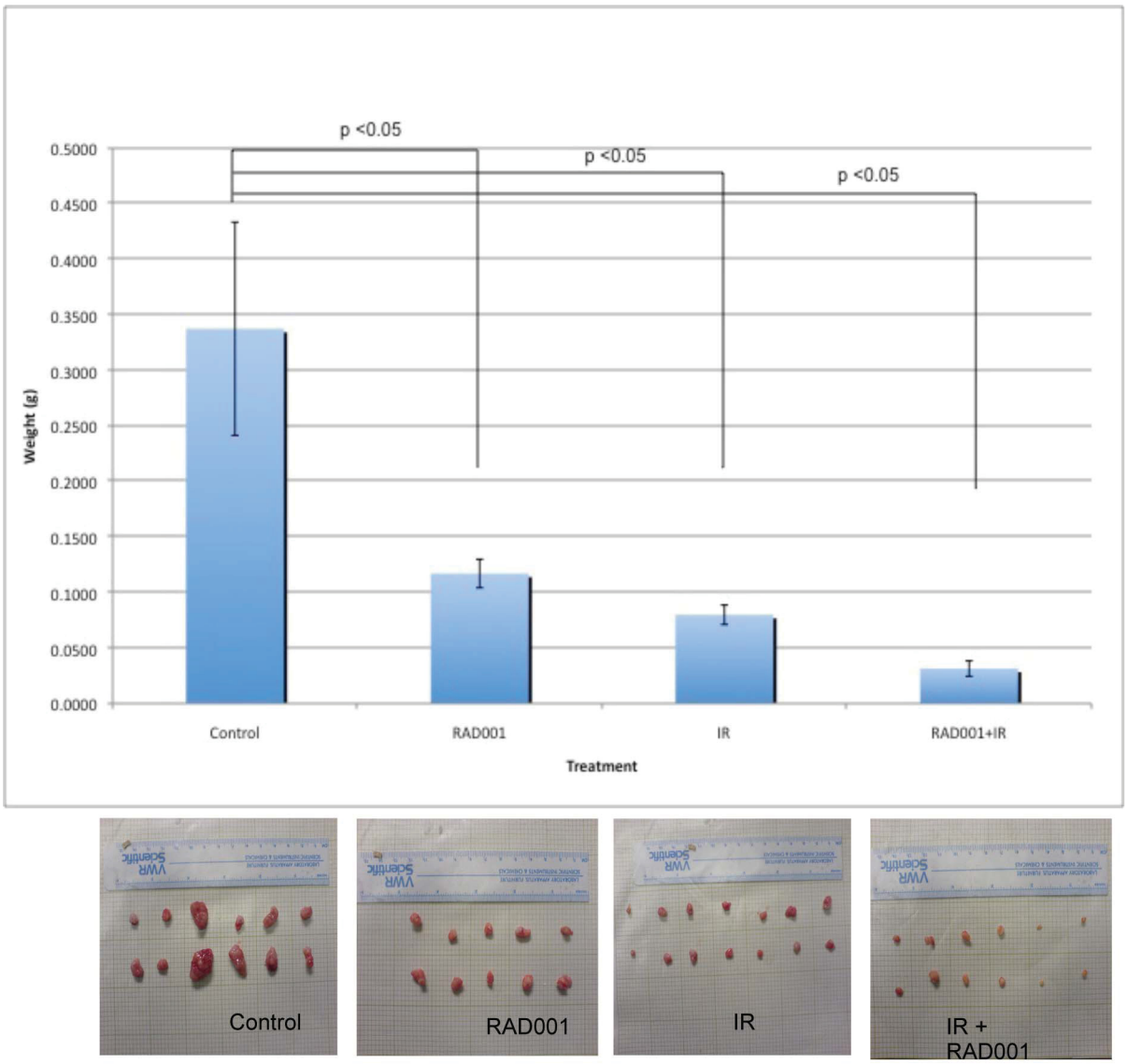

Figure 6. Effect of RAD001 and ionizing radiation on bladder cancer tumor weight *in vivo*. The athymic mice bladder tumor model of KU7 was used as described in Methods. Tumor weights (in grams) reached at the experimental endpoint and expressed as mean weight of tumors harvested for each group of mice in the 4 treatment arms, as indicated. Similar findings obtained using 253J-BV cells (data not shown).
doi:10.1371/journal.pone.0065257.g006

damage checkpoint that was shown to be involved in either a cell cycle arrest or apoptosis depending on the levels of p53 in the cell and the status of the p53 gene [41]. That being said, it has been demonstrated that the activation of p53 results in the activation of p21 leading to cell cycle arrest [42]. In our tested cell lines, we noticed higher baseline levels of p21 in cells that are p53-WT (253J-BV and UM-UC6) compared to p53-mutant cells (KU7 and UM-UC3) (data not shown). Surprisingly, 253J-BV and UM-UC6 had a significantly lower GI50 for RAD001 compared to KU7 and UM-UC3. While the mechanism of actions of RAD001 and ionizing radiation together is not fully understood, these observations indicate a possible cross talk between the p53/p21 pathway (which is activated by radiation) and the mTOR pathway.

Our *in vitro* results seemed to be well echoed in our *in vivo* xenograft model where we report a significantly slower growth rate that translated into smaller tumor weights ($p<0.05$) observed at the end of treatment in all treated groups compared to the untreated group. More interestingly, we observed the lowest tumor weights ($p<0.05$) in the group treated with the combination arm of RAD001 and ionizing radiation compared to all other groups. When untreated, the tumors grew at a much faster pace and weighed more than tumors of mice treated with RAD001 and ionizing radiation alone.

Our study shows clearly that RAD001, alone and in combination of radiation therapy, exhibits a cytostatic effect on tumor cells. Our previously published report showed also that no apoptosis is induced following the treatment of RAD001 alone when measured by propidium iodide uptake. Of interest, we have remarked an induction of autophagy as measured by levels of the autophagic marker, the light chain 3A (LC3) protein, in the cells following the treatment with RAD001 and in combination with ionizing radiation. Future research in our laboratory will further evaluate other types of cell death in the combination arm including autophagy and mitotic catastrophe.

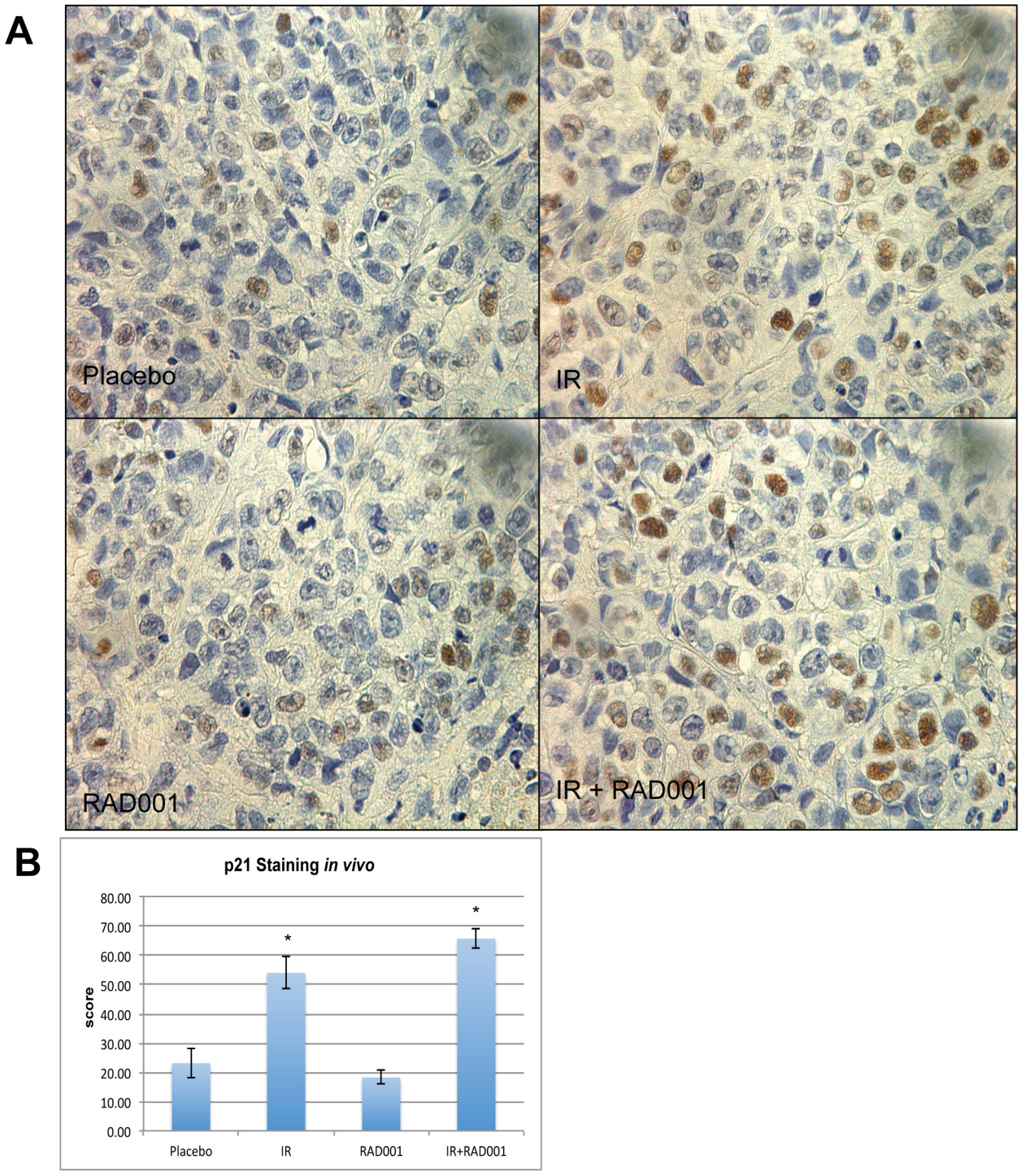

Figure 7. Immunohistochemical p21 levels in mouse xenograft paraffin sections. (**A**) Immunohistochemistry was used to detect the levels of p21 in paraffin-embedded mouse xenograft bladder cancer tissues treated with placebo, IR, RAD001 and in combination. (**B**) Quantification of the immunohistochemistry data revealed a significant increase in p21 expression as observed in tumors treated with ionizing radiation and in combination compared to the placebo and RAD001 treatment.
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Conclusion

To our knowledge, this is the first report of an enhance effect when combining RAD001 with radiation in bladder cancer *in vitro* and *in vivo*. The proposed treatment regimen is very promising and may potentially provide a remarkable advancement in the management of bladder cancer to improve clinical outcomes. These findings formed a platform on which a phase II homegrown clinical trial evaluating RAD001 combined with chemoradiation is now open at the McGill University Health Center targeting patients with invasive bladder cancer.

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Author Contributions

Conceived and designed the experiments: RN JJM SC FC WK. Performed the experiments: RN JJM FC WK. Analyzed the data: RN JJM SC WK. Contributed reagents/materials/analysis tools: RN JJM SC FC WK. Wrote the paper: RN JM SC WK.

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