The Effects of Hypoxia on Centrosome Function in Prostate Cancer

By

Daria Taiakina

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

© Copyright by Daria Taiakina 2015
The Effects of Hypoxia on Centrosome Function in Prostate Cancer

Daria Taiakina

Master of Science
Department of Medical Biophysics
University of Toronto

2015

Abstract

Hypoxia and centrosome aberrations are both prominent features of prostate cancer and have been shown to be markers of poor clinical prognosis. Hypoxia has been linked to increased genomic instability and aggressive tumor phenotype. Centrosome aberrations have also been directly linked to chromosomal instability. I hypothesized that hypoxia causes centrosome aberrations in prostate cancer cells, as a potential mechanism of hypoxia-induced genomic instability. Analyses of centrosome gene expression in DU14 prostate cancer cells demonstrated that hypoxia causes centrosome gene deregulation. Centrosome structure assays revealed that hypoxia leads to accumulation of centriolar satellites in a cell line and re-oxygenation independent manner. If centrosome aberrations are specific to hypoxic cancer cells, centrosome and mitosis targeting drugs could provide a high therapeutic ratio and better outcome for patients with high hypoxic tumor fraction.
This thesis is dedicated to my mother Irina Taiakina, and my father Fedor Taiakin, for always believing in me and encouraging me. For showing real courage and doing what seemed impossible, to get their daughters to where opportunity is plentiful. Your unconditional love continues to nourish, and I am forever indebted to you.
I would like to thank my supervisor, Dr. Robert Bristow, for guiding me along this research project. Rob, you have provided me with the firm encouragement and kind support I needed along this journey. I couldn’t have asked for a better supervisor. Thank you to my supervisory committee members, Drs. Fei Fei Liu and Vuk Stambolic, for their helpful feedback and comments. I am also grateful to my collaborators Drs. Laurence Pelletier, Steffen Lawo and David Comartin for all their help in mitotic/centrosome labelling and biology.

Thank you to all the Bristow lab members, past and present, for helping me along the way with the science, as well as inspiration and support. I have been very fortunate to work with such a talented team of scientists. Special thank you to Ken, Gaetano, Shane, Ramya, Alice, Alan, Stefania, Winnie, Cihan, Jon, Agata, Gerben and Rita. Ken, you have been so instrumental in getting me through so many experiments (and can’t forget all the fishing lessons).

I am grateful to all my mentors and teachers that inspired me and taught me science and much more. Mr. Manias, thank you for showing me the awe that is biology. Dr. Ian Orchard, thank you for taking me on as a thesis student, I have learned much from you. Vicky TeBrugge, your enthusiasm for science inspired me. Thank you for being an amazing mentor.

Thank you to all my friends, the new ones I made in the process of graduate school, and the old friends that stuck by me through all of it. My friends have been my armor, my therapists and my inspiration, I couldn’t have done it without all of you. Игорь, спасибо за поддержку, я никогда не забуду и очень благодарна за время которое мы провели вместе. Твоя дружба и вера в меня мне много значит. Special thank you to Катя, Оля and Юлька. Always knowing you are there for me means the world to me. Thank you to James, for being my pillar of strength and biggest supporter. You have given me the courage to do the things that seemed so daunting. You also make the best brunch, and I will never think of one as complete without a Caesar or Mimosa thanks to you.

Thank you to my family. Val, stay amazing big sis. Mom and Dad, thank you for your continued support and unconditional love. I will always find a way home to you.
Contents

ACKNOWLEDGMENTS.......................................................................................................................... IV

LIST OF TABLES.................................................................................................................................... VII

LIST OF FIGURES ................................................................................................................................... VIII

LIST OF ABBREVIATIONS ..................................................................................................................... IX

CHAPTER 1 INTRODUCTION AND RATIONALE...................................................................................... 1

1.1 TUMOR MICROENVIRONMENT AND CANCER METABOLISM ....................................................... 2

1.1.1 Impact of hypoxia in prostate cancer progression and treatment response ................................... 2

1.1.2 Chromosomal instability and prostate cancer prognosis ............................................................... 6

1.1.3 The relationship between hypoxia and genomic instability ....................................................... 9

1.2 THE REPAIR OF DNA DAMAGE IN HYPOXIC CELLS........................................................................ 9

1.2.1 The effect of hypoxia on DNA DSB repair: The HR and NHEJ subpathways ............................... 9

1.2.2 Other DNA repair pathways modified by hypoxia: MMR and NER ......................................... 10

1.2.3 Using hypoxia-mediated DNA repair defects as an Achilles’ heel for cancer treatment: The concept of “contextual synthetic lethality”............................................................................... 11

1.3 CONTROL OF MITOTIC FUNCTION AND SIGNIFICANCE FOR CIN .................................................. 12

1.3.1 Causes and consequences of CIN ................................................................................................. 12

1.3.2 Mitotic regulation and implications for chromosome stability .................................................. 12

1.3.3 The centrosome as a major regulator of chromosome integrity ............................................... 15

1.3.4 Centrosome structure and related genes ..................................................................................... 16

1.3.5 Assays for centrosome structure .................................................................................................. 17

1.4 THE ROLE OF THE CENTROSOME COMPLEX IN CANCER AND HYPOXIA-INDUCED GENOMIC INSTABILITY ................................................................. 20

1.4.1 The relationship between centrosome function and cancer progression ................................... 20

1.4.2 The link between the DNA-damage response and centrosome function: a possible mechanism of hypoxia-induced genomic instability ........................................................................... 21

1.5 TARGETING CENTROSOMES AS AN ANTI-CANCER STRATEGY ..................................................... 25

1.6 SUMMARY AND HYPOTHESIS ..................................................................................................... 25

CHAPTER 2 - HYPOXIA LEADS TO ABERRANT CENTROSOME FUNCTION AND CENTRIOLAR SATELLITES IN PROSTATE CANCER CELLS ........................................................................ 29

2 HYPOXIA LEADS TO ABERRANT CENTROSOME FUNCTION AND CENTRIOLAR SATELLITES IN PROSTATE CANCER CELLS .................................................................................... 31
2.1 INTRODUCTION .................................................................................................................................. 31

2.2 MATERIALS AND METHODS ............................................................................................................ 33
  2.2.1 Cell culture, hypoxic gassing and irradiation treatments ................................................................. 33
  2.2.2 Immunofluorescence microscopy ...................................................................................................... 33
  2.2.3 Cell growth and cell cycle analysis .................................................................................................... 34
  2.2.4 Clonogenic survival assays ............................................................................................................. 35
  2.2.5 RNA extraction and real-time quantitative PCR (qRT-PCR) ............................................................ 35
  2.2.6 Western Blot Analysis .................................................................................................................... 36
  2.2.7 Statistical Analysis ......................................................................................................................... 37

2.3 RESULTS ................................................................................................................................................. 37
  2.3.1 IR-induced exogenous DNA damage increases centrosome aberrations in mitotic DU145 prostate cancer cells .................................................................................................................................. 37
  2.3.2 The DU145 prostate cancer cell line as a model to study centrosome aberrations under hypoxia. 40
  2.3.3 Centrosome aberrations in mitotic DU145 cells in response to exogenous DNA damage after hypoxia treatment ................................................................................................................................. 44
  2.3.4 Increased frequency of extra CETN spots in response to hypoxia treatment is not a cell-line specific effect. ........................................................................................................................................ 47
  2.3.5 Extra CETN spots most likely represent accumulation of centriolar satellites. ............................ 47
  2.3.6 Hypoxia modulates centrosome complex gene expression. ............................................................ 53

2.4 DISCUSSION ........................................................................................................................................ 55

2.5 SUPPLEMENTARY TABLES AND FIGURES .................................................................................... 58

CHAPTER 3 .............................................................................................................................................. 61

3 DISCUSSION AND FUTURE WORK ..................................................................................................... 61
  3.1 SUMMARY OF FINDINGS AND DISCUSSION ................................................................................. 62
  3.2 FUTURE WORK ................................................................................................................................... 66
    3.2.1 Correlating centrosome aberrations and development of CIN in primary human tissues .......... 66
    3.2.2 Studies of centrosome ultrastructure in hypoxic cells ................................................................. 68
  3.3 CANCER IMPACT ............................................................................................................................. 70

REFERENCES ............................................................................................................................................. 71
List of Tables

Chapter 1

Table 1.1: Selected clinical studies of hypoxia in prostate cancer……………………………3
Table 1.2: Selected clinical studies of aneuploidy in prostate cancer……………………… 7
Table 1.3: DNA repair proteins that may have a role in centrosome function and are also
down-regulated by hypoxia…………………………………………………………………23

Chapter 2

Table S1: List of primers for SYBRGreen qPCR analyses………………………………………58
Table S2: List of probes for TaqMan qPCR analyses……………………………………………58
List of Figures

Chapter 1

Figure 1.1: Hypoxia and aneuploidy are markers of poor clinical prognosis………………5
Figure 1.2: Cellular pathways responsible for regulating chromosomal stability…………14
Figure 1.3: Representation of the centrosome markers I used in immunofluorescence (IF) studies……………………………………………………………………………………… 19
Figure 1.5: Mitotic and centrosome abnormalities in cancer and their potential relationship with intratumora hypoxia……………………………………………………. 26
Figure 1.6: Summary of hypoxia studies carried out in this thesis……………………….. 28

Chapter 2

Figure 2.1: Centrosome aberrations in response to radiation treatment in mitotic DU145 prostate cancer cells……………………………………………………………………… 39
Figure 2.2: Proliferation and cell cycle response of DU145 cells to hypoxic (0.2% O2) treatment……………………………………………………………………………. 41
Figure 2.3: Biological response to hypoxia and anoxia treatment in DU145 cells……… 42
Figure 2.4: Representative IF images of the different types of centrosome aberrations observed in mitotic DU145 cells in response to radiation and hypoxia treatment………… 45
Figure 2.5: Centrosome aberrations in mitotic DU145 cells in response to radiation treatment following oxia and hypoxia pre-treatment………………………………………. 46
Figure 2.6: Extra CETN spots in response to hypoxia treatment in four cell lines (U2OS, PC3, DU145, and BPH1)………………………………………………………………… 48
Figure 2.7: Extra CETN spots in response to hypoxia treatment in interphase DU145 cells colocalize with PCM1 but not Cep135 or NEDD1…………………………………… 50
Figure 2.8: Centrosome aberrations in response to hypoxia treatment in interphase DU145 cells at various time points post re-oxygenation…………………………………… 52
Figure 2.9: Changes in mRNA expression in response to hypoxia treatment…………… 54

Figure S1: Distribution of centrosome aberrations within IR-treated cells……………… 59
Figure S2: Representative images of cell cycle analysis in oxic, hypoxic and re-oxygenated cells……………………………………………………………………………… 59
Figure S3: Centrosome aberrations in response to IR and hypoxia treatment………….. 60

Chapter 3

Figure 3.1: Inducible shRNA knockdown system for the HAUS1 gene………………… 65
Figure 3.2: RAD51 (red) and EF5 (green) co-staining in histologic sections (RKO xenograft) …………………………………………………………………………………… 67
List of Abbreviations

ACC – Augmin centrosome complex
ADT – androgen deprivation therapy
AR – androgen receptor
BER – base-excision repair
BrdU – 5-bromo-2’-deoxyuridine
CETN – centrin
CI – confidence interval
CIN – chromosomal instability
CNA – copy number alterations
DAPI – 4’,6-diamidino-2-phenylindole
DDR – DNA damage repair
DMEM – Dulbecco’s Modification of Eagle's Medium
DNA – deoxyribonucleic acid
DSB – double strand break
EM – electron microscopy
FA – Fanconi Anemia
FBS – fetal bovine serum
GFP – green fluorescent protein
H2B – histone H2B
H3P – phospho-histone H3
HIF – hypoxia-inducible factor
HR – homologous recombination
IHC – immunohistochemistry
IR – ionizing radiation
LOH – loss of heterozygosity
MEM – minimum essential medium
MIN – microsatellite instability
MMR – mismatch repair
mRNA – messenger ribonucleic acid
MTOC – microtubule organizing center
NaOAc – sodium acetate
NER – nucleotide-excision repair
NHEJ – non-homologous end joining
NIR – non-irradiated
OER – oxygen enhancement ratio
OPN – osteopontin
PARP – poly (ADP-ribose) polymerase
PCM – pericentriolar material
PCNT - pericentrin
PHD – prolyl-4-hydroxylase
PI – propidium iodide
PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase
PIMO – pimonidazole
PIN – prostatic intraepithelial neoplasia
PLK – polo-like kinase
pO2 – partial pressure O2
PSA – prostate-specific antigen
PVDF – polyvinylidene difluoride
qPCR – quantitative polymerase chain reaction
RFP – red fluorescent protein
RT – radiation therapy
SAC – spindle assembly checkpoint
SEM – standard error of the mean
SDS-PAGE - sodium dodecyl sulfate – polyacrylamide gel electrophoresis
shRNA – short hairpin ribonucleic acid
SSB – single strand break
TBS-T – tris-buffered saline and Tween 20
VEGF – vascular endothelial growth factor
γ-TuRC – γ-tubulin ring complex
Chapter 1

Introduction and Rationale

The following chapter is a modified version of a published book chapter:


I wrote the majority of the material contained within this chapter. Co-author Alan Dal Pra wrote sections 1.1.1 and 1.1.2, and produced Tables 1 and 2. All other Figures and Tables are my own work. Robert G Bristow was instrumental in guiding the direction of the review and editing the final work.
1.1 Tumor microenvironment and cancer metabolism

1.1.1 Impact of hypoxia in prostate cancer progression and treatment response

The tumor microenvironment consists of sub-regions of abnormal cell metabolism with dynamic and differential gradients of oxygen consumption. Chronic (diffusion-limited) tumor hypoxia develops in solid tumors because of the limited diffusion of oxygen through the tumor interstitium with high levels of hypoxia measured at distances of more than 150 µm from the surrounding vasculature. Acute or cycling hypoxia occurs due to fluctuating anoxia and subsequent re-oxygenation as a consequence of vascular instability and transient variability in microregional tumor perfusion (Chan et al., 2007). The biological and clinical effects of tumor hypoxia include increased rates of genomic instability, increased capacity for systemic metastases, and resistance to chemotherapy and radiotherapy (Bristow and Hill, 2008).

Historically, hypoxic cells were documented as being resistant to ionizing radiation, since the presence of oxygen renders cells up to 2-3 times more susceptible to DNA damage and cell kill following exposure to ionizing radiation (e.g. the oxygen enhancement ratio (OER) is 2-3) (Chan et al., 2007). Increased resistance to chemotherapy occurs due to a decrease in perfusion of agents across diminished oxygen gradients, reduced cell death of hypoxic cells in G0-G1 state when treated by proliferation-dependent drugs, and altered multi-drug resistance and DNA repair (Chan et al., 2007). However, recent data suggests hypoxia can modify the DNA damage response. In some cases, hypoxic cells are rendered DNA repair-deficient with reduced OER values and differential sensitivity to certain types of chemotherapy and radiotherapy (Bristow and Hill, 2008; Chan et al., 2008; Sprong et al., 2006). An increased capacity for metastases in hypoxic tumor cells is associated with multiple mechanisms including increased hypoxia-activated genes involved in metastasis and angiogenesis (e.g. VEGF, LOX) and selection of genetically-unstable metastatic clones during tumor progression (Bristow and Hill, 2008).

When taken together, these aggressive biological properties of hypoxic cells lead to the clinical scenario whereby the presence of intratumoral hypoxia is an adverse prognostic factor in cancer (Bristow and Hill, 2008). This is particularly true in prostate cancer (the most common
non-cutaneous, malignancy in men) where several clinical studies have shown an association between hypoxia and poor clinical outcome following radiotherapy or radical prostatectomy (see Table 1).

Table 1.1 Selected clinical studies of hypoxia in prostate cancer.

<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Risk group</th>
<th>Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Turaka et al., 2012)</td>
<td>57</td>
<td>cT1-3</td>
<td>pO2 probe</td>
<td>Lower prostate/Muscle pO2 ratio predicted early biochemical failure after brachytherapy</td>
</tr>
<tr>
<td>(Milosevic et al., 2012)</td>
<td>247</td>
<td>cT1-2</td>
<td>pO2 probe</td>
<td>Largest study showing that hypoxia predicted early biochemical relapse after radiotherapy and local recurrence</td>
</tr>
<tr>
<td>(Vergis et al., 2008)</td>
<td>201 (RT)</td>
<td>cT1-3</td>
<td>IHC – VEGF, HIF-1 alpha, OPN</td>
<td>Increased expression of VEGF, HIF-1 alpha, and, for patients treated with surgery, OPN, identified patients at high risk of biochemical failure</td>
</tr>
<tr>
<td></td>
<td>289 (surgery)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Carnell et al., 2006)</td>
<td>43</td>
<td>cT1-3</td>
<td>IHC – PIMO</td>
<td>A positive correlation of PIMO +3 binding with Gleason score was demonstrated</td>
</tr>
<tr>
<td>(Boddy et al., 2005)</td>
<td>149</td>
<td>cT1-3</td>
<td>IHC – VEGF, HIF-1α</td>
<td>There was a significant correlation between HIF-1 α and HIF-2 α expression, and with AR and VEGF expression. VEGF was also significantly related to the androgen receptor, whereas PHD2 was inversely related to HIF-2a expression. No significant association was shown between HIF-1a or HIF-2a and time to PSA recurrence.</td>
</tr>
<tr>
<td>(Green et al., 2007)</td>
<td>50</td>
<td>cT3</td>
<td>IHC</td>
<td>High VEGF expression was associated with lower disease specific survival</td>
</tr>
<tr>
<td>(Thoms et al., 2012)</td>
<td>199 (T1-3) 37 (M1)</td>
<td>cT1-T3 and M1</td>
<td>ELISA – OPN</td>
<td>Within localized prostate cancers plasma OPN was not predictive of more aggressive disease. For CRPC-MET patients, OPN was significantly higher, and was as good as PSA at predicting treatment response after chemotherapy.</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>---------------</td>
<td>--------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Weber et al., 2012)</td>
<td>103</td>
<td>cT1-3</td>
<td>IHC</td>
<td>High nuclear expression of HIF1α and low EGFR expression was associated with a good prognosis in diagnostic biopsies of prostate cancer patients treated with RT ± ADT.</td>
</tr>
</tbody>
</table>


As can be seen in Table 1.1, different methodologies have been used to assess intraglandular tumor hypoxia including direct pO2 electrode measurements, use of the hypoxia biomarker, pimonidazole (PIMO), and immunohistochemistry (IHC) for hypoxia-activated protein expression. Using a needle–electrode technique, Turaka (2012) and co-workers studied 57 patients with more than 8 years of follow-up and showed that a decreased prostate-to-muscle oxygen ratio was an important predictor of early biochemical recurrence following brachytherapy. These authors suggested that hypoxia was driving early recurrence as it was associated with an increased likelihood of occult metastases at the time of treatment (Turaka et al., 2012). Using a similar methodology, Milosevic et al. (2012) have directly measured intraprostatic O2 levels in the largest study to date of 247 prostate cancer patients with localized intermediate risk disease (Milosevic et al., 2012). This large study showed that hypoxia is associated with early biochemical relapse and local recurrence in the prostate gland (see Figure 1.1A).
Figure 1.1: Hypoxia and aneuploidy are markers of poor clinical prognosis. (A) Prostate cancer patients with more hypoxic tumors had higher rates of biochemical relapse after radiation therapy (adapted from Milosevic et al., 2012). (B) Patients with tetraploid or aneuploid tumors had increased disease relapse rates after radical prostatectomy relative to patients with diploid tumors (adapted from Pretorius et al., 2009).
Prostate tumor hypoxia can also be assessed in situ using IHC on biopsies or post-surgical specimens. Tumor cells adapt to a hypoxic microenvironment via up-regulation of transcription factor hypoxia-inducible factor 1α (HIF1α) (Semenza, 2012). HIF1α stabilization leads to increased transcription of several genes responsible for tumor cell survival in the low-oxygen environment, including VEGF, GLUT-1 and OPN (Wilson and Hay, 2011). Using IHC, Vergis et al. (2008) have shown that an increased expression of the hypoxia-induced proteins (e.g. HIF1α, VEGF and OPN for surgical patients and HIF1α and VEGF for radiotherapy patients) predicted treatment failure, independent of clinical factors such as tumor stage, Gleason score, serum PSA and radiotherapy dose (Vergis et al., 2008). The latter study’s observation that the prognostic value of low pO2 and increased expression of hypoxia-associated markers in situ is independent of radiation dose suggest that eradication of the aggressive hypoxic sub-fraction may require escalation in both local and systemic therapies. For example, this could lead to the use of combined modality therapies using precision surgery or radiotherapy plus androgen-deprivation therapy (Milosevic et al., 2007) and/or selective and hypoxia-targeted systemic agents (Ahn and Brown, 2007; Chan and Bristow, 2010; Chan et al., 2010; Meng et al., 2012).

1.1.2 Chromosomal instability and prostate cancer prognosis

In order to understand the link between hypoxia and genetic instability in prostate cancer, one first needs to define “genome instability”. Although often used interchangeably, chromosomal instability (CIN) and tumor cell aneuploidy are not the same. CIN is the dynamic process of constant loss or gain of chromosomes (or parts of chromosomes) while aneuploidy defines a more static concept of chromosomal alteration (Geigl et al., 2008). Both CIN and aneuploidy are associated with cancer progression and poor prognosis (McGranahan et al., 2012). For example, Pretorius et al. (2009) have shown that prostate cancer patients with tetraploid or aneuploid tumors had decreased disease-free survival following radical prostatectomy when compared to patients with diploid tumors (see Figure 1.1B) (Pretorius et al., 2009). Table 1.2 summarizes the many clinical studies that have linked aneuploidy to poor prognosis in prostate cancer.
<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Risk group</th>
<th>Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Pretorius et al., 2009)</td>
<td>186</td>
<td></td>
<td>Image cytometry of Feulgen stained tissue.</td>
<td>On multivariate analysis, DNA ploidy was shown to be an independently predictor of disease recurrence. In Gleason score 7 cases (N=68), DNA ploidy was a significant predictor of disease recurrence.</td>
</tr>
<tr>
<td>(Wirth et al., 1991)</td>
<td>80</td>
<td>Stage C</td>
<td>DNA flow cytometry</td>
<td>DNA ploidy was a strong prognostic indicator independent of tumor grade and tumor stage. Patients with diploid tumors did significantly better than those with aneuploid or tetraploid tumor pattern.</td>
</tr>
<tr>
<td>(DiSilverio et al., 1996)</td>
<td>85</td>
<td>Stage C-D1</td>
<td>DNA flow cytometry</td>
<td>DNA aneuploidy conferred a relative risk 2.3 times higher than diploidy for local and distant recurrences.</td>
</tr>
<tr>
<td>(Ross et al., 1994)</td>
<td>89</td>
<td>Early clinical stage (A2-B2)</td>
<td>Image analysis of Feulgen stained tissue sections</td>
<td>DNA content analysis of needle biopsy specimens directly correlated with radical prostatectomy specimen ploidy status and is associated independently with the presence of metastasis, disease recurrence, and extracapsular extension.</td>
</tr>
<tr>
<td>(Amling et al., 1999)</td>
<td>108</td>
<td>pT2-4N0-1</td>
<td>DNA flow cytometry</td>
<td>DNA ploidy predicted cancer specific and progression-free survival.</td>
</tr>
<tr>
<td>(Song et al., 1992)</td>
<td>65</td>
<td>cT1-3N0</td>
<td>DNA flow cytometry and image analysis of Feulgen stained tissue sections</td>
<td>DNA content was the most important independent variable for cancer specific survival.</td>
</tr>
<tr>
<td>(Pollack et al., 1994a)</td>
<td>76</td>
<td>cT1-3 N0</td>
<td>DNA flow cytometry</td>
<td>In a RT cohort, DNA ploidy (near-diploid vs. diploid and non-diploid tumors) was an independent prognostic factor for recurrence.</td>
</tr>
<tr>
<td>(Pollack et al., 1994b)</td>
<td>76</td>
<td>cT1-3N0</td>
<td>DNA flow cytometry</td>
<td>In the same RT cohort (above), a significant correlation of DNA-ploidy with PSA-DT was observed. Nondiploid tumors were associated with shorter PSA-DT (higher actuarial rates of disease relapse at 3 years).</td>
</tr>
<tr>
<td>------------------------</td>
<td>----</td>
<td>---------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Pollack, 2003)</td>
<td>149</td>
<td>T2-3N0-N1</td>
<td>Image analysis of Feulgen stained tissue sections</td>
<td>Based on the RTOG 8610 (RT alone vs. RT plus short-course-ADT) nondiploidy was associated with shorter overall survival, which seemed to be related to reduced response to salvage hormone therapy for those previously exposed to short-term ADT.</td>
</tr>
</tbody>
</table>

cT1: Clinical (pre-radiotherapy) T-category; pT: Pathologic (post-surgery) T-category; RT: radiotherapy; ADT: androgen deprivation therapy;

As hypoxia leads to down-regulation of DNA damage repair mechanisms and increased genomic instability (Bristow and Hill, 2008; Vergis et al., 2008), repair-deficient hypoxic tumor cells could adapt to low oxygen levels and acquire an aggressive “mutator” phenotype. Clonal selection could then arise leading to aggressive and resistant phenotypes (Bristow and Hill, 2008; Luoto et al., 2013a). As such, there is a rationale to explore the intersection and mechanism between intratumoral hypoxia, genomic instability and aneuploidy in prostate cancer cells.
1.1.3 The relationship between hypoxia and genomic instability

One model of hypoxia-mediated tumor progression incorporates the concept of hypoxia driving the accumulation of mutations and chromosome aberrations during hypoxic cellular adaption and subsequent proliferation. These hypoxic cells continue to proliferate under low oxygen conditions with an increased likelihood of generating an unstable genome if genetic alterations accumulate during DNA replication and mitosis. For example, CIN can occur as a result of defects in DNA double-strand break (DSB) repair and mitotic aberrations (McGranahan et al., 2012). DNA mutations can also occur secondary to microsatellite instability (MIN) due to defects in hypoxia-induced mismatch repair (MMR) (Geiersbach and Samowitz, 2011; Michor et al., 2005). Hypoxia can alter DNA damage repair (DDR) through alterations in DDR gene expression. I will now discuss experimental data in which hypoxia was shown to lead to defects in DNA repair.

1.2 The Repair of DNA Damage in Hypoxic Cells

1.2.1 The effect of hypoxia on DNA DSB repair: The HR and NHEJ subpathways

DSBs are lethal to the cell if not correctly repaired and can occur as a result of exogenous DNA damage in the form of radiotherapy or chemotherapy (e.g. bleomycin) or endogenous DNA damage during DNA replication (which can produce unrepaired, replication stress-induced DNA breaks) (Kuzminov, 2001; Mills et al., 2003). Unrepaired DSBs can lead to the loss or gain of partial or whole individual chromosomes, and chromosome translocations can occur due to DSB misrepair (Helleday et al., 2007; Jeggo and Löbrich, 2007).

There are two sub-pathways of DSB repair. The rapid, but error-prone non-homologous end-joining (NHEJ) pathway is active throughout the cell cycle. The second pathway of homologous recombination (HR) is a more time-consuming and stringent error-free mechanism that requires an intact homologous chromosome (acquired during DNA replication in S-phase) as a template. HR is therefore only active during the S and G2 phases of the cell cycle (Helleday et al., 2007; Rothkamm et al., 2003).
Hypoxia causes down-regulation in the expression of a number of HR and NHEJ genes including RAD51, BRCA1, BRCA2 and DNA-PKcs genes, and their protein products and has been linked to functional HR defects (Chan et al., 2008; Meng et al., 2005). In our laboratory, we found that despite lower levels of initial DSB formation following ionizing radiation (IR) in hypoxic cells, these hypoxia-induced DSB defects led to increased residual and unrepaired DSBs and associated chromosomal aberrations at first mitosis following IR (Kumareswaran et al., 2012). Hypoxia is also known to induce common fragile sites (chromosomal regions prone to chromosomal breakage and deletions), leading to increased chromosome aberrations, which over time drives genomic instability and chromosomal instability. CIN would then lead to aneuploidy and loss of heterozygosity (LOH) for specific tumor suppressor or oncogenic genes (Michor et al., 2005). Indeed, LOH can elevate the rate of tumor suppressor gene inactivation and contribute to cancer progression (Michor et al., 2005). It would be of interest to compare rates of LOH in oxic versus hypoxic cells to support or refute this hypothesis.

### 1.2.2 Other DNA repair pathways modified by hypoxia: MMR and NER

Hypoxia also causes defects in other DNA damage repair pathways, including MMR, nucleotide-excision repair (NER) and the Fanconi anemia (FA) DNA repair pathway. MMR is the mechanism of repair for mismatch and misalignment of bases that can occur during DNA replication (Hsieh and Yamane, 2008). The MMR pathway is suppressed by hypoxia due to down-regulation of the MMR proteins, including MLH1 and MSH2 (Mihaylova et al., 2003; Nakamura et al., 2008; Shahrzad et al., 2005). Defects in MMR have been shown to cause MIN due to accumulation of unrepaired replication errors. Microsatellites are repeat sequences 1 to 6 base pairs in length, and MIN presents as changes in the number of microsatellite repeats. MIN occurs due to defects in MMR during the process of DNA replication which causes elevated nucleotide-level mutation rates (Kinzler & Vogelstein, 1996). Tumors with MIN generally have less large-scale genomic alteration and gene mutation profiles distinct from those observed in CIN tumors (Geiersbach and Samowitz, 2011).

Bulky DNA adducts or crosslinks caused by chemotherapy such as cisplatin can be repaired by the NER pathway (Nouspikel, 2009). Importantly, HIF1α binds to the hypoxia-responsive elements within the gene promoters of two NER proteins, XPC and XPD, and exhibits negative transcriptional regulation on these genes under hypoxic conditions (Rezvani et al., 2010).
HIF1α also down-regulates the NER protein RAD23B under hypoxia via activation of the microRNA miR373 (Crosby et al., 2009). Fanconi anemia (FA) is a hereditary cancer predisposition disorder caused by mutations in any of 14 FANC genes which participate in DNA inter-strand crosslink repair (Kitao and Takata, 2011). Less is known about the function of the FA pathway under hypoxic conditions, but FANCD2-deficient cells have differential DNA repair and radiosensitivity under hypoxia versus oxia (Kuhnert et al., 2009; Sprong et al., 2006). The global reduction in numerous DNA repair pathways in hypoxic cells can therefore contribute to an accumulation of different mutations and translocations that, if not lethal, could lead to aggressive mutator cell variants.

1.2.3 Using hypoxia-mediated DNA repair defects as an Achilles’ heel for cancer treatment: The concept of “contextual synthetic lethality”

Treatment resistant and aggressive tumor phenotypes associated with DNA repair-deficient hypoxic cells may be uniquely targeted using the knowledge of the DNA repair defect. For example, our laboratory and others have suggested that DDR-deficient hypoxic tumor cells can be targeted using the concept of contextual synthetic lethality (Chan and Bristow, 2010; Chan et al., 2010). Two genes are synthetic lethal if a mutation of either alone is compatible with viability but mutation of both genes leads to cell death (Kaelin, 2005). This is termed “genetic synthetic lethality” as it was originally based on yeast genetics (Kaelin, 2005). However, this observation could also apply to hypoxic cells deficient in HR or MMR (Chan and Bristow, 2010; Chan et al., 2010). For example, hypoxic cells deficient in HR can be sensitized by inhibitors of poly(ADP-ribose) polymerase (PARP) proteins, which function in single-strand break (SSB) and base-excision repair (BER) (Chalmers et al., 2010; Chan et al., 2010; Hegan et al., 2010). This is similar to the use of PARP inhibitors in ovarian cancers which are defective in HR due to a loss of BRCA1 or BRCA2 gene function (Fong et al., 2009). Contextual synthetic lethality can also potentially be utilized to target MMR down-regulation in hypoxic cells. Genetic disruption of DNA polymerases Pol-γ and Pol-β has been shown to be synthetically lethal in cells with deficiency in MSH2 and MLH1, the MMR proteins known to be down-regulated by hypoxia (Martin et al., 2010). In addition, repair-deficient hypoxic tumor cells can have increased sensitivity to specific drugs which are selectively toxic to
repair-deficient cells (e.g. mitomycin C or cisplatin with HR-defective cells) (Chan and Bristow, 2010; Chan et al., 2008). Therefore defining the presence and fraction of repair-defective hypoxic cells in human tumors using biomarkers of hypoxia and DNA repair defects could lead to personalized treatments with these agents. Such approaches could achieve a high therapeutic ratio given these hypoxic abnormalities are likely to be tumor-specific (Bristow and Hill, 2008; Chan and Bristow, 2010).

1.3 Control of mitotic function and significance for CIN

1.3.1 Causes and consequences of CIN

CIN is highly prevalent in cancer and leads to aneuploidy (Geigl et al., 2008). Numerical CIN is classified as deletion or addition of whole chromosomes, whereas structural CIN is associated with intra-chromosome breaks (Geigl et al., 2008). Structural CIN can occur in tumor cells when there is a genetic inactivation of one or more cell cycle checkpoints that normally act to prevent genome instability (e.g. abrogation of the G1 checkpoint in cells mutated for p53), while numerical CIN generally occurs as a result of mitotic defects (Gisselsson, 2003; Smith and Fornance, 1995; Thompson et al., 2010). Structural chromosome rearrangements can result in gene fusion products and both types of CIN can lead to gene amplification/deletion (McGranahan et al., 2012). A link between intratumoral hypoxia and abnormal mitotic biology resulting in CIN could lead to loss of heterozygosity (LOH). LOH is a prominent mechanism by which a functional loss in tumor suppressor genes occurs during prostate tumor progression (Baker et al., 2009; McGranahan et al., 2012).

1.3.2 Mitotic regulation and implications for chromosome stability

In normal cells, mitosis is carefully choreographed to ensure the fidelity of sister chromatid segregation to opposite daughter cells to maintain chromosomal stability (Thompson et al., 2010). Disruption of mitotic function, such as defects in sister chromatid cohesion, bypass of the spindle assembly checkpoint (SAC), and centrosome aberrations, can lead to numeric and structural CIN (see Figure 1.2) (Thompson et al., 2010).
Central to proper chromosome segregation is sister chromatid cohesion, which is maintained by the cohesin complex (Rieder et al., 1994). Cohesion of sister chromatids must be maintained during G2 and M phase until the onset of anaphase at which point it must be abruptly disrupted. The precise timing of the disruption of sister chromatid cohesion is accomplished by activity of cyclin-dependent kinases and the spindle assembly checkpoint (SAC). Chromosomes with kinetochores that are not properly attached to mitotic spindles (and therefore are not ready for anaphase) emit a signal that inhibits the onset of anaphase (Rieder et al., 1994, 1995). The SAC functions to ensure correct anaphase onset, so that sister chromatid segregation does not occur until all chromosomes are aligned and kinetochores are properly attached to microtubules.

Defects in sister chromatid cohesion or the SAC can potentially lead to inappropriate sister chromatid separation and CIN (Thompson et al., 2010). Although large-scale tumor genome sequencing reveals that cohesin gene mutations are rare (Thompson et al., 2010), there is evidence of higher expression levels of separase, the protein responsible for cleaving the cohesin complex, in breast cancer tumor tissue compared with normal tissue (Zhang et al., 2008). Mutations in the SAC protein Mad2 have been identified in breast and gastric cancer cell lines (Kim et al., 2005; Percy et al., 2000), and decreased levels of Mad2 have been observed in other tumor cell lines (Li and Benezra, 1996; Wang et al., 2000, 2002). In addition, mutations in the SAC protein Bub1 have been identified in colon cancer cell lines (Cahill et al., 1998). A defective SAC in prostate cancer cells could permit the aberrant onset of anaphase and increase the frequency of chromosome missegregation as a contributor to CIN.
Figure 1.2: Cellular pathways responsible for regulating chromosomal stability. When these pathways are deregulated, the result is chromosomal instability and aneuploidy. Deregulation of these pathways can occur through oncogene activation, and tumor suppressor deactivation at the major upstream regulating proteins. Additionally, hypoxia may modify the expression and function of many of the downstream effector proteins. Adapted from Duijf and Benezra (2013).
1.3.3 The centrosome as a major regulator of chromosome integrity

The centrosome is the main microtubule-organizing center (MTOC) in mammalian cells. The centrosome has many functions in the cell, such as ciliogenesis, cytoskeleton maintenance, and intracellular signaling, but its major importance in cancer biology is the centrosome’s vital role during mitosis. Centrosomes are crucial for mitotic spindle assembly, proper sister chromatid segregation, and cytokinesis, processes that are essential for maintenance of chromosome stability (reviewed in Pihan, 2013). Centrosome number and structure are stringently regulated in normal cells. During mitosis, each daughter cell inherits one centriole pair-containing centrosome from the mother cell. The process of centrosome duplication (e.g. the centrosome cycle) is tightly coupled to the cell cycle and DNA replication. The cell duplicates centrioles once per cell cycle in S-phase, and the centriole pairs separate to form two centrosomes in late G2-M-phase (Nigg and Stearns, 2011). A “licensing” signal in late M/early G1-phase permits the disengagement of the centriole pair in the inherited centrosome to allow a new centriole duplication cycle to begin (Tsou et al., 2009). Disruption of the cell cycle can lead to decoupling of the centrosome duplication cycle from the DNA replication cycle, causing centrosome over-duplication; the latter is a major cause of numerical centrosome aberrations.

Centrosome aberrations in cancer are heterogeneous with both numerical and structural abnormalities documented (Krämer et al., 2005; Pihan et al., 1998, 2001) The most commonly documented centrosome aberration in cancer cells is the presence of supernumerary centrosomes, also referred to as centrosome amplification. Cells can acquire supernumerary centrosomes via a number of mechanisms: cell fusion, failure in cytokinesis, deregulation of the centrosome duplication cycle, and de novo centrosome biogenesis (Vitre and Cleveland, 2012). Centrosome amplification directly links to CIN by promoting aberrant kinetochore microtubule attachments (Ganem et al., 2009). Mitotic cells with extra centrosomes undergo a transient state of multipolar mitotic spindles, followed by centrosome clustering. During the intermediate multipolar spindle state the cell accumulates merotelic kinetochore-microtubule attachment errors that persist into anaphase. The kinetochore-microtubule attachment errors cause chromosome missegregation and lagging chromosomes in anaphase (Ganem et al., 2009). Since multipolar mitoses in cells with centrosome amplification are rare, and usually produce inviable progeny when they do occur, it has been postulated that centrosome
clustering is the major mechanism leading to increased CIN in cells with centrosome amplification (Ganem et al., 2009; Krämer et al., 2011; Marthiens et al., 2012). Consequently, the protein machinery that promotes centrosome clustering has become an attractive target for cancer therapy (Ogden et al., 2012).

1.3.4 Centrosome structure and related genes

The centrosome consists of a pair of orthogonally-oriented, microtubule-based cylindrical structures called centrioles. A complex protein matrix surrounds the centrioles and is called the pericentriolar material (PCM). Centrioles consist of centriolar microtubules that include α- and β- tubulin, followed by the addition of ε- and δ- tubulin as the centriole matures (Brito et al., 2012). The centriole lumen also contains a large variety of functional proteins, including Centrin 2/3, Cep135, Plk4 and γ-tubulin. Centrin 2 (CETN2) is the protein used to mark centrioles in IF experiments in this study. Although most of the CETN2 protein is found throughout the cell cytoplasm, it is highly concentrated at the distal luminal end of centrioles and has been shown to be important in centriole duplication (Paoletti et al., 1996; Salisbury et al., 2002). The centriole outer walls include a set of appendages containing hSas6, Cep164 and other proteins, and are important in microtubule anchoring (Brito et al., 2012).

The PCM is highly organized, with different proteins occupying specific domains within the PCM structure (Lawo et al., 2012). Recent work by Lawo et al. (2012) demonstrated that centrosome components (CEP120, CEP192, CEP152, NEDD1 and TUBG1) adopt a toroidal pattern around the centrioles. In addition, γ-tubulin ring complexes (γ-TuRCs), ring-like structures found in the PCM, are responsible for mediating the anchoring and nucleation of microtubules (Moritz et al., 1995). Proteins implicated in the recruitment and/or attachment of γTuRCs include pericentrin (PCNT) and CEP192 (Dictenberg et al., 1998; Gomez-Ferreria et al., 2007). Additionally, the Augmin Centrosome Complex (ACC), which consists of 8 subunits (HAUS1-8), was recently found to interact with γTuRCs, and knockdown experiments demonstrated that the interaction is important in proper microtubule assembly. PCNT, the protein used to label the PCM in this study, was found to be an elongated molecule that spans the width of the PCM, with its C terminus in the vicinity of centrioles and N terminus extending out to the periphery. PCNT is necessary for PCM scaffold assembly and formation of mature mitotic centrosomes (Lawo et al., 2012).
Further observations of centriole structure with electron microscopy (EM) identified electron dense granules localizing around the centrosome, later identified as centriolar satellites (Reviewed in Bärenz, Mayilo, and Gruss 2011a). Centriolar satellites are approximately 70–100 nm in diameter as observed by EM. These structures were initially observed next to newly synthesized daughter centrioles during centriole duplication. Later it was shown that centriolar satellites were also associated with microtubules originating from the centrosome, and around ciliary basal bodies in epithelial cells. Further work by Kubo et al. (1999) demonstrated that these previously obscure granules contain a protein named pericentriolar material-1 (PCM-1), and others have shown centriolar satellites to also contain Cep72, Cep90, and centrin (Bärenz et al., 2011; Dammermann and Merdes, 2002; Kubo et al., 1999; Oshimori et al., 2009). The PCM-1–containing centriolar satellites move along microtubules toward centrosomes (microtubule minus ends) in live cells (Kubo et al., 1999). Additionally, centriolar satellites were shown to have a role in the assembly of centrin, pericentrin and ninein at the centrosome, and are required for centrosome mediated microtubule nucleation (Dammermann and Merdes, 2002; Oshimori et al., 2009). It has been suggested that a possible function of centriolar satellites is to serve as assembly platforms for centriolar and PCM proteins and mediate their transport from the cytoplasm to the centrosome along microtubules (Bärenz et al., 2011; Dammermann and Merdes, 2002).

1.3.5 Assays for centrosome structure

To determine the presence of centrosome aberrations, it is possible to label the centrosomal proteins in several ways, including immunofluorecence (IF) in fixed cells, live cell imaging of green or red fluorescent protein (GFP or RFP)-tagged centrosomal proteins, and IHC analysis in tissues. IF labelling of fixed cells with antibodies manufactured against centrosomal proteins is the most common method of observing supernumerary centrosomes, as well as centrosome structure. Centrioles and the PCM can be distinguished by using antibodies against components found in only one or the other structure (Figure 1.3). For the purpose of this thesis, I used CETN2 to identify centrioles, as it is particularly concentrated in the lumen of centrioles (Paoletti et al., 1996). To specifically label the PCM, I used antibodies for PCNT, as this protein is an important structural component throughout the PCM and is not found in centrioles (Lawo et al., 2012). Previous studies have demonstrated that co-staining for CETN, PCNT, and...
NEDD1 (another PCM component) is effective for identifying centrosome structure aberrations (Lawo et al., 2009, 2012). In this study I also used localization of an additional centriole protein Cep135, found in the centriole lumen, for additional structural studies in centrosomes.

Live cell imaging has an advantage over IF in that it can track centrosome dynamics over time in living cells to study mitotic spindle assembly, mitotic function and daughter cell biology following cytokinesis. Previous studies have successfully observed centrosome dynamics in live cells expressing GFP-NEDD1, GFP-centrin2, GFP-pericentrin, GFP-Cep192, GFP-α-tubulin (mitotic spindle) or GFP-H2B (chromosomes) (Dictenberg et al., 1998; Ganem et al., 2009; Inanç et al., 2010; Lawo et al., 2009; Moser et al., 2013; Saladino et al., 2009). Cells expressing GFP tagged mitotic proteins can also be transfected to simultaneously express a second RFP tagged mitotic protein, allowing for simultaneous observations of mitotic spindles and centrosomes and/or chromosomes to analyze mitotic function.
**Figure 1.3: Representation of the centrosome markers I used in immunofluorescence (IF) studies.** Shown is a cartoon representation of the centrosome, demonstrating the location of centrin (CETN) and pericentrin (PCNT) within the centrioles and pericentriolar material (PCM), respectively. Top left is an IF image of a cell IF-labelled with antibodies to show DNA (blue), CETN (yellow-green) and PCNT (red).
1.4 The role of the centrosome complex in cancer and hypoxia-induced genomic instability

1.4.1 The relationship between centrosome function and cancer progression

Chromosome abnormalities (aneuploidy) and centrosome amplification are both key features of cancer cells. German zoologist Theodor Boveri was the first to suggest that normal cells become malignant when they acquire chromosomal abnormalities due to the aberrant replication and activity of centrosomes. Boveri’s theory was largely influenced by his colleague David Hansemann, who made extensive observations on the incidence of anomalous mitotic figures in human tumors (Bignold et al., 2006). Subsequently, a number of studies have correlated CIN with centrosome abnormalities in cancer cells (Anderhub et al., 2012; Chan, 2011; Lingle et al., 2005; Vitre and Cleveland, 2012). In addition, clinical studies have associated increased centrosome aberrations with poor clinical prognosis in breast, urogenital, lung, neural and other cancers (Chan, 2011; D’Assoro et al., 2002). Collectively, these studies have shown that the majority of cancers harbor centrosome amplification, and centrosome amplification correlates with cancer aggressiveness and poor survival (Anderhub et al., 2012; Chan, 2011; D’Assoro et al., 2002). Pihan et al. (2001) demonstrated that centrosome abnormalities are evident in the majority of prostate carcinomas and are enhanced in poorly differentiated tumors; as such, higher levels of centrosome aberrations may also lead to prostate cancer aggression.

In-vitro and in-vivo experiments have also provided direct evidence demonstrating a link between centrosome amplification and cancer progression. Godinho et al. (2014) used a three-dimensional human mammary epithelial cell model to show that centrosome amplification can trigger invasive cell behaviour similar to that induced by oncogene ERBB2 overexpression. The centrosome amplification-induced increase in centrosomal microtubule nucleation causes an increase in Rac1 activity and results in disrupted cell-cell adhesion and increased cell invasiveness (Godinho et al., 2014). Spindle and kinetochore-associated protein 1 (Ska1),
recently shown to also localize to centrosomes during mitosis, was shown to be over-expressed in prostate cancer precursors – prostatic intraepithelial neoplasia (PIN) – as well as in prostate adenocarcinomas and other human cancers (Li et al., 2014). Over-expression of Ska1 leads to centriole over-duplication and centrosome amplification in epithelial cell cultures and DU145 prostate cancer cell cultures. Importantly, Ska1 overexpression was shown to cause spontaneous formation of mouse-PIN in a transgenic mouse prostate model and tumor formation in immunodeficient mice injected with Ska1 overexpressing cells (Li et al., 2014).

1.4.2 The link between the DNA-damage response and centrosome function: a possible mechanism of hypoxia-induced genomic instability

As we have just discussed, disrupted centrosome function has been recognized as one of the leading causes of CIN in many cancer types, potentially contributing to cancer aggression. Centrosome amplification can occur as a pre-mitotic stress in response to DNA damage during prolonged G2-phase arrest (Dodson et al., 2004). During G2 phase, DNA damage response and repair proteins such as RAD51, BRCA1, BRCA2, XRCC2, MDC1, and BRIT1 colocalize to centrosomes (Cappelli et al., 2011; Rai et al., 2006, 2008). Depletion or mutation of these genes enhances centrosome amplification (see Table 1.3) (Dodson et al., 2004; Ko et al., 2006; Rai et al., 2008; Saladino et al., 2009; Shimada et al., 2010). ATM and ATR are phosphatidylinositide 3-kinases (PI3K)-like kinases that are involved in DSB sensing and subsequent activation of cell-cycle arrest and DNA repair protein recruitment to the sites of DNA damage. These two kinases have complementary roles in limiting centrosome amplification in response to DNA damage (Bourke et al., 2007; Dodson et al., 2004).

The link between the DNA damage repair pathways and centrosome amplification is of interest in the context of tumor hypoxia. Since DNA damage repair protein disruption can lead to centrosome amplification, we speculate that hypoxia may also lead to centrosome aberrations. Centrosome aberrations and subsequent mitotic abnormalities may be an additional mechanism of hypoxia-induced genomic instability (see Figure 1.4). However, there are few studies that have investigated the effect of hypoxia on centrosome function. Nakada et al. (2011) have shown an increase in centrosome amplification in cells overexpressing miR-210, a microRNA
up-regulated by the HIF-1α pathway and hypoxic conditions (Nakada et al., 2011). Another link between the biological response to hypoxia and centrosome function was observed when Moser et al. (2013) demonstrated that prolyl-4-hydroxylase 1 (PHD1) is required for centrosome duplication and maturation. PHD1 hydroxylates centrosomal protein Cep192 to target it for ubiquitination and subsequent proteasomal degradation (Moser et al., 2013). PHD1-3 are important in HIF transcription factor stabilization, which occurs due to a decrease in PHD activity in response to hypoxia (Fandrey et al., 2006). It is unclear whether the decrease in PHD activity in response to hypoxia has a direct effect on centrosome function. Therefore the study of hypoxia on centrosome function in prostate cancer cells could provide novel mechanistic data to the existing literature.
Table 1.3. DNA repair proteins that may have a role in centrosome function and are also down-regulated by hypoxia.

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Genetic model</th>
<th>Protein function</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td>Knockdown</td>
<td>HR</td>
<td>Increase in centrosome amplification; Colocalizes with centrosomes</td>
<td>(Cappelli et al., 2011; Dodson et al., 2004)</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Null mutant/knockout</td>
<td>HR</td>
<td>Increased centrosome amplification in the absence of any treatment; Enhanced IR-induced centrosome amplification; Colocalizes with centrosomes</td>
<td>(Cappelli et al., 2011; Hsu and White, 1998; Saladino et al., 2009)</td>
</tr>
<tr>
<td>BRCA2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-PK, Ku70</td>
<td>Null mutant/knockout</td>
<td>DDR</td>
<td>IR-induced centrosome amplification is reduced</td>
<td>(Saladino et al., 2009)</td>
</tr>
<tr>
<td>DNA-PK, Ku70</td>
<td>Knockout</td>
<td>NHEJ</td>
<td>Slightly increased IR-induced centrosome amplification</td>
<td>(Shimada et al., 2010)</td>
</tr>
<tr>
<td>NBS1</td>
<td>Knockout</td>
<td>DSB sensing</td>
<td>Slightly increased IR-induced centrosome amplification</td>
<td>(Shimada et al., 2010)</td>
</tr>
<tr>
<td>XRCC2</td>
<td>Deficient (irs1 hamster cells)</td>
<td>HR</td>
<td>Centrosome disruption (without IR-induced DNA damage); Co-immunoprecipitates with BRCA1 and γ-tubulin; Colocalizes with centrosomes</td>
<td>(Cappelli et al., 2011)</td>
</tr>
</tbody>
</table>

HR: homologous recombination; NHEJ: non-homologous end-joining; IR: ionizing radiation; DDR: DNA damage response; DSB: double-strand break
Figure 1.4: Mitotic and centrosome abnormalities in cancer and their potential relationship with intratumoral hypoxia. Hypoxia down-regulates DNA damage repair proteins (e.g. RAD51, BRCA1, BRCA2) involved in both homologous recombination (HR) and centrosome biology. Defective HR and centrosome disruption leads to increased mutation burden. As a cell progresses through mitosis, the presence of supernumerary centrosomes and centrosome aberrations can lead to improper microtubule-kinetochore attachments and result in lagging chromosomes and DNA-chromosomal breaks. As chromosome aberrations accumulate, chromosomal instability and aneuploidy increase during tumor progression.
1.5 Targeting centrosomes as an anti-cancer strategy

If hypoxic sub-regions of tumors harbor cancer cells with defective mitotic function, then mitosis- and centrosome-targeting drugs that are already under investigation could be potential cancer therapeutics (Mazzorana et al., 2011). Centrosome-associated kinases such as polo-like kinase 1 (PLK1), Aurora A and cyclin-dependent kinase 1 (CDK1), which regulate centrosome function, are all targets of compounds that are in advanced stages of clinical trials for cancer therapy (Mazzorana et al., 2011).

A more recent strategy that has come under investigation targets centrosome clustering proteins as an anti-cancer therapy (Ogden et al., 2012, 2014). Cancer cells cluster supernumerary centrosomes to prevent multipolar mitosis and increase cellular survival in the presence of extra centrosomes (Ganem et al., 2009). It has been proposed that centrosome clustering contributes to CIN and decreases mitotic catastrophe caused by multipolar mitosis. This provides a direct link between centrosome amplification, CIN and aggressive phenotypes in cancer (Krämer et al., 2011; Marthiens et al., 2012). Centrosome de-clustering drugs can increase the occurrence of multipolar mitosis and result in mitotic catastrophe-induced cell death in cancer cells with supernumerary centrosomes. Since the majority of cancers harbor supernumerary centrosomes, while normal cells do not, the strategy has potential to achieve a high therapeutic ratio given that hypoxia is rarely present in normal tissues.

1.6 Summary and Hypothesis

Experimental evidence suggests that hypoxia can lead to increased metastasis and poor clinical prognosis as a result of a “mutator” tumor phenotype aligned with cellular adaptation and selection. The role of centrosome aberrations in CIN and cancer progression is important as it could be a potential mechanism by which hypoxia leads to genetic instability (including hypoxia-induced CIN) and the observed aggressive clinical phenotypes. A potential model of the interaction between hypoxia, CIN and mitotic function that leads to cancer aggression is shown in Figure 1.5.
Figure 1.5: A mechanistic model for the effect of intratumoral hypoxia on genomic instability and cancer metastasis. One model of hypoxia-induced genomic instability suggests that the decrease in protein expression and function associated with DNA damage response and repair leads to increased chromosomal aberrations and instability and drives abnormal mitosis. The clonal selection and expansion of these unstable mutant cells leads aggressive tumor phenotypes and an increased capacity for metastasis.
In this chapter, I have described potential links between hypoxia and centrosome function in the form of centrosome-associated DDR genes that are also down regulated by hypoxia, as well as other studies hinting at effects of hypoxia on centrosome function. I have also summarized the roles of centrosome aberrations and hypoxia in tumor progression and clinical prognosis. It seems appropriate to ask a fundamental question: is aberrant mitotic function enhanced or specific to hypoxic tumor cells and do these abnormalities lead to increased tumor CIN?

Specifically, *I hypothesized in this thesis that prostate tumor cells exposed to hypoxia would develop increased centrosome complex abnormalities.*

The second chapter (Chapter 2) describes my experimental studies on the effects of hypoxia on centrosome structure (outlined in Figure 1.6). I demonstrate that hypoxia induces an increase in aberrant CETN spots in mitotic DU145 prostate cancer cells. In further experiments I show that an increase in extra CETN spots also occurs in interphase cells in DU145, BPH1 and U2OS cells. Additionally, these CETN spots occur in response to hypoxia treatment, not as a byproduct of re-oxygenation. To identify the structures of the extra CETN spots observed in response to hypoxia, I immunofluorescently labelled cells for other centrosome and centriole markers (NEDD1, Cep135, and PCM1). The CETN spots colocalized with centriolar satellite marker PCM1, but not other centrosome markers. This led me to conclude that hypoxia induces an increase in centriolar satellites in mitotic DU145 and interphase BPH1, U2OS and DU145 cells. I also investigated the effects of hypoxia on centrosome complex gene expression, demonstrating that hypoxia deregulates several centrosome-associated genes, including CETN2 and 3, PCM1, and some of the Augmin Centrosome Complex (ACC) members.

In Chapter 3, I summarize the thesis findings and discuss the caveats of using the in-vitro system and the experiments performed using the DU145 prostate cancer cell line. I outline future work that can stem from the findings of this thesis, including the use of an isogenic cell line I have produced for the inducible knockdown of ACC genes. I end the last chapter of my thesis with a discussion of the clinical impact of my findings in the context of recent literature on centrosome aberrations and CIN in prostate cancer.
Figure 1.6: Summary of hypoxia studies carried out in this thesis. DU145 cells were used to demonstrate the validity of the hypoxia model, centrosome complex gene expression and centrosome aberrations in response to hypoxia in mitotic cells. Interphase cells of other cell lines were used for further IF studies on the effects of hypoxia on centrosome biology.
Chapter 2 - Hypoxia Leads to Aberrant Centrosome Function and Centriolar Satellites in Prostate Cancer Cells

Work presented in this chapter is being prepared for publication.

I intellectually drove and carried out the experiments described here, including the writing of the manuscript and producing the figures. Jenna Sykes carried out all the statistical analyses and created Figure S1; James Stewart created Figure 2.9. Robert Bristow was instrumental in guiding the direction of the project and final manuscript editing.
Abstract

Hypoxia and centrosome aberrations are both prominent features of prostate cancer and have been shown to be markers of poor clinical prognosis. Hypoxia has been linked to increased genomic instability and aggressive tumor phenotype. We have previously shown that hypoxia down-regulates the expression and function of DNA damage repair genes such as RAD51, BRCA1 and BRCA2; these proteins are also involved in centrosome function. Centrosome aberrations have also been directly linked to chromosomal instability (CIN). Despite the potential link between tumor hypoxia and centrosome aberrations, the effect of hypoxia on centrosome function during mitosis has not been directly investigated. In this study we demonstrate that hypoxia leads to an increase in centriolar satellites in mitotic DU145 cells. This was also observed in interphase in BPH1, U2OS and DU145 cells. Centriolar satellites were observed as extra CETN spots co-localizing to centriolar satellite marker PCM1, but not other centrosome markers. We also demonstrated the deregulation of centrosome complex genes in hypoxia treated DU145 cells. If centrosome aberrations are specific to hypoxic cancer cells, centrosome and mitosis targeting drugs could provide a high therapeutic ratio and better outcome for patients with high hypoxic tumor fraction.
2 Hypoxia Leads to Aberrant Centrosome Function and Centriolar Satellites in Prostate Cancer Cells

2.1 Introduction

Cells have acquired numerous ways to protect the integrity of the genome. These include sensing and repairing DNA damage (as part of the DNA damage response; DDR), initiating cell cycle checkpoints in response to DNA breaks, and exquisite control over mitosis given the need to maintain chromosomal stability during cell division. The centrosome is a cellular organelle essential for the maintenance of genomic stability. As the main microtubule organizing center (MTOC) of mammalian cells, the centrosome is crucial in orchestrating proper separation of genetic material to daughter cells during mitoses. Centrosomes are very complex organelles, consisting of a pair of orthogonally oriented microtubule-based cylindrical structures called centrioles, surrounded by the pericentriolar material (PCM) protein matrix. The structure and number of centrosomes are tightly regulated in normal cells, with the centrosome duplication cycle coupled to the DNA replication cycle (Nigg and Stearns, 2011; Pihan, 2013).

Centrosome dysfunction is frequently observed in malignant cells, whereby structural and numeric alterations are associated with chromosomal instability (CIN) in cancer cells. Centrosome amplification can occur as a pre-mitotic DNA damage-induced stress response during prolonged Chk1-dependent G2-phase arrest (Bourke et al., 2007; Dodson et al., 2004). DNA damage repair proteins RAD51, BRCA1, BRCA2, XRCC2, MDC1, and BRIT1 colocalize to centrosomes at various points through-out the cell cycle (Cappelli et al., 2011; Hsu and White, 1998; Rai et al., 2008). Depletion or mutation of these DNA repair genes enhances centrosome amplification (Dodson et al., 2004; Ko et al., 2006; Rai et al., 2008; Saladino et al., 2009; Shimada et al., 2010). ATM and ATR are PI3K-like kinases that are involved in double strand break (DSB) sensing and the DDR. These two kinases have complementary roles in limiting centrosome amplification in response to DNA damage (Bourke et al., 2007; Dodson et al., 2004).
Hypoxic sub-regions exist in all solid tumors. Increased levels of hypoxia portends an adverse clinical course due to radio-or chemoresistance and/or increased metastatic capability (Bristow and Hill, 2008). The acquisition of these resistant phenotypes is thought to be due, in part, to cellular adaptation and decreased DNA repair leading to a mutator phenotype and increased genetic instability. Many of the DDR proteins that accumulate at centrosomes are down-regulated at the transcriptional and translational level under hypoxia, including RAD51, BRCA1, BRCA2 and other DSB repair proteins (Chan et al., 2008, 2010). These hypoxia-mediated changes are functional losses as hypoxic cells can have up to a 3 times decrease in homologous recombination (HR) and an increased sensitivity to agents which are toxic to HR-defective cells including ionizing radiation (IR), Mitomycin C and PARP inhibitors (Chan and Bristow, 2010; Luoto et al., 2013b). Whether hypoxia mediates its effects on genetic instability by interfering with centrosome function and mitotic integrity is not known (Bristow and Hill, 2008; Kumareswaran et al., 2012; Meng et al., 2005; Taiakina et al., 2014).

As genetic disruption of DDR protein activity in cells can lead to centrosome aberrations, we speculated that hypoxia-induced functional losses in DNA repair may also lead to centrosome aberrations. Centrosome aberrations and subsequent mitotic abnormalities could then be an additional mechanism for observed hypoxia-induced genomic instability. Both CIN and tumor hypoxia, on their own, are prognostic for poor outcome in many tumors, including prostate cancer (Milosevic et al., 2012; Turaka et al., 2012; Vergis et al., 2008). Furthermore prostate cancers that have both increased hypoxia and increased genetic instability have a worse prognosis than having hypoxia or genetic instability alone (Lalonde et al., 2014).

Despite the potential role of hypoxia-induced mitotic abnormalities in cancer, there are currently only a handful of studies that have made links between hypoxia and centrosome function. Cells overexpressing miR-210, a microRNA up-regulated by the HIF-1α pathway and hypoxic conditions, have been shown to have increased centrosome amplification (Nakada et al., 2011). Additionally, Moser et al. (2013) demonstrated that prolyl-4-hydroxylase 1 (PHD1), which decreases in activity in response to hypoxia leading to HIF stabilization, is required for centrosome duplication and maturation. (Fandrey et al., 2006; Moser et al., 2013). It is unclear whether the decrease in PHD activity in response to hypoxia has a direct effect on centrosome function.
Herein, we describe the effects of hypoxia on centrosome function in mitotic and interphase prostate cancer cells under endogenous and exogenous DNA damage conditions. We describe a novel observation in which hypoxic exposure leads to altered centrosome gene expression and an increase in centriolar satellites. We discuss these findings in the context of genome instability based on potential centrosome dysfunction in both mitotic cells and daughter cells following cytokinesis.

2.2 Materials and Methods

2.2.1 Cell culture, hypoxic gassing and irradiation treatments

Cell lines were obtained and cultured as previously described (Meng et al., 2005) and checked frequently for lack of genetic variance by STR assays. In brief, cells were cultured in the following media: DU145 and PC3 - α-Modification of Minimum Essential Medium (α-MEM) with 10% fetal bovine serum (FSB); U2OS - Dulbecco's Modification of Eagle's Medium (DMEM)/Ham's F12 50/50 mix with 10% FSB; BPH1 - RPMI-1640 with 10% FSB. Cell cultures were incubated at 37 °C in 5% CO2. Asynchronous, logarithmically growing cell populations were used in all experiments. For hypoxic/anoxic treatment cells were gassed in microenvironmental chambers (Don Whitley Scientific workstation, model H35) with 0.2% O2 for 72 hours for hypoxia or <0.01% O2 for 16 hours for anoxia. Gassing always included 5% CO2 balanced with N2. In the case of oxic treatment, cells were maintained in atmospheric oxygen (21% O2). To induce exogenous DNA damage, cells were treated with ionizing radiation (IR) using a Cesium-137 irradiator (Gammacell® 40 Exactor, Best Theratronics) at room temperature. Following IR treatment, cellular aliquots were taken for study during re-oxygenation (21% O2).

2.2.2 Immunofluorescence microscopy

Immunofluorescence (IF) imaging was used for centrosome analyses. Cells were grown on square 18mm glass coverslips (N 1.5) within the wells of 6-well tissue culture dishes. Following treatment, cells were fixed with 100% methanol at -20°C for 5-10 minutes. Cells were blocked in 0.2% cold water fish skin gelatin (G7765 Sigma) followed by incubation with
appropriate primary antibodies for 60-75 minutes at the following concentrations, diluted in blocking solution: 1/1000 mouse anti-centrin (Millipore clone 20H5, 04-1624); 1/2000 rat anti-α-tubulin (Abd Serotec, MCA77G); 1/1000 rabbit anti-pericentrin (Abcam, ab4448); 1/1000 goat anti-pericentrin (Santa Cruz clone N-20, sc-28143); 1/1000 mouse anti-NEDD1 (Abcam, ab57336); 1/250 rabbit anti-NEDD1 (Santa Cruz clone H-300, sc-67263); 1/1500 rabbit anti-PCM1 and 1/1000 rabbit anti-cep135 antibodies (received from Dr. Laurence Pelletier and previously validated in Lawo et al., 2009). After washing with blocking solution, cells were incubated with the appropriate Alexa Fluor conjugated secondary antibodies (Invitrogen) diluted 1/500 in block solution. Cells were also stained with 0.1 µg/ml 4’,6-diamidino-2-phenylindole (DAPI) for DNA content and visualization. Coverslips were then mounted on slides with Vectashield anti-fade solution (Vector Laboratories) and sealed with clear nail polish.

Slides were imaged using the Olympus IX81 Spinning Disk Confocal Microscope, in wide-field setting, with the following objective lenses, depending on experiment: LUCPlanFL N 40x/0.60 RC3, PlanApo 60x/1.42 Oil, UPlanSApo 100x/1.4 Oil. Approximately 30-60 mitotic or interphase cells per treatment were imaged to produce z-stacks consisting of 0.2-0.3 µm optical sections. In the case of some experiments, only a few representative images were acquired, while quantification was performed visually on the microscope. For image analysis, maximum intensity projections were produced from z-stacks using Image Pro Plus software. Cells were determined to be mitotic or interphase based on DAPI staining, whereby cells with a DNA pattern consistent with condensed chromosomes were classified as mitotic.

2.2.3 Cell growth and cell cycle analysis

Growth curves for cell proliferation analyses under oxia and hypoxia used a TC-20 automated cell counter (Bio-Rad) to quantify cell proliferation over time. Cell cultures were grown in 10cm tissue culture dishes and gassed with hypoxia or maintained in atmospheric oxygen conditions. Cell dishes were trypsinized and counted in triplicate at 0 hours (prior to gassing) and then at 24, 48 and 72 hours of treatment. After 72 hours in hypoxia, cells were re-oxygenated and allowed to continue to proliferate further for up to 72 hours following re-oxygenation.
For flow cytometry analyses, cells were grown in 10cm dishes and gassed in hypoxia or oxia for 72 hours, then fixed with 75% ethanol at -20°C for at least 20 min, immediately post gassing (no re-oxygenation), or 24, 48, and 72 hours post re-oxygenation. Cells were treated with 10µM 5-bromo-2'-deoxyuridine (BrdU) for 1 hour prior to fixation. Cells were prepared for labelling with anti-BrdU antibodies as previously described in (Chan et al., 2008). Antibodies used include: mouse anti-BrdU conjugated to Alexa 647 (clone MoBU-1, Invitrogen, B35140) for S phase analysis (nucleic acid incorporation) and Anti-Phospho-Histone H3 (Ser10) (anti-H3P) conjugated to Alexa 488 (clone D2C8, Cell Signaling, 3465S) for mitotic cell fraction. Cells were stained with propidium iodide (PI) for DNA content. Multi-parameter (PI, BrdU and H3P) cell cycle analysis was performed on a Canto II Becton Dickinson (BD) analyzer with FacsDiva v.6.1.3 software.

2.2.4 Clonogenic survival assays

Cells were treated with hypoxia, anoxia or oxia as above and irradiated with 0-15 Gy under oxic, hypoxic, or re-oxygenation (30 min and 48 hour) gassing conditions. Immediately post IR treatment, cells were trypsonized at room temperature, counted with the TC-20 automated cell counter (Bio-Rad) and plated at appropriated densities, in triplicate, in 6-well tissue culture dishes (Greiner). Cells were incubated for 9 days for colony formation before fixation and staining with 1% Methylene Blue in 50% ethanol. Colonies consisting of 50 or more cells were scored and the plating efficiency was calculated as the average number of colonies in 3 wells divided by the number of cells seeded in each well. The surviving fraction was calculated as the plating efficiency of irradiated cells divided by the plating efficiency of non-irradiated cultures and plotted as a function of dose on a semi-logarithmic plot. A linear quadratic model was fitted to the data for plotting purposes as previously described (Harding et al., 2013). Oxygen enhancement ratios (OERs) were calculated by dividing the radiation dose that produces a surviving fraction of 0.1 for hypoxic (or anoxic) cells by the radiation dose required to produce the same 0.1 surviving fraction in control oxic cells.

2.2.5 RNA extraction and real-time quantitative PCR (qRT-PCR)

Cells were grown on 10cm tissue culture dishes and treated with hypoxia or oxia for 72 hours. Total RNA was extracted using TRI Reagent (Sigma-Aldrich) as previously described (Meng
et al., 2005). Isolated RNA was treated with DNase I (Invitrogen) to eliminate DNA contamination and purified again with phenol/chloroform extraction followed by NaOAc/ethanol precipitation. The products were reverse transcribed with Maxima Reverse Transcriptase (Thermo Scientific) and oligo dT<sub>16</sub> and dN<sub>6</sub> primers. cDNA was then amplified for target genes using the appropriate primers (synthesized by Sigma-Aldrich, Table S1) with the SYBRGreen assay PCR master mix, or the appropriate TaqMan probes (Table S2) and TaqMan Master mix (Applied Biosystems). PCR reactions were detected using an Applied Biosystems StepOne Plus Realtime PCR system with StepOne v2.2.2 software. The fluorescence intensity threshold was set automatically with the software and the reaction cycle-threshold (C<sub>T</sub>) was obtained. Analysis was performed using the comparative ΔΔC<sub>T</sub> quantification method.

2.2.6 Western Blot Analysis

Protein expression was analyzed using Western blotting as previously described (Kumareswaran et al., 2012). Briefly, 10% bis-acrylamide gel wells were loaded with 50 µg of whole-cell lysate for each sample. Samples were resolved with SDS-PAGE performed at room temperature, at 100-120V for 2 hours and then transferred onto PVDF membranes (Life Sciences) at 100mA for 1 hour at room temperature. Staining of the membrane with Ponceau solution confirmed optimal protein transfer. Membranes were then blocked using Odyssey blocking buffer (LI-COR Biosciences) for 1 hour at room temperature and incubated with primary antibodies overnight (~16 hours) at 4°C. Primary antibodies were diluted in 50/50 blocking buffer and Tris-Buffered Saline and Tween 20 (TBS-T) buffer: 1/200 rabbit anti-RAD51 (Santa Cruz); 1/5000 mouse anti-β-actin (Sigma, clone AC-74). Following washes with TBS-T buffer to remove unbound primary antibodies, membranes were incubated with the appropriate secondary antibodies diluted to 1/2000 in 50/50 blocking buffer/TBS-T buffer for 1 hour at room temperature: anti-rabbit Alexa Fluor 680 (Invitrogen) and anti-mouse IRdye-800 (Rockland Immunochemicals). Membranes were imaged and analyzed on the LI-COR Odyssey machine.
2.2.7 Statistical Analysis

Differences in centrosome counts and clonogenic survival were statistically analyzed as previously described (Kumareswaran et al., 2012). All centrosome count statistical analyses were performed using a negative binomial model with random effects. Although count data is generally modeled with a Poisson regression, the negative binomial model was used because the high frequency of zero counts encountered in our data violates the assumptions of a Poisson regression. A random intercept term was used to account for the correlation between counts within an experiment. In the experiment testing the effect of hypoxia and IR on mitotic DU145 cells, to save on statistical power, we tested for the statistical interaction between condition (hypoxia and oxia) and IR dose (NIR, 5gy, 10gy). Dose was modeled in a linear way with NIR<5Gy<10Gy. Paired two-tailed t-tests were performed to determine the effect of hypoxia with each dose individually, while using the Bonferroni adjustment for multiple comparisons. In the case of testing the effects of hypoxia on centrosome aberrations in interphase cells of U2OS, PC3, DU145 and BPH1 cell lines, the models were run separately for each cell line. For clonogenic survival statistical analysis, we fit a linear quadratic regression model as previously described (Franken et al., 2006). The intercept term was fixed to remain at 1 for all treatment groups. To determine if any treatment caused survival to decrease at a faster rate compared to another treatment, a 2-df Wald test was calculated testing for the difference in both the linear and quadratic terms simultaneously. All analyses were performed using SAS version 9.3 and the glmmADMB package in the open-source software R version 3.0.1. All tests are two-sided and assessed at a significance level of 0.05.

2.3 Results

2.3.1 IR-Induced exogenous DNA damage increases centrosome aberrations in mitotic DU145 prostate cancer cells.

To study the effect of exogenous DNA damage on centrosome aberrations in DU145 cells under oxic conditions, cells were fixed 48 or 72 hours post IR treatment, and IF-labeled for the centrosome components centrin (CETN) and pericentrin (PCNT). A wide range of different
centrosome aberrations were observed in DU145 cells following exposure to IR (Figure 2.1A). This included: PCNT spots that did not colocalize with CETN; extra centrosomes (observed as >2 PCNT and CETN colocalized); CETN spots that do not colocalize to PCNT; centrosomes with abnormally intense PCNT staining; and, centrosomes with >2 CETN spots (which might be over-duplicated centrioles). In addition to centrosome aberrations, IF labelling for mitotic spindles (α-tubulin) revealed a series of mitotic spindle aberrations.

Aberrations were initially scored by counting >2 PCNT spots, regardless of co-localization with CETN: a scoring method that quantifies structural and numerical centrosome aberrations. When treating with 1 to 10Gy, the percent of cells with centrosome aberrations increased proportionally with increasing radiation dose (Figure 2.1B). Aberrations were more likely to occur at 48 hours post IR treatment as compared to 72 hours. A maximum aberration count was observed at 10 Gy, with more than 70% of cells having >2 PCNT signal spots. Not only did the percent of cells with centrosome aberrations increase, but the number of extra PCNT spots per cell increased with increasing IR dose, suggesting more complex centrosome damage with increased DNA damage (Figure S1).
Figure 2.1: Centrosome aberrations in response to radiation treatment in mitotic DU145 prostate cancer cells. (A) Normal mitotic cell (left) with two centrosomes, immunofluorescently labelled for pericentrin (PCNT), centrin (CETN), and DAPI for DNA visualization. Right panel shows a mitotic DU145 cell 48 hours post treatment with 5Gy, with an accumulation of a variety of centrosome aberrations (arrows). Scale bars represent 5µm. (B) Dose response of aberrations accumulated post IR treatment in mitotic DU145 cells. Cells were treated with various IR doses and fixed 48 or 72 hours post treatment. Aberrations were more likely to occur 48 hours post treatment (p<0.01). Mean percent of cells with aberrations (>2 PCNT spots) ± SEM are plotted. (N=2)
2.3.2 The DU145 prostate cancer cell line as a model to study centrosome aberrations under hypoxia.

To study the effects of hypoxia on centrosome aberrations in a prostate cancer cell line, we first studied the proliferation and cell cycle responses of the DU145 prostate cancer cells following exposure to hypoxia. We did not observe proliferative differences for cells under hypoxia versus oxia in the presence of re-oxygenation (Figure 2.2A). Cell cycle distribution analyses (Figure 2.2B and S2) suggested that following 72 hours of hypoxia treatment (i.e. 0 hours re-oxygenation), the G1-phase fraction was larger in hypoxic cells relative to oxic cells, suggesting an initial G1 accumulation in response to hypoxia. However, by 48 hours after re-oxygenation, the cell cycle distribution returned to normal due to adaption or cell cycle re-initiation.

To show that we achieved radiobiological hypoxia, we determined oxygen enhancement ratios (OER) for relative clonogenic survival under hypoxic and anoxic conditions (Figure 2.3A). Not surprisingly, clonogenic survival in cells irradiated 30 min or 48 hours post re-oxygenation (e.g. after anoxia or hypoxia pre-treatment), did not significantly differ from cells maintained in oxic conditions. Plating efficiency remained similar in all conditions. However, cells irradiated under 0.2% O2 (hypoxia) or <0.01% O2 (anoxia) showed increased survival, with anoxic cells having the highest radiation resistance. The OER was calculated to be 2.5 for anoxia exposure and 1.5 for hypoxia exposure consistent with previous work from our laboratory (Chan et al., 2008). We also assessed the expression of the hypoxia/HIF1α-inducible genes, VEGF and CA9, using qRT-PCR following hypoxia in DU145 cells. Both genes were highly up-regulated after exposure to 72 hours of hypoxia; mRNA levels of VEGF increased by ~10 fold and ~80 fold for CA9 (Figure 2.3B).

Consistent with previous data from our laboratory, expression of the centrosome-associated HR protein, RAD51, was decreased following hypoxia (Meng et al., 2005) with reductions to ~65% of that of oxic levels after 72 hours of hypoxia gassing (Figure 2.3 C and D). Taken together, we conclude that the hypoxic response is intact in DU145 cells with acquired radioresistance (i.e. increased OER) and HIF1α-associated gene expression; yet, minimal effects on cell proliferation or cell cycle arrest.
Figure 2.2: Proliferation and cell cycle response of DU145 cells to hypoxia (0.2% O₂) treatment. (A) Growth curves of DU145 cells grown under hypoxic or normoxic (21% O₂) conditions, followed by re-oxygenation after 72 hours of treatment in the case of hypoxic cells. (N=3; means ± 95% confidence intervals are plotted) (B) Cell cycle analysis of DU145 cells post hypoxia pre-treatment. Cells were pre-treated with 72 hours of 0.2% O₂ and analyzed for cell cycle distribution at 0, 24 and 48 hours post re-oxygenation. (N=2; means are plotted)
Figure 2.3: Biological response to hypoxia and anoxia treatment in DU145 cells. (A)
Clonogenic survival assays of DU145 cells pretreated with hypoxia (72 hours of 0.2% O₂) or anoxia (16 hours of <0.01% O₂). Cells in the hypoxia and anoxia groups were irradiated under hypoxic/anoxic conditions, while all other groups were irradiated under oxic conditions with 30 minutes or 48 hours of re-oxygenation prior to radiation treatment. Cells irradiated in anoxic or hypoxic conditions were more resistant to radiation than all other groups (p<0.0001). Anoxic (16h) cells were more resistant than hypoxic (72 hours) cells (p=0.0019). The mean surviving fractions ± SEM are plotted. N=2
Figure 2.3: continued... (B) Up-regulation of mRNA expression of hypoxia-inducible genes VEGF and CA9 post hypoxia pre-treatment, based on qRT-PCR analysis. Means of change in mRNA expression ± SEM are plotted. N=2 (C) Change in protein expression of centrosome-associated DNA-damage repair gene RAD51 post hypoxia pre-treatment, based on Western blotting analysis. Mean of change in protein expression is plotted. N=2 (D) Representative Western blot of RAD51 expression in oxia and hypoxia treated DU145 cells.
2.3.3 Centrosome aberrations in mitotic DU145 cells in response to exogenous DNA damage after hypoxia treatment

To determine whether hypoxia can modulate centrosome aberrations following exogenous DNA damage, DU145 cells were pre-treated with 72 hours of hypoxia and irradiated immediately following re-oxygenation and scored for centrosome abnormalities. Mitotic DU145 cells exhibited a wide range of centrosome aberrations under oxia and hypoxia. The most commonly observed aberrations included extra centrosomes, extra PCNT spots, extra CETN spots and multipolar mitoses (Figure 2.4). Quantification of these aberrations demonstrated that increasing DNA damage led to increasing percentage of cells with aberrations for all aberrations scored (Figure 2.5). Gassing treatment was not a significant factor for percent of cells with extra centrosomes, extra PCNT spots, and multipolar mitoses. However, in contrast to cells gassed under oxia, we observed a significant increase in the percent of hypoxic cells with extra CETN spots. When cells were irradiated under oxic conditions, the effect of dose was significant (p<0.0001); but this was not the case for hypoxic cells (p=0.78) given that hypoxia induced extra CETN spots even in non-irradiated cells. In fact, when DU145 cells were pre-treated with hypoxia and fixed 48 hours post re-oxygenation in the absence of exogenous DNA damage, the percent of cells with extra CETN spots increased dramatically (Figure 2.5B).
Figure 2.4: Representative IF images of the different types of centrosome aberrations observed in mitotic DU145 cells in response to radiation and hypoxia treatment. (A,B,C) are stained to show PCNT, CETN and DNA and (D) is stained to show PCNT, α-tubulin and DNA. Arrows point to aberrations. Scale bar represents 5µm. (A) Extra centrosomes observed as extra spots of colocalized PCNT and CETN. (B) Extra CETN spots, not colocalized to PCNT. (C) Extra PCNT spots, not colocalized to CETN. (D) Multipolar mitotic spindle (four spindle poles in this case).
Figure 2.5: Centrosome aberrations in mitotic DU145 cells in response to radiation treatment following oxia and hypoxia pre-treatment. Cells were treated with hypoxia or oxia, and radiation (NIR (i.e. 0Gy), 5Gy and 10Gy); fixed 48 hours post re-oxygenation and radiation. Shown are means (± SEM, N=3) of percent of cells with (A) extra centrosomes, (B) extra CETN spots, not colocalized to PCNT, (C) extra PCNT spots, not colocalized to CETN, and (D) multipolar (>2) mitotic spindles. Modeling the data with a negative binomial regression revealed that radiation dose, but not gassing treatment, has a statistically significant effect on percent of cells with aberrant spindles (p=0.001), extra PCNT spots (p<0.0001) and supernumerary centrosomes (p<0.0001). In the case of extra CETN spots, both radiation dose and gassing treatment have a significant effect (p<0.0001). Paired, two-tailed t-tests, performed with the Bonferroni adjustment, demonstrated a significant difference in % of cells with >2 spindle poles in hypoxic non-irradiated cells (p=0.00046).
2.3.4 Increased frequency of extra CETN spots in response to hypoxia treatment is not a cell-line specific effect.

Next, we determined if the increase in cells harboring extra CETN spots in response to hypoxia treatment was a cell line-specific effect. We treated four cell lines with hypoxia and analyzed occurrence of extra CETN spots in interphase cells. The cell lines included prostate cancer cells (DU145 and PC3), an SV40-immortalized prostate epithelial cell line (BPH1), and a human bone osteosarcoma cell line (U2OS). Interphase cells of DU145, U2OS and BPH1 all had statistically significant increases in the frequency of cells with extra CETN spots in response to hypoxia treatment (Figure 2.6). Other centrosome aberrations were not significantly different in hypoxia treated cells, with the exception of >2 CETN spots within centrosomes in BPH1 cells (Figure S3).

2.3.5 Extra CETN spots most likely represent accumulation of centriolar satellites.

To attempt to understand the biological significance of the observed extra CETN spots, we analyzed if these structures co-localize to other centrosomal proteins. The extra CETN spots in hypoxic cells colocalized to the centriolar satellite protein PCM1 (Pericentriolar material 1), but not to the centrosomal proteins cep135 and NEDD1 (Figure 2.7). CETN spots that did not colocalize to PCM1 were observed to be rare (Figure 2.8). We conclude that the extra CETN spots represent centriolar satellites.

To determine if the appearance of extra CETN/PCM1 colocalized spots in response to hypoxia is solely a product of re-oxygenation, we treated cells with hypoxia and fixed either without re-oxygenation or 30 min following re-oxygenation (Figure 2.8). The significant increase in the percent of cells with extra CETN/PCM1 spots was independent of re-oxygenation. Supernumerary centrosomes occurred very rarely in oxic and non-re-oxygenated cells, but became significant (though still low) in cells re-oxygenated for 30 min. Extra CETN spots within centrosomes (potential extra centrioles) were significantly increased in both hypoxia treatments; again independent of re-oxygenation.
Figure 2.6: continued on next page...
Figure 2.6: Extra CETN spots in response to hypoxia treatment in four cell lines (U2OS, PC3, DU145, and BPH1). Cells were treated with hypoxia and fixed 48 hours post re-oxygenation. (A) The percent of interphase cells with extra CETN spots that do not colocalize with PCNT was quantified in IF-labelled cells. Shown are means ± SEM, N=3. Asterisks denote a statistically significant difference between oxia and hypoxia treated cells. (*) p<0.01; (**) p<0.001; (***) p<0.0001 (B) Representative images of interphase cells with extra CETN spots, not colocalized to PCNT. (i) U2OS (ii) PC3 (iii) DU145 and (iv) BPH1. Arrows point to extra CETN spots. Scale bar represents 5µm.
Figure 2.7: Extra CETN spots in response to hypoxia treatment in interphase DU145 cells colocalize with PCM1 but not cep135 or NEDD1. Cells were treated with hypoxia and fixed 48 hours post re-oxygenation. Cells were analyzed for CETN spots that did not colocalize with PCNT to observe colocalization to other centrosome markers.
**Figure 2.7: continued...** (A) Representative images of a cell with CETN spots colocalizing with PCM1 (yellow) but not PCNT. (Arrows point to CETN spots) (B) Same images, with fluorescence channels shown separately in black and white for better visualization of CETN and PCM1 colocalization. (C) Representative images of cells with extra CETN spots, not colocalizing to cep135 or NEDD1. Scale bars represent 5µm.
Figure 2.8: Centrosome aberrations in response to hypoxia treatment in interphase
DU145 cells at various time points post re-oxygenation. Cells were treated with hypoxia and fixed without re-oxygenation (0h) or 30 min post re-oxygenation. IF-labelling was done for visualization of DNA, CETN, PCNT and PCM1. The percent of interphase cells with extra centrosomes, extra centrioles, and extra CETN spots was quantified (CETN spots stratified based on colocalization with PCM1). Shown are means ± SEM, N=3. Asterisks denote a statistically significant difference between treatments. (*) p<0.05; (***)) p<0.0001
2.3.6 Hypoxia modulates centrosome complex gene expression.

Given our observations of differential CETN staining patterns under hypoxia, we hypothesized that hypoxia may effect centrosome function by modifying centrosome complex gene expression. To test this hypothesis, we performed real-time qPCR analysis of centrosome-associated genes in hypoxic cells to compare mRNA expression relative to oxic cells. DU145 cells showed differential centrosome gene mRNA levels under hypoxia (Figure 2.9). Many of the analyzed genes had up-regulated expression under hypoxia, while others were down-regulated. Notably, centrosomal proteins CETN2, CETN3, PCNT, SASS6, NEDD1 and PCM1 were found to be increased in mRNA expression under hypoxia. Conversely, Aurora kinase B and some members of the Augmin centrosome complex had decreased levels of mRNA expression in hypoxic cells.
Figure 2.9: continued on next page…
Figure 2.9: Changes in mRNA expression in response to hypoxia treatment. qPCR analysis was performed on cDNA from DU145 cells treated with 72 hours of 0.2% O₂. Shown are values of individual experiments (N=2), where experiment 1 is represented with black squares and experiment 2 with red triangles. To better represent fold change in mRNA levels, genes were grouped in plots based on effect of hypoxia on mRNA expression relative to oxia: (A) decreased expression or (B) increased expression.

2.4 Discussion

Previous studies in our lab and others have demonstrated that intratumoral hypoxia leads to increased genomic instability and aggressive tumor phenotypes (Kumareswaran et al., 2012; Taiakina and Bristow, 2012). Additionally, centrosome aberrations are a common feature of prostate cancer and occur more frequently in poorly differentiated tumors (Pihan et al., 2001). Recent studies have demonstrated that hypoxia down-regulates centrosome-associated genes, and that in-vitro depletion or mutation of these genes causes increases in centrosome aberrations (Dodson et al., 2004; Ko et al., 2006; Rai et al., 2008; Saladino et al., 2009; Shimada et al., 2010). Despite this enticing potential mechanistic link between hypoxia leading to genomic instability via centrosome aberrations, until now there have been no studies directly observing centrosome function in hypoxic cells. Our aim was to investigate how hypoxia modulates centrosome function in prostate cancer cells.

We demonstrated that DU145 prostate cancer cells are a germane model to study hypoxia in the absence of cell cycle changes. The cells responded to 72 hours of 0.2% O₂ gassing with no observed growth delay, increased hypoxia-induced gene expression, and achieved an OER similar to our previous experiments in other cell lines (Chan et al., 2008).

The most important finding of our studies was an increase in extra CETN spots observed in hypoxia treated cells (Figure 2.5B). The extra CETN spots observed in response to hypoxia pre-treatment did not colocalize to PCNT and therefore represented a unique centrosome aberration. To determine if the effect was cell-line specific, we looked at the effect of hypoxia
pre-treatment on extra CETN spots in interphase cells in three other cell lines (U2OS, BPH1 and PC3). A significant increase in extra CETN spots was found in interphase DU145, U2OS and BPH1 cell lines (Figure 2.6). This demonstrated to us that the effect is not specific to only DU145 cells, and is likely a generalized and specific response to hypoxia in many cancer cell types.

To further determine the biological significance of the increase in extra CETN spots in response to hypoxia, we analyzed for co-localization to other centrosome markers to determine if they represented centrosomes, centrioles, centrosome fragmentation or something else entirely. Co-staining for CETN with centrosome markers NEDD1 or Cep135 revealed that the CETN spots do not colocalize to these centrosome components and therefore likely do not represent supernumerary centrosomes (Figure 2.7C). Instead, the majority of the extra CETN spots colocalized with the centriolar satellite protein PCM1 (Figure 2.7 A and B). This is an interesting finding as a recent study by Löffler, et. al. (2013) observed centrosome amplification in response to DNA-damage through initial formation of centriolar satellites. These were identified as \textit{de novo}-generated atypical CETN spots co-localizing with PCM1; but not with other centrosome markers. The authors concluded that excessive formation of centriolar satellites, which then act as assembly platforms for centrosomal proteins, leads to centrosome amplification (Löffler et al., 2013). We speculate that if hypoxia also leads to increased formation of centriolar satellites, this could contribute to centrosome amplification at later time points following hypoxia. This is a testable hypothesis for future studies as re-oxygenation for 30 minutes increased the percent of cells with extra CETN spots and slightly elevated the number of supernumerary centrosomes (Figure 2.8). Cells may continue to generate the CETN-containing bodies even after re-oxygenation. If the CETN-containing bodies are in fact centriolar satellites, as PCM1 staining indicates, we expect that cells should also acquire supernumerary centrosomes at later time points as the centriolar satellites mature into centrosomes. Further studies are needed to investigate this possibility.

One potential mechanism for our observations is the finding that hypoxia modifies the expression of centrosome complex genes, in addition to our previous data linking hypoxia to decreased expression of the HR- and centrosome-associated protein, RAD51 (Chan et al., 2008). The observed increase in expression of PCNT, CETN2, CETN3 SASS6, NEDD1, and PCM1, could lead to altered protein stoichiometry and centrosome abnormalities. Importantly,
we also observed decreased mRNA expression of the Augmin centrosome complex (ACC) members HAUS 2, 4, 5, 7 and 8. Genetic studies (i.e. knock-down of these genes with RNAi) caused disruption in centrosome integrity and microtubule nucleation (Lawo et al., 2009). Hypoxia-induced deregulation of centrosome-associated genes may be leading to the observed increase in centrosome aberrations.

In conclusion, we have demonstrated that hypoxia leads to increased centriolar satellite formation and centrosome gene deregulation. This provides a mechanistic link between hypoxia and genomic instability. Centrosome function may be an attractive therapeutic target for cancer treatment if it can be used to target hypoxic cells. This may be accomplished by taking advantage of hypoxia-induced centrosome aberrations that can be amplified and pushed to the point of cell death.
2.5 Supplementary Tables and Figures

Table S1: List of primers for SYBRGreen qPCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expected product size (bp)</th>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA9</td>
<td>273</td>
<td>FOR</td>
<td>5'-GCTTGGAAGAAATCGCTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV</td>
<td>5'-TCACTCGCCCATTCAGAAG-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>261</td>
<td>FOR</td>
<td>5'-GGGCAGAATCATCAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV</td>
<td>5'-CATCTCTCTCCATGCTGCC-3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>386</td>
<td>&quot;243&quot;</td>
<td>5'-CGTGCGGGTCTTTTCACCAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;244&quot;</td>
<td>5'-AATTATGGCGAGACTGAACGTC-3'</td>
</tr>
<tr>
<td>HSP90AB1</td>
<td>210</td>
<td>FOR</td>
<td>5'-AGCAACAGGATGATGACAG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV</td>
<td>5'-CCAAATATCGGTGATGAG-3'</td>
</tr>
<tr>
<td>RPL13A</td>
<td>232</td>
<td>FOR</td>
<td>5'-AAACCCGGATGACACCAAC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV</td>
<td>5'-AGGCAAACCTTTTTGATGAG-3'</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>248</td>
<td>FOR</td>
<td>5'-AAATGTGTTGAGGAGCCGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REV</td>
<td>5'-CCTCAGCCAGTAACCGTAG-3'</td>
</tr>
</tbody>
</table>

Table S2: List of probes for TaqMan qPCR analyses.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>TaqMan Assay ID</th>
<th>Gene Symbol</th>
<th>TaqMan Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>AURKB</td>
<td>Hs00945858 g1</td>
<td>HAUS8</td>
<td>Hs00928622 m1</td>
</tr>
<tr>
<td>BUB1</td>
<td>Hs01557695 m1</td>
<td>MADIL1</td>
<td>Hs00269119 m1</td>
</tr>
<tr>
<td>CEP120</td>
<td>Hs00537880 m1</td>
<td>MAD2L2</td>
<td>Hs01057448 m1</td>
</tr>
<tr>
<td>CEP152</td>
<td>Hs00942690 m1</td>
<td>NEDD1</td>
<td>Hs01110695 m1</td>
</tr>
<tr>
<td>CEP164</td>
<td>Hs00209047 m1</td>
<td>NUMA1</td>
<td>Hs01096494 m1</td>
</tr>
<tr>
<td>CEP192</td>
<td>Hs00259717 m1</td>
<td>PCM1</td>
<td>Hs00196390 m1</td>
</tr>
<tr>
<td>CEP57</td>
<td>Hs01100372 m1</td>
<td>PCMNT</td>
<td>Hs00195774 m1</td>
</tr>
<tr>
<td>CEP70</td>
<td>Hs00225806 m1</td>
<td>PLK2</td>
<td>Hs01573405 g1</td>
</tr>
<tr>
<td>CETN2</td>
<td>Hs00942570 g1</td>
<td>PLK3</td>
<td>Hs01085655 g1</td>
</tr>
<tr>
<td>CETN3</td>
<td>Hs00189076 m1</td>
<td>PLK4</td>
<td>Hs00179514 m1</td>
</tr>
<tr>
<td>HAUS1</td>
<td>Hs00937737 g1</td>
<td>SASS6</td>
<td>Hs01591578 m1</td>
</tr>
<tr>
<td>HAUS2</td>
<td>Hs01077859 m1</td>
<td>VEGF</td>
<td>Hs00900055 m1</td>
</tr>
<tr>
<td>HAUS3</td>
<td>Hs00989405 m1</td>
<td>CA9</td>
<td>Hs00154208 m1</td>
</tr>
<tr>
<td>HAUS4</td>
<td>Hs00250407 m1</td>
<td>HPRT1</td>
<td>Hs02800695 m1</td>
</tr>
<tr>
<td>HAUS5</td>
<td>Hs00294007 m1</td>
<td>HSP90AB1</td>
<td>Hs04194349 g1</td>
</tr>
<tr>
<td>HAUS6</td>
<td>Hs01550439 g1</td>
<td>RPL13A</td>
<td>Hs01926559 g1</td>
</tr>
<tr>
<td>HAUS7</td>
<td>Hs03831989 m1</td>
<td>YWHAZ</td>
<td>Hs00852925 sH</td>
</tr>
</tbody>
</table>
**Figure S1:** Distribution of centrosome aberrations within IR-treated cells. Cells were treated with different radiation doses and fixed 48 or 72 hours post treatment. The number of PCNT spots was quantified. The plot demonstrates the increase in extra PCNT spots in individual cells with increasing IR dose, indicating increasing aberration complexity.

**Figure S2:** Representative images of cell cycle analysis in oxic, hypoxic and re-oxygenated cells. Cells were stained with PI, anti-BrdU and anti-H3P for visualization of G1 (light blue), S (green), G2 (dark blue) and M -phase (red) cells.
Figure S3: Centrosome aberrations in response to IR and hypoxia treatment. Interphase U2OS, PC3, BPH1 or DU145 cells were pre-treated with hypoxia, irradiated immediately post re-oxygenation and fixed for IF 48 post radiation. Asterisks denote a statistically significant difference between oxia and hypoxia treated cells. (*) p<0.05; (**) p<0.001; (***) p<0.0001
Chapter 3

3 Discussion and Future Work
3.1 Summary of Findings and Discussion

In Chapter 1 of this thesis, I outlined how the centrosome plays a major role in maintaining chromosome integrity during mitosis, and centrosome aberrations correlate to poor prognosis in prostate cancer patients. I also discussed the role of hypoxia in inducing genomic instability and the clinical relevance for prostate cancer.

To further understand the connection between genomic instability, hypoxia, and centrosome function, in Chapter 2 I investigated the effects of hypoxia treatment on centrosome aberrations in prostate cancer cells. I attempted to support or refute the hypothesis that hypoxia-induced centrosome aberrations occur as an additional mechanism by which hypoxia drives genomic instability in cancer cells. My experiments in mitotic DU145 prostate cancer cells and interphase cells of multiple cell lines, have demonstrated that hypoxia leads to an accumulation of centrosome aberrations and deregulation of centrosome complex genes.

Löffler, et. al. (2013) revealed how the mechanism of centrosome amplification in response to DNA-damage occurs through initial formation of centriolar satellites, identified as de novo-generated atypical CETN spots colocalizing with PCM1 but not with other centrosome markers. The authors concluded that excessive formation of centriolar satellites, which then act as assembly platforms for centrosomal proteins, leads to centrosome amplification (Löffler et al., 2013). Similarly, I have demonstrated an increase in extra CETN spots in response to hypoxia and found them to colocalize with PCM1, but not other centrosome markers. Additionally, this phenotype is not cell line dependent and occurs in interphase cells of at least three cell lines. The observation is consistent with the original hypothesis that hypoxia leads to increased centrosome aberrations. If the increase in centriolar satellites post hypoxia treatment results in centrosome amplification in a manner similar to the mechanism described by Löffler, et. al. (2013), then centriolar satellites may be a mechanistic link between hypoxia and increased CIN.

The experiments I carried out to analyze centrosome aberrations in hypoxic cells were performed in asynchronous cell cultures. However, to determine the specific effects of hypoxia on mitotic cells, further studies would carry out an experiment that uses early G2-synchronized
cell cultures at hypoxic gassing with live-cell or multiple time-point studies using fixed cells, to track DNA damage-induced centrosome aberrations in cells that have undergone first mitosis under hypoxic conditions. By synchronizing cells and following the cell cycle throughout treatment, it could be determined at what point in the cell cycle hypoxic treatment induces accumulation of centriolar satellites.

Our laboratory has previously shown that hypoxia down-regulates the expression of a number of centrosome-associated DDR genes including RAD51, BRCA1, BRCA2 (Chan et al., 2008; Meng et al., 2005). Additional studies have shown that depletion or mutation of these genes enhances centrosome amplification (Dodson et al., 2004; Ko et al., 2006; Rai et al., 2008; Saladino et al., 2009; Shimada et al., 2010). In agreement with this data, I have demonstrated that hypoxia deregulates mRNA expression of a number of centrosome complex genes, including up-regulation of CETN2, CETN3 and PCM1 and down-regulation of some members of the Augmin centrosome complex. This result supports the observation of increased CETN and PCM1 spots in hypoxia-treated cells, and potentially represents the mechanism of increased centriolar satellites. Additionally, the observed effects of hypoxia on centrosome function were not the result of re-oxygenation, as we observed gene deregulation and increases in centriolar satellites even in cells fixed or lysed without re-oxygenation. These findings are consistent with the previously described literature on hypoxia-induced deregulation of centrosome associated genes (e.g. RAD51, BRCA1, etc.) with the resulting effects on centrosome function.

To determine if the deregulation of centrosome complex genes in hypoxic cells is the mechanism responsible for the accumulation of centriolar satellites, siRNA screens and isogenic inducible shRNA expressing cell lines can be used in hypoxia experiments. As part of my work with centrosome-associated genes, I have developed a prostate cancer cell line (22RV1) with inducible shRNA expression for knockdown of Augmin centrosome complex genes (Figure 3.1). Since some of the Augmin complex genes were found to be down-regulated by hypoxia, this system could be used determine if the centrosome aberrations observed in hypoxia-treated cells can be replicated by controlled knockdown of the same genes. Furthermore, using this gene knockdown system in combination with hypoxia treatment gives us the opportunity to simulate “contextual loss of heterozygosity”. Our lab has previously described a theoretical model of hypoxia-mediated defects in DNA repair that could cause a
contextual loss of heterozygosity (LOH) (Chan and Bristow, 2010). An example of contextual LOH might occur when a cell with single allelic functional loss of a DDR gene (eg. RAD51, BRCA1, BRCA2, etc.), has a further hypoxia-induced decrease in the expression of the gene. Since some DDR genes also have a role in centrosome function, it would be interesting to see if contextual hypoxia-induced LOH effects centrosome function. For example, our lab has also developed an inducible RAD51 knockdown system in 22RV1 and DU145 cells; these cells could be used to test my hypothesis using isogenic systems which are, or are not, competent for HR.

Although the use of cell lines allowed us to carry out controlled studies of centrosome aberrations and gene expression, the in-vitro models of prostate cancer are not without limitations. Although the increase in centriolar satellites was observed in three very different cell lines (BPH1, U2OS and DU145), the majority of experiments, including the mRNA expression analysis, were performed on the DU145 cell line. This prostate cancer cell line is derived from a brain metastasis of prostate adenocarcinoma and therefore is rapidly proliferating and is already aneuploid. In contrast, the majority of clinically-localized prostate cancer cases are slow growing tumors with lower levels of genomic instability. To determine if the results obtained using DU145 cells apply in the clinic, we plan to perform analysis of primary patient samples for correlation between hypoxia, centrosome aberrations and CIN, as further outlined in the next section.
Figure 3.1: Inducible shRNAi knockdown system for the HAUS1 gene. (A) Cartoon demonstrating the concept behind doxycycline (Dox)-inducible Tet-Repessor (TetR) and Tet-Operator (TetO) expression system. TetR binds to TetO blocking hairpin expression, until Dox is introduced and binds to TetR releasing it from TetO and inducing hairpin expression. (B) qPCR analysis of HAUS1 mRNA in 22RV1 isogenic clones, after 100nM Dox treatment, relative to untreated cells.
3.2 Future Work

3.2.1 Correlating centrosome aberrations and development of CIN in primary human tissues.

To determine if the findings in this thesis can be related to clinical cases, a study of human primary tumors in patients that have ingested the hypoxia marker pimonidazole (PIMO) prior to surgery would allow the study of centrosome-associated biology within oxic and hypoxic subregions of prostate glands. Using histologic sections and antibodies against PIMO adducts (only found in hypoxic cells) and against centrosome proteins could conclusively associate hypoxia with centrosome aberrations in situ using clinical material. Previous studies have demonstrated the potential for histological staining for centrosomal markers such as centrin, γ-tubulin and pericentrin to identify centrosomal abnormalities in tumor sections (Krämer et al., 2005; Lingle et al., 1998; Pihan et al., 2001). Our lab has previously shown a decrease in RAD51 expression in correlation with cell hypoxia in EF5 (hypoxia marker) treated mouse xenograft tumors using a similar method (Figure 3.2) (Chan et al., 2010). If the centrosome function changes that we observed in-vitro also occur in patient samples, we would be able to conclude that the increase in centriolar satellites in response to hypoxia is a clinically relevant feature of hypoxic tumors.
Figure 3.2: Representation of histological (IHC) staining for centrosome markers and hypoxia gradient. Upper two images show RAD51 (red) and EF5 (green) co-staining in histologic sections (RKO xenograft). The line profile for staining intensity demonstrates that RAD51 decreases in areas with increased hypoxia (EF5). (Adapted from Chan and Bristow 2010) Similar staining patterns are expected to be observed in PIMO labelled histological section co-stained for centrosome markers, with increasing centrosome aberrations in hypoxic regions (cartoon – red area represents hypoxic cells with centrosome amplification).
Furthermore, tumor sections can be micro-dissected to isolate hypoxic (PIMO positive) cells for mRNA expression and comparative genomic hybridization array (aCGH) analysis. The results of such an analysis will demonstrate any correlations between hypoxia and centrosome gene deregulation in patient tumor samples. Our lab has previously used aCGH in patient tumor samples to show that copy number alterations (CNA) in c-Myc, NKX3.1 and PTEN are prognostic (Locke et al., 2012; Zafarana et al., 2012). The same technique can be used to determine if any CNAs in mitotic genes are also prognostic or whether cells with abnormal centrosomes also contain these CNAs. Such a finding would support a direct link between tumor hypoxia, genomic instability and patient outcome.

Recent studies by Gao et al. (2014) and Karthaus et al. (2014) have developed methods for generating patient-derived 3D cell cultures that support long-term expansion of primary human prostate organoids derived from either healthy prostate epithelial tissue or advanced prostate cancer. The patient derived prostate cancer organoids more effectively recapitulate the genetic and phenotypic diversity of prostate cancer cells as observed in patient tumors than 2D in-vitro cell cultures (Karthaus et al., 2014). Using these methods, it would be possible to investigate the effects of hypoxia on centrosome complex gene expression and centrosome aberrations in primary patient samples to validate our findings in DU145 cells. The 3D cell cultures can also be used for mFISH analysis to determine the direct effects of hypoxia on CIN, using methods previously described in (Gao et al., 2014; Karthaus et al., 2014). Moreover, using 3D cell cultures derived from healthy prostate epithelium provides the opportunity to compare the effect of hypoxia on mitotic function in genetically stable cells as compared to cancer cells with genomic instability.

3.2.2 Studies of centrosome ultrastructure in hypoxic cells.

To gain further insight into the effects of hypoxia on centrosome function, it would be prudent to confirm the identity of the observed extra CETN/PCM1 spots as centriolar satellites. Although PCM1 is thought to represent a general marker and scaffold protein for centriolar satellites, it is also present in mature centrosomes (Bärenz et al., 2011). Centriolar satellites were initially observed in various cell types with electron microscopy (EM) as 70–100 nm electron dense granules that commonly localize around the centrosome (Bärenz et al., 2011).
To establish that we have been observing increases in centriolar satellites in response to hypoxia, it would be important to perform EM imaging in hypoxic cells to investigate accumulation of similar electron dense granules. EM studies can also be used to elucidate any structural changes that may occur in the centrosomes present in interphase and mitotic cells following collection of cells under hypoxia treatment and that are not easily observed with IF staining alone.

Furthermore, to determine if the accumulation of centriolar satellites in response to hypoxia leads to centrosome amplification, we would perform experiments to analyze centrosome structure and quantity abnormalities in hypoxia pre-treated cells at a number of time points. In the experiments described in this thesis which demonstrated increases in centriolar satellites, cells were treated with 72 hours of hypoxia followed by 48 hours of re-oxygenation before fixing and IF analysis. To study the dynamics of increases in centrosome amplification as a consequence of centriolar satellite formation, we would fix and stain for centrosomal proteins at 24, 48, 72, 96, 120 and 144 hours post hypoxia pre-treatment. We would expect that following the initial increase in centriolar satellites, there would be an increase in centrosome amplification at later time points.

Live cell imaging experiments can further confirm *de-novo* formation of centriolar satellites and reveal if these structures act as protein scaffolds for centrosomal proteins and lead to formation of supernumerary centrosomes. Live cell imaging experiments have been successfully utilized by several groups to follow the dynamics of centriolar satellites and centrosomes, as well the mitotic function and the fates of daughter cells (Bärenz et al., 2011). To study centrosome dynamics in live cells, cells can be transfected with plasmids to express GFP or RFP tagged centrin-2, γ-tubulin, NEDD1 and other centrosomal proteins. In addition, histone H2B tagged with GFP can be used to track chromosomes in live cells to observe the frequency of chromosome missegregation, anaphase bridges and other markers of CIN (Ganem et al., 2009; İnanç et al., 2010; Kuriyama et al., 2007; Löffler et al., 2013; White et al., 2000). Live cell experiments will allow us to directly observe centriolar satellite accumulation, centrosome amplification, CIN and daughter cell survival in response to hypoxia.
3.3 Cancer Impact

Our lab has shown that patients with combined hypoxia and genomic instability have a worse prognosis than patients with either phenotype alone. It would be of interest to investigate if these patients also have mitotic abnormalities that link hypoxia to CIN. Given that mitotic aberrations such as accumulations of centriolar satellites occur more frequently in hypoxic cells, these aberrations could turn out to be an important novel IHC-based prognostic factor. If centrosome aberrations in hypoxic cells also lead to CIN, it could be predictive of patient outcome and an indication for treatment intensification or the use of anti-hypoxia therapies.

Further investigation may elucidate how tumor cells with high CIN as a result of hypoxia could be specifically targeted by taking advantage of mitotic function abnormalities. Targeting hypoxic cells with CIN may provide a mechanism to target the most aggressive cancer cells to improve patient outcome. It is worth investigating if patients with increased centrosome aberrations in hypoxic tumors would have an augmented response to treatment with mitotic kinase inhibitors. Initial studies of mitotic kinase inhibitors in hypoxic in-vitro and ex-vivo cell cultures would provide insight into personalizing medicine in prostate cancer clinics.
References


