HYPOXIA-INDUCED CENTROSOOME LOSS IN EPITHELIAL CELLS

by

Emily Loertscher

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A Thesis Submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN GENETICS

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2022
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Master’s Committee, we certify that we have read the thesis prepared by Emily Loertscher, titled Hypoxia-Induced Centrosome Loss in Epithelial Cells and recommend that it be accepted as fulfilling the dissertation requirement for the Master’s Degree.

Gregory Rogers
Date: Jan 7, 2022

Anne Cress
Date: Jan 7, 2022

Nathan Ellis
Date: Jan 12, 2022

Final approval and acceptance of this thesis is contingent upon the candidate’s submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master’s requirement.

Gregory Rogers
Date: Jan 7, 2022

Master’s Thesis Committee Chair
Genetics GIDP
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Abstract

Centrosome loss has recently been reported as a phenotype of prostate cancer. Hypoxia, an environmental condition seen commonly in prostate cancer, can cause centrosome loss in the immortalized prostate epithelial cell line, RWPE1. Little is known about hypoxia-induced centrosome loss, including how commonly it occurs in other cell types and the mechanism behind centrosome loss. This thesis further characterizes hypoxia-induced centrosome loss as seen in RWPE1 cells as well as in two other epithelial cell lines, MCF10A and HaCaT. Hypoxia-induced centrosome loss is affected by cell density and is reversible upon return to oxygen in some cell lines. Disassembly of centrosomes in hypoxia may happen through a two-step process, first with the removal of pericentriolar material and then this the disassembly of centrioles.
Introduction

The centrosome is the major microtubule organizing center (MTOC) of the cell. Centrosomes are membraneless organelles composed of two barrels of microtubule bundles organized in 9-fold radial symmetry. These barrels, or centrioles, are surrounded by a protein cloud called the pericentriolar material (PCM). Proteins in the PCM bind γTubulin Ring Complexes (γTuRCs) from which microtubules nucleate and polymerize. During mitosis, centrosomes define the shape of the mitotic spindle. When there is a proper number of centrosomes (two during mitosis) the spindle will be bipolar which ensures the replicated genome is segregated equally into the two daughter cells. Strict regulation of centrosome number during the cell cycle is critical to the correct formation of the bipolar spindle. Centrosome number is maintained through centrosome duplication. During duplication one daughter centriole will grow orthogonally from the wall of a mother centriole. The centrosome duplication cycle ensures that each daughter cell inherits a single centrosome during cell division, each containing two centrioles that are able to carry out subsequent duplication events.

Centrosome amplification, the presences of too many centrosomes within a cell, is known to cause genomic instability and is seen often in cancers. Supernumerary centrosomes during mitosis can change the shape of the spindle, creating a multipolar spindle. Multipolar spindles lead to improper genome segregation which can cause aneuploidy and death in daughter cells. Cancer cells that experience centrosome amplification can correct multipolar spindles by clustering centrosomes into a pseudo-bipolar spindle. Even when centrosomes cluster together there are often merotelic attachments which may also cause aneuploidy or chromosome lagging. Thus, centrosome amplification is detrimental to cells and their genomes.

Centrosome loss is another example of abnormal centrosome number and is as harmful as centrosome amplification. Cells can undergo mitosis without centrosomes by organizing a microtubule basket around chromosomes to segregate the genome. Even though these spindles can become bipolar, cells often spend a prolonged time in mitosis which often results in lagging chromosomes. As the name suggest, these lagging chromosomes arrive in the daughter later than the rest of the genome. As a result, lagging chromosomes miss nuclear envelope formation and are sequestered in micronuclei, a nuclear envelope-encased structure outside of the main nucleus. Micronuclei are fragile and they often burst, exposing the micronuclear chromosome(s) to the cytoplasm and initiating chromothripsis (shredding and restitching of DNA). This single mutation event can result in further mutations within future progeny, even if the progeny cells undergo normal mitosis. Thus, understanding how and why centrosomes are lost in cycling cells is just as relevant to the field of genomic instability as understanding centrosome amplification.

While the effects of centrosome loss can be catastrophic to the genome, mechanisms of centrosome loss are poorly characterized. This is largely because centrosome loss is not a phenomenon that is seen often clinically, as centrosome amplification is. It was only recently that centrosome loss was characterized as a phenotype seen in prostate cancer. Wang et al. showed centrosome loss in human prostate tumors, with severity of loss increasing as Gleason score increased. Further work in our lab has identified a Hif1α-dependent hypoxia response as a causal factor of centrosome loss in RWPE1 cells, an immortalized prostate epithelial cell line.
Cells that experience hypoxia, or sub-physiological oxygen levels, must adapt to their hypoxic environment to survive. For example, Hif1α (Hypoxia-induced factor 1α) is an oxygen sensitive transcription factor that regulates metabolism, cell proliferation, and angiogenesis to promote survival in hypoxia. Hif1α is constitutively expressed in cells, but under normoxic (regular oxygen levels) conditions Hif1α is enzymatically hydroxylated, mainly by the hydroxylase PHD2. Hydroxylated Hif1α is then ubiquitinated and degraded by the proteosome. Only under hypoxic conditions is PHD2 unable to hydroxylate Hif1α which leaves Hif1α free to complex with another hypoxia factor, Hif1β. This complex moves into the nucleus and initiates transcription of hypoxia response genes.

Hypoxia-induced centrosome loss mechanistically links hypoxia as an environmental factor seen in prostate cancer and other solid tumors to genomic instability, a hallmark of cancer. Understanding the molecular mechanism of how hypoxia contributes to centrosome loss and subsequent genomic instability will further our understanding of how hypoxia contributes to cancer progression. This thesis will further characterize hypoxia-induced centrosome loss as seen in RWPE1 cells as well as in MCF10A and HaCaT cells.

**Results**

*Hypoxia-Induced Centrosome Loss in Epithelial Cell Lines*

Hypoxia-induced centrosome loss in RWPE1 cells is dependent on Hif1α activity. RWPE1 cells will lose centrosomes when Hif1α is chemically stabilized in normoxic conditions similar to loss seen in hypoxic conditions. Additionally, CRISPR Hif1αΔ/Δ RWPE1 cells retain their centrosomes in hypoxia. After the identification of hypoxia as a causal factor of centrosome loss in RWPE1 cells, we started to look for other cell lines that would also lose centrosomes under hypoxic conditions. Epithelial cells were incubated in hypoxia for 6, 12, 24, and 48 hours. After incubation cells were fixed and then stained for centrosomes using the protocol described in Wang et al. adapted for use in cell culture. The number of centrosomes per cell was quantified by counting the number of centrioles (CEP135) as they localized with PCM (γTub) where the expected number of centrioles is two. While not every cell line tested lost centrosomes in hypoxia (Table 1), I identified two epithelial cell lines that experience hypoxia-induced centrosome loss: MFC10A, a breast epithelial line, and HaCaT, a spontaneously immortalized epidermal keratinocyte line. The dynamics of centrosome loss in these cells differed from RWPE1 cells. Roughly half of the cells in a population of MCF10A or HaCaT cells lose their centrosomes after 24 hours of hypoxia. This is in contrast to RWPE1 cells where roughly half the population loses their centrosomes after 6 hours in hypoxia.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Centrosome Loss in Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWPE1</td>
<td>Immortalized prostate epithelial cell line</td>
<td>Yes</td>
</tr>
<tr>
<td>MCF10A</td>
<td>Breast epithelial cell line</td>
<td>Yes</td>
</tr>
<tr>
<td>HaCaT</td>
<td>Spontaneously immortalized skin keratinocyte cell line</td>
<td>Yes</td>
</tr>
<tr>
<td>SUM159*</td>
<td>Triple negative breast cancer cell line</td>
<td>Yes</td>
</tr>
<tr>
<td>MIA PaCa-2**</td>
<td>Pancreatic ductal adenocarcinoma cell line</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 1 Cell lines tested for centrosome loss in hypoxia. All cell lines were plated at 50,000 cells/cm², 100,000 cells/cm², 200,000 cells/cm², and 500,000 cells/cm². Cells were then incubated at 1% oxygen for 48 hours then fixed and stained for centrosomes. * Centrosome loss experiments were conducted by Dr. John Ryniawec (ryniawecj@arizona.edu) ** Centrosome loss experiments were conducted by Matthew Coope (mcoope@arizona.edu).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Description</th>
<th>Hypoxia Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrEC Hahn</td>
<td>Prostate epithelial cell line</td>
<td>No</td>
</tr>
<tr>
<td>DU145*</td>
<td>Prostate cancer brain metastasis cell line</td>
<td>No</td>
</tr>
<tr>
<td>RPE1*</td>
<td>Retinal pigment epithelial cell line</td>
<td>No</td>
</tr>
<tr>
<td>HeLa*</td>
<td>Endocervical adenocarcinoma cell line</td>
<td>No</td>
</tr>
<tr>
<td>U2OS*</td>
<td>Osteosarcoma cell line</td>
<td>No</td>
</tr>
<tr>
<td>Capan-2</td>
<td>Pancreatic ductal adenocarcinoma cell line</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 1 MCF10A and HaCaT cells lose centrosomes in hypoxia. (a) Immunofluorescent visualization of centrosomes in MCF10A cells plated at 500,000 cells/cm² incubated in normoxia and 6, 12, 24 hours of hypoxia with (b) centriole quantification.
As we tested cells for centrosome loss in hypoxia, we controlled for number of cells plated per cm². Varying numbers of cells (50,000, 100,000, 200,000, or 500,000) were plated in a 1 cm² culture well, incubated in normoxic conditions for 48 hours then incubated in hypoxia for 48 hours. Cells were then fixed and stained for centrosomes. We found that cells become sensitive to hypoxia-induced centrosome loss as they are plated at higher numbers per area. The sensitivity to cell density varies between cell lines. RWPE1 cells will lose centrosomes when plated at 100,000 cells/cm², though more cells within the population experience hypoxia-induced centrosome loss as density increases (Fig 2A). MCF10A and HaCaT cells are very sensitive to cell density and will only lose centrosomes in hypoxia when plated at 500,000 cells/cm² (Fig 2B, Fig 2C). The difference in sensitivity to density that we see between these cell lines could be explained by different cell sizes. A cell line with smaller sized cells would need numerically more cells to reach the same confluency level as a cell line with comparatively larger sized cells.

To assess the affect of cell size on how these different cell lines experience hypoxia-induced centrosome loss at different plating densities future measurements of cell size and surface area cells cover when adhered will be taken.

The sensitivity to cell density adds another level of complexity to understanding the mechanism behind hypoxia-induced centrosome loss. The density dependent component of centrosome loss could be caused by several different reasons. One hypothesis is that the Hif1α mediated hypoxia response in these cells cause secretion of a factor that, when at high enough concentrations, leads to centrosome loss. More cells would increase the concentration of the secreted factor in the media until the factor reaches a threshold which then induces centrosome loss. Another hypothesis is that centrosome loss is caused by a combination of the Hif1α response and a signaling pathway that is activated through cell-to-cell contact. Then increased cell density and the subsequent cell-to-cell contact activates signaling that is also required for centrosome loss. Yet another possible explanation is hypoxia-induced centrosome loss is triggered by a mechanosensing pathway which could be activated through the increased tension of increased cells. In this case, growing cells on plating substrates with varying stiffnesses could also affect how cells lose centrosomes in hypoxia. While the mechanism behind hypoxia-induced centrosome loss is unclear it is difficult to explain the dependency on cell density for centrosome loss.

After Loss: How Hypoxia and Centrosome Loss Affect the Cell Cycle

Physiologically, not all hypoxic events are chronic. One outcome of the hypoxia response is to upregulate angiogenesis in order to return oxygen levels to a normal range. Thus, it is informative to look at how cells behave upon a return to normoxic conditions in addition to hypoxic conditions. Cells were plated at 500,000 cells/cm² and then incubated in hypoxia, returned to normoxia, and fixed and stained for centrosomes. Centrosome loss is reversible in RWPE1 cells, which recover their centrosomes after 48 hours in normoxia (Fig 3A). Centrosome counts do not change significantly when counts from binucleated cells are excluded (data not shown) which suggests that the increase in centrosomes upon return to normoxia is not due to
Figure 2 Hypoxia-induced centrosome loss is cell density dependent. (a) DIC of RWPE1 cells plated at 50,000, 100,000, 200,000, and 500,000 cells/cm². Immunofluorescence images of centrosomes in RWPE1 cells at all densities in both normoxia and hypoxia. Quantification of centriole counts. (b) DIC of MCF10A cells plated at 50,000, 100,000, 200,000, and 500,000 cells/cm². Immunofluorescence images of centrosomes in MCF10A cells at all densities in both normoxia and hypoxia. Quantification of centriole counts. (c) DIC of HaCaT cells plated at 50,000, 100,000, 200,000, and 500,000 cells/cm². Immunofluorescence images of centrosomes in HaCaT cells at all densities in both normoxia and hypoxia. Quantification of centriole counts.
cytokinesis failure but is instead a result of de novo centriole assembly. De novo centriole assembly has been shown to occur during S-phase in many different cell models including in HeLa cells\textsuperscript{17, 18}. If centriole recovery in RWPE1 cells is happening during S-phase as cells return to normoxia, it is reasonable that the population recovers within 48 hours, which is just in excess of their doubling time. Centrosome loss was not reversible in MCF10A and HaCaT cells (Fig 3B, Fig 3C). If we expect recovery to occur during S-phase it is likely that failure to recover could be caused by cell cycle arrest either during or after hypoxia. To determine this, we need to understand how hypoxia and re-oxygenation is affecting the cell cycle.

**Figure 3** Post hypoxia recovery varies between cell types. (a) Immunofluorescence images of RWPE1 cells plated at 500,000 cells/cm\textsuperscript{2} during normoxia, hypoxia, and recovery. Quantification of centriole counts. (b) Immunofluorescence images of MCF10A cells plated at 500,000 cells/cm\textsuperscript{2} during normoxia, hypoxia, and recovery. Quantification of centriole counts. (c) Immunofluorescence images of HaCaT cells plated at 500,000 cells/cm\textsuperscript{2} during normoxia, hypoxia, and recovery. Quantification of centriole counts. (d) Representative images of MCF10A cells which are positive for p53, p21, and Casp3. Quantification of p53, p21, and Casp3 in MCF10A cells plated at 500,000 cells/cm\textsuperscript{2} at normoxia, 48hr hypoxia, and 72hr recovery. (e) Representative images of HaCaT cells which are positive for p53, p21, and Casp3. Quantification of p53, p21, and Casp3 in HaCaT cells plated at 500,000 cells/cm\textsuperscript{2} at normoxia, 48hr hypoxia, and 72hr recovery.

Understanding how the cell cycle is progressing in these cells while they experience hypoxia is also critical for understanding the ramifications of hypoxia-induced centrosome loss on genomic stability. Centrosome loss could only cause lagging chromosomes, micronuclei, and chromothripsis if centrosomes are lost during mitosis in cycling cells. Further, this instability will only result in a mutant population if daughter cells from the acentrosomal division are viable and continue to divide. The mitotic surveillance pathway is one mechanism that could lead to cell cycle arrest. The mitotic surveillance pathway is triggered when cells spend an extended time in mitosis. When triggered, p53 levels rise in a USP28- and 53BP1-dependent manner. p53 then activates p21 which leads to G1-phase arrest and apoptosis\textsuperscript{19, 20}. Previous work in our lab showed that RWPE1 cells do not show an increase of apoptosis in hypoxia\textsuperscript{10} but this is a limited characterization of the cell cycle. To further examine the cell cycle during and after a hypoxic event, cells were plated at 500,000 cells/cm\textsuperscript{2}, incubated in hypoxia for 48 hours, returned to
normoxia for 72hr, and fixed and stained for markers in the mitotic surveillance pathway (p53 and p21) as well as for cleaved Caspase3 (an apoptotic marker) (Figure 3D, E). This is not a standard assay, and results will need to be confirmed through western blot analysis to be conclusive.

MCF10A cells (Fig 3D) show an increase of p53 in hypoxia which decreases upon return to normoxia. As p53 is stabilized in hypoxia this is to be expected. Because p21 levels in MCF10A cells stay stable through normoxia, hypoxia, and recovery it seems unlikely that the mitotic surveillance pathway is being activated in response to centrosome loss. This evidence suggests that cells are arresting in response to hypoxia or as a result of the high number of cells plated. If cells are not dividing in hypoxia, there would be no prolonged mitosis and the mitotic surveillance pathway would not be activated. However, an increase of cleaved Casp3 upon return to normoxia indicates that cells are dying after hypoxia. All together this indicates that MCF10A cells experience apoptosis after a hypoxic event and that apoptosis is initiated through a mechanism separate from the mitotic surveillance pathway.

HaCaT cells (Fig 3E) levels of p53 do not change in hypoxia or during recovery. Even with elevated p53 levels, p21 levels stay consistent across all the conditions, similar to the MCF10A cells. It seems that hypoxia-induced centrosome loss does not trigger the mitotic surveillance pathway in HaCaT cells, again suggesting that these cells are not proliferating in hypoxia. Unlike the MCF10A cells, apoptosis levels do not increase in HaCaT cells upon return to normoxia. Because apoptosis is not occurring, it is possible that HaCaT cells are senescing as a response to hypoxia or as a response to the high number of cells plated.

The data presented here only provides a limited understanding on how the cell cycle progresses in these cell lines under hypoxic conditions. Further data, such as DNA content and proliferation markers, is required to determine exactly how hypoxia is affecting the cell cycle.

Exploring the Molecular Mechanism of Centrosome Loss

As hypoxia-induced centrosome loss is only now being characterized, the molecular mechanism behind centrosome loss is still unknown. Centrosome loss in RWPE1 cells starts after just 6 hours in hypoxia, which is a fraction of these cell’s doubling time. At 24 hours (half their doubling time) in hypoxia the majority of a population of RWPE1 cells will have lost their centrosomes. This timing makes it unlikely that centrosome loss is a result of failed centrosome duplication. We hypothesize, instead, that centrosomes are being disassembled during hypoxia. There is no well-established molecular mechanism for how centrosomes disassemble, but removal of centrosomes is a common event during oogenesis in metazoan oocytes. The standing model for removal of centrosomes from Drosophila oocytes is through a two-step disassembly process. In this model, PCM is first depleted from centrosomes which results in unstable centrioles. The centrioles then disassemble, and the oocyte is left without centrosomes.

To further explore the molecular mechanism behind hypoxia-induced centrosome loss, we performed RNA sequencing on WT RWPE1 cells and our CRISPR Hif1αΔΔ RWPE1 cells which do not lose centrosomes in hypoxia. RNA was harvested from cells that experienced hypoxia for 6, 12, and 24 hours (Fig 4A). To find potential targets which contribute to
Table 2  **Differentially expressed centrosome genes in WT RWPE1 cells.** RNA sequencing data from RNA isolated from RWPE1 cells which had been grown in hypoxia for 6, 12, or 24 hours. Fold change is normalized relative to RNA from RWPE1 cells grown in normoxia for all time points.

<table>
<thead>
<tr>
<th>Genes</th>
<th>6 Hours Hypoxia</th>
<th>12 Hours Hypoxia</th>
<th>24 Hours Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log$_2$ Fold Change</td>
<td>Adjusted p-value</td>
<td>Log$_2$ Fold Change</td>
</tr>
<tr>
<td>CACYBP</td>
<td>2.402055</td>
<td>1.67E-52</td>
<td>2.396032</td>
</tr>
<tr>
<td>MPHOSPH9</td>
<td>-1.80364</td>
<td>1.69E-41</td>
<td>-1.53099</td>
</tr>
<tr>
<td>AKAP12</td>
<td>4.026879</td>
<td>7.60E-78</td>
<td>3.8803</td>
</tr>
<tr>
<td>PJA1</td>
<td>1.861335</td>
<td>2.37E-52</td>
<td>1.914713</td>
</tr>
<tr>
<td>EHD1</td>
<td>3.204459</td>
<td>1.82E-75</td>
<td>2.63337</td>
</tr>
<tr>
<td>LYAR</td>
<td>2.120689</td>
<td>1.51E-41</td>
<td>1.897534</td>
</tr>
<tr>
<td>PPP2R2A</td>
<td>1.296104</td>
<td>4.91E-26</td>
<td>1.408428</td>
</tr>
<tr>
<td>CEP250</td>
<td>-1.45762</td>
<td>3.23E-12</td>
<td>-1.38389</td>
</tr>
<tr>
<td>CCDC113</td>
<td>-2.04157</td>
<td>1.84E-18</td>
<td>-1.61431</td>
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<td>TUBGCP3</td>
<td>-1.34406</td>
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<td>-1.19633</td>
</tr>
<tr>
<td>CCDC15</td>
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<td>1.44E-16</td>
<td>-1.81481</td>
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<td>HOOK2</td>
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<td>7.23E-13</td>
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</tr>
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<td>CEP126</td>
<td>-1.45898</td>
<td>1.50E-12</td>
<td>-1.01855</td>
</tr>
</tbody>
</table>

Figure 4  **RNA sequencing of RWPE1 cells in hypoxia.** (a) Graphic explaining how RNA was harvested for sequencing. (b) Z-scores of three genes of interest for both WT RWPE1 cells and ΔHif1α RWPE1 cells.

centrosome loss differentially expressed genes were filtered using multiple criteria. First, from a list of 195 genes that have been associated with centrosomes in the literature, we found 18 genes (Table 2) which were differentially expressed in WT RWPE1 cells at all time points. Since Hif1α
generally acts as an activator rather than a suppressor, we next looked only at centrosome genes that were upregulated throughout hypoxia, which narrowed the list down to 6 genes. Finally, we isolated our top genes by choosing only those that were not differentially expressed in ΔHif1α RWPE1 cells. Combined, we discovered three candidate genes that may contribute to hypoxia-induced centrosome loss: PJA1, PPP2R2A, and EHD1 (Fig 4B).

This is preliminary data, and we can only use it to guide hypotheses on the molecular mechanism of hypoxia-induced centrosome disassembly. The target genes from this screen, however, do fit nicely into the two-step disassembly hypothesis described above. EHD1 participates in membrane reorganization and endocytosis. EHD1 has been shown to be essential for the removal of pericentriolar material from the centrosome after mitosis to allow for centrosome duplication. PJA1 is an E3 ubiquitin ligase shown to interact with centriolar satellites, which transport centrosome components to and from the centrosome. Thus, it could localize to centrosomes and ubiquitinate centrosomal components causing centrosome instability during hypoxia. PPP2R2A is a regulatory subunit of the phosphatase PP2A. Dephosphorylation of PCM components by PP2A, regulated by PPP2R2A, after mitosis structurally weakens the PCM and is a first step in the mitotic spindle shedding its expanded PCM.

As depletion of pericentriolar material is the first step in the hypothesized two-step disassembly of centrosomes and centriole instability is the second, these targets support this hypothesized model of disassembly. Further work to show the functionality of these and other genes in centrosome disassembly is needed to define the molecular mechanism of hypoxia-induced centrosome loss.

Discussion

Hypoxia promotes the loss of centrosomes in certain epithelial cell lines. Some aspects of hypoxia-induced centrosome loss are cell type specific, such as dynamics of loss and centrosome recovery after hypoxia. Loss happening in a cell-density-dependent manner is common between all three cell types characterized here. Without a definite molecular mechanism for centrosome loss, it is difficult to explain the differences and similarities of hypoxia-induced centrosome loss in these cell lines. As de novo centriole assembly is linked to the cell cycle, it is likely that the differences in centrosome recovery are due to the cell lines regulating the cell cycle differently during hypoxia. Differences in cell cycle progression could also be caused by cells senescence in response to the high cell density or the hypoxic environment. Future work to determine how these cells regulate their cell cycle under the experimental conditions will elucidate these differences and provide insight into the molecular mechanism behind centrosome loss and recovery.

Understanding the similarities between the cell lines will also help to clarify the mechanism behind centrosome loss. One of the novel causes of centrosome loss in hypoxia presented here is high cell density. While this could be caused by secretion of a paracrine or autocrine signaling factor, results from preliminary conditioned media experiments (data not shown) make this hypothesis unlikely. Because the three cell lines characterized here are all basal-like epithelial cells, it is more plausible that the mechanism behind density-dependent loss is mediated through
cell-to-cell interactions. One potential signaling pathway regulating this is the Hippo pathway, which is a mechano-sensing signaling pathway used to regulate cell growth and proliferation. This is an attractive hypothesis because components of the Hippo pathway are differentially regulated within RWPE1 cells in hypoxia. Defining these aspects of hypoxia-induced centrosome loss becomes important as we start to think about hypoxia in a physiological setting.

Just as centrosome amplification is a driving mechanism of genomic instability in cancer, the centrosome loss in prostate cancer could also drive genomic instability. If the centrosome loss in prostate cancer is driven by hypoxia like it is in RWPE1 cells in cell culture conditions, centrosome loss may contribute to genomic instability at very early stages of prostate cancer. Prostate cancer is unique from other cancers because there are very few driver mutations found in common within primary prostate tumors. Instead, primary prostate cancer is characterized by large scale genomic instability which could be driven by centrosome loss. Age is one of the biggest risk factors for prostate cancer in men and age is correlated with decreased blood flow to the prostate. This would create a hypoxic environment where cells could lose centrosomes and thus promote genomic instability which would in turn increase the risk of cancer incidence.

Centrosome loss in hypoxia occurs in other cell types as well, so there is the potential for hypoxia to have similar effects in tissues besides the prostate. However, there is currently no evidence connecting centrosome loss to disease outside of the prostate. As shown here while hypoxia can cause loss in breast and skin cells, they behave differently in and out of hypoxia than prostate cells do. Future work to identify the mechanism behind centrosome loss and how hypoxia-induced centrosome loss affects the cell cycle will be critical in informing how applicable these findings are to both prostate and other tissues.

### Methods and Materials

#### Cell Culture

RWPE1 cells were grown in Keratinocyte Serum Free Media (K-SFM) (GIBCO, Kit Catalog Number 17005-042). K-SFM was supplemented with 0.05 mg/ml BPE (provided with the K-SFM kit), 5 ng/ml EGF (provided with the K-SFM kit), 100 IU penicillin, 100 mcg/ml streptomycin, and 0.25 mcg/ml amphotericin (MP Biomedicals, Cat# 1674049). MCF10A cells were grown in Mammary Epithelial Cell Growth Basal Medium (MEBM) (Lonza, Catalog Number CC-3151). MEBM was supplemented with the SingleQuots Kit (Lonza, CC-4136) (BPE, hEGF, Insulin, Hydrocortisone, and GA-1000) as well as 0.1µg/mL cholera toxin (Sigma C8052) and 100 IU penicillin, 100 mcg/ml streptomycin, and 0.25 mcg/ml amphotericin (MP Biomedicals, Cat# 1674049). HaCaT cells were grown in Dermal Cell Basal Medium (ATCC PCS-200-030) supplemented with the Keratinocyte Growth Kit (ATCC PCS-200-040) (0.4% Extract P, 0.5ng/mL rh TGF-alpha, 6mM L-Glutamine, 100 ng/mL Hydrocortisone, 5µg/mL Insulin, 1.0 µM Epinephrine, 5µg/mL Apo-transferrin) as well as 100 IU penicillin, 100 mcg/ml streptomycin, and 0.25 mcg/ml amphotericin (MP Biomedicals, Cat# 1674049). RPE1, HeLa, and Capan-2 cells were cultures in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Corning, product #10-013-CV) supplemented with 10% FBS. MIA PaCa-2 cells were cultured in DMEM supplemented with 10% FBS and 2.5% horse serum (HS). PrEC Hahn and DU145
cells were cultured in Iscove’s DMEM (Corning, product #10-016-CV) supplemented with 10% FBS. SUM159 cells were cultured in Ham’s F-12 media (Corning, product #10-080-CV) supplemented with 5% FBS, 0.5% insulin, 0.1% hydrocortisone, and 10mM HEPES. U2OS cells were cultured in McCoy’s 5A media (Corning, product #10-050-CV) supplemented with 10% FBS. RWPE1, MCF10A, and HaCaT cells were passaged using 0.05% trypsin EDTA in PBS and incubated at 37°C for 15-30 minutes. Trypsin was inactivated using soybean trypsin inhibitor (1mg/mL). Cells were stained with trypan blue and counted with BioRad TC20 Automated Cell Counter. For experiments cells were plated on 8-well culture plates (MatTek CCS-8) with 1 cm² wells.

**Hypoxia**

Cells incubated in hypoxia were grown in media supplemented with 10mM HEPES buffer and 100µM n-Acetyl Cystine. All hypoxia incubations were done at 1% O₂, 5% CO₂, and 94% N₂ using a Whitley H35 Hypoxystation.

**Immunofluorescence**

Cells were fixed in methanol at -20°C for 15 minutes, rehydrated in 1x PBS for 5 minutes, permeabilized in PBS-tritonX (0.25%) for 5 minutes, and blocked in blocking buffer (5% NGS, 0.1% tritonX, 2mM NaN₃ in PBS) for 30 minutes at room temperature. Cell were then incubated in primary antibodies diluted in blocking buffer for either 1 hour at room temperature or overnight at 4°C. Cells were washed thrice with PBS-tritonX for 5 minutes each wash and then incubated in secondary antibodies and Hoechst 33342 [diluted 1:5000] diluted in blocking buffer for 30 minutes at room temperature. Slides were then mounted with ProLong Glass Antifade Mountant (Invitrogen, Catalogue Number P36980) and left to set overnight. Slides were imaged using a DeltaVision Core system (GE Healthcare Bio-Sciences) equipped with an Olympus IX71 microscope, a 60X objective (NA 1.42), and a cooled charge-coupled CoolSNAP HQ2 camera (Photometrics) and softWoRx v1.2 software (Applied Science).

**Antibodies used**

- Cep135: custom made with PRF&L against the peptide CSTLRSPSHSPEHRNV diluted 1:3000
- γTubulin: Sigma-Aldrich GTU-88 diluted 1:1500
- p53: Abcam ab26 diluted 0.5mg/mL
- p21: Abcam ab188224 diluted 1:500
- Cleaved Caspase3: Cell Signaling Technology #9661 diluted 1:400

**RNA Sequencing**

RNA was isolated using the Qiagen RNeasy mini kit (catalog number 74104). Genomic DNA was removed from samples using Qaigen RNase-Free DNase Set (catalog number 79254) as instructed in the RNeasy protocol. RNA was sequenced at the University of Arizona Genomics Core. Bioinformatics analysis was done by the Padi lab in the University of Arizona Cancer Center.

**Statistical Analysis**
Centriole count graphs represent the average of replicates (n=3) for each condition. Graphs were made using GraphPad Prism 9. Significance of differences between conditions was determined using unpaired t-tests where P < 0.05 is considered significant. In graphs, “ns” indicates not significant, “*” indicates P ≤ 0.05, “**” indicates P ≤ 0.01, and “***” indicates P ≤ 0.001. Error bars represent standard deviation.

Acknowledgements

I express my thanks to Dr Greg Rogers for his mentorship and support while I learned in his lab. I am also grateful to my lab mates, especially Dr John Ryniawec, who spent much of their time teaching me and helping with this research.

References


