Response To Stress By The Tumor Microenvironment

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Response To Stress By The Tumor Microenvironment

Abstract

Fibroblasts are typically quiescent and tumor suppressive in adult mammals but secrete factors upon activation in benign and malignant diseases such as wound healing and tumor growth. As residents of the tumor microenvironment (TME), fibroblasts are subjected to the same stresses that tumor cells experience including nutrient deprivation that activates the integrated stress response (ISR) pathway. This eventually leads to either apoptosis due to intense or prolonged stress or autophagy to promote cellular survival in the absence of essential nutrients. Studies have shown that nutrient deprivation also activates the tumor suppressor p53 in fibroblasts. However, little is known about the role of other tumor suppressors such as the INK4a locus of the CDKN2A gene, the second most commonly mutated tumor suppressor gene in human cancers. The INK4a locus encodes two tumor suppressor genes including the p19 Alternative reading frame (p19Arf) that activates p53 by sequestering the ubiquitin ligase MDM2. While P19Arf regulation has been well characterized in cancer cells, its role in the TME has not been investigated. Endothelial cells in the TME are also subjected to stress such as shear stress that is altered upon changes in blood flow as a consequence of tumor vascularization as well as activities such as aerobic exercise. Exercise is commonly prescribed to cancer patients to enhance quality of life however, the effect of exercise during chemotherapy has not been extensively investigated. My work shows that the growth of transplanted tumor cells in the flank of mice is significantly upregulated in p19Arf-/- mice as compared to wild-type littermate controls indicating a role for P19Arf in the TME. My studies show that primary murine adult lung fibroblasts (ALFs) induce P19Arf expression upon nutrient deprivation and that prolonged leucine deprivation triggers apoptosis in wild-type ALFs. However, p19Arf-/- ALFs demonstrate enhanced proliferation, migration and survival in response to long-term leucine deprivation due in part to upregulation of the ISR pathway and increased autophagic flux. My data also suggests that loss of p19Arf in fibroblasts promotes survival during nutrient deprivation through increased proliferation and autophagy. My studies investigating the effect of acute aerobic exercise on endothelial cell activation and subsequent tumor vascular normalization show that a single round of acute exercise enhances chemotherapeutic efficacy when administered after exercise in melanoma xenograft tumor models independent of tumor vascular normalization. My data suggests that acute exercise may provide an opportunity to enhance chemotherapeutic efficacy.
RESPONSE TO STRESS BY THE TUMOR MICROENVIRONMENT

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Dedication page

To my ancestors and those that will follow after me, thank you for dreaming and believing. This is for you Mama.
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ABSTRACT

RESPONSE TO STRESS BY THE TUMOR MICROENVIRONMENT

Kerry Roby

Sandra Ryeom

Fibroblasts are typically quiescent and tumor suppressive in adult mammals but secrete factors upon activation in benign and malignant diseases such as wound healing and tumor growth\(^1\). As residents of the tumor microenvironment (TME), fibroblasts are subjected to the same stresses that tumor cells experience including nutrient deprivation that activates the integrated stress response (ISR) pathway. This eventually leads to either apoptosis due to intense or prolonged stress or autophagy to promote cellular survival in the absence of essential nutrients\(^2-\)\(^6\). Studies have shown that nutrient deprivation also activates the tumor suppressor p53 in fibroblasts. However, little is known about the role of other tumor suppressors such as the INK4a locus of the \(CDKN2A\) gene, the second most commonly mutated tumor suppressor gene in human cancers\(^6-\)\(^8\). The INK4a locus encodes two tumor suppressor genes including the p19 Alternative reading frame (\(p19Arf\)) that activates p53 by sequestering the ubiquitin ligase MDM2. While P19\(^{\text{Arf}}\) regulation has been well characterized in cancer cells, its role in the TME has not been investigated. Endothelial cells in the TME are also subjected to stress such as shear stress that is altered upon changes in blood flow as a consequence of tumor vascularization as well as activities such as aerobic exercise. Exercise is commonly prescribed to cancer patients to enhance quality of life however, the effect of exercise during chemotherapy has not been extensively investigated. My work shows that the growth of transplanted tumor cells in the flank of mice is significantly upregulated in \(p19Arf^{\text{−/−}}\) mice as compared to wild-type
littermate controls indicating a role for P19Arf in the TME. My studies show that primary murine adult lung fibroblasts (ALFs) induce P19Arf expression upon nutrient deprivation and that prolonged leucine deprivation triggers apoptosis in wild-type ALFs. However, p19Arf−/− ALFs demonstrate enhanced proliferation, migration and survival in response to long-term leucine deprivation due in part to upregulation of the ISR pathway and increased autophagic flux. My data also suggests that loss of p19Arf in fibroblasts promotes survival during nutrient deprivation through increased proliferation and autophagy. My studies investigating the effect of acute aerobic exercise on endothelial cell activation and subsequent tumor vascular normalization show that a single round of acute exercise enhances chemotherapeutic efficacy when administered after exercise in melanoma xenograft tumor models independent of tumor vascular normalization. My data suggests that acute exercise may provide an opportunity to enhance chemotherapeutic efficacy.
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LIST OF ABBREVIATIONS

AAD  Amino acid deprivation
ALF  Adult lung fibroblast
ARF  Alternate reading frame
ATF4 Activating transcription factor 4
ATF6 Activating transcription factor 6
ATG  Autophagy related gene
α-SMA Alpha smooth muscle actin
Baf  Bafilomycin
BME  Basement membrane extract
CAF  Cancer associated fibroblasts
CAR  Chimeric antigen receptor
CDKN2A Cyclin Dependent Kinase Inhibitor 2A
CHOP C/EBMP homologous protein
Cq   Chloroquine
DDP  Dipeptidyl peptidase
Dox  Doxorubicin
dsRNA Double stranded
eIF2α Eukaryotic initiation factor 2-alpha
ECM  Extracellular matrix
eNOS Endothelial nitric oxide synthase
ER   Endoplasmic reticulum
FAP  Fibroblast activation protein
FGF  Fibroblast growth factor
Gem  Gemcitabine
GCN2 General non-depressible protein 2
**GRP78** Glucose regulated protein-78  
**HCC** Hepatocellular carcinoma  
**HCV** Hepatitis virus C  
**HGF** Hepatocyte growth factor  
**HIF** Hypoxia inducible factor  
**HK2** Hexokinase 2  
**HPF** High powered field  
**HRI** Heme-regulated inhibitor  
**IFN-γ** Interferon gamma  
**IRE-1** Inositol-requiring enzyme  
**LAT1** L-type amino acid transporter 1  
**LC3** Light chain 3B  
**LD** Leucine Deprivation  
**LLC** Lewis lung carcinoma  
**M1** Macrophage type 1  
**M2** Macrophage type 2  
**MDM2** Mouse double minute 2  
**MEF** Mouse embryonic fibroblast  
**Mito C** Mitomycin C  
**MMP** Matrix metalloproteinase  
**mTOR** Mammalian target of rapamycin  
**mTORC1** mTOR complex 1  
**NFAT** Nuclear factor of activated T-cells  
**PPAR** Peroxisome proliferator-activated receptor gamma  
**PGC1** Peroxisome proliferator-activated receptor gamma coactivator 1 alpha  
**PKR** RNA-dependent protein kinase  
**PERK** PKR-like ER Kinase
smARF small mitochondrial ARF
TAM Tumor associated macrophages
TGF-β Transforming growth factor-β
TME Tumor Microenvironment
tRNA Transfer ribonucleic acid
TSP-1 Thrombospondin-1
UPR Unfolder Protein Response
VEGF Vascular endothelial growth factor
pVHL von Hippel-Lindau protein
WT Wildtype
CHAPTER 1: Introduction

Over 90% of all cancer related-deaths are due to metastatic progression\(^9\). Initial studies investigating cancer treatment focused solely on the primary tumor, but in 1889 Dr. Stephen Paget introduced a concept that shifted our understanding of cancer progression. Paget’s theory of metastasis suggested that upon entering the bloodstream, cancer cells (seeds) are selective in terms of the organ site where they will metastasize and grow. He proposed that the landing site or colonization of circulating metastatic cancer cells is regulated by the conditions in a distal organs microenvironment (soil)\(^{10,11}\). The tumor microenvironment (TME) is the local area surrounding the tumor cells consisting of different cellular populations that include immune cells, endothelial cells, and fibroblasts as well as extracellular matrix proteins (Figure 1.1).
Cellular Populations in the Tumor Microenvironment

*Immune Cells*

In response to antigens expressed on the surface of tumor cells, immune cells, such as CD8⁺ cytotoxic T lymphocytes, are primed and activated to induce cancer cell death via the release of cytotoxic granules or Fas Ligand-mediated apoptosis. Activated T-cells proliferate rapidly to exert effector functions that require the consumption of large amounts of nutrients and resources that are scarce in the TME, such as amino acids. T-cells accomplish this by increasing the expression of amino acid transporters on their cell surface that are required for different processes such as T-cell...
activation, homeostasis, differentiation, and memory\textsuperscript{13}. One particular amino acid transporters is the L-type amino acid transporter 1 (LAT1) which is responsible for transporting neutral amino acids including leucine, isoleucine, and valine into the cell\textsuperscript{14}. LAT1 has been shown to be highly expressed on cancer cells but expressed at low levels on normal cells with the exception of activated T-cells. In activated human T-cells, upregulation of LAT1 expression correlated with high cytotoxic cytokine production that was abrogated when LAT1 was specifically targeted and deleted or inhibited using a LAT1 selective inhibitor, JPH203\textsuperscript{15}. While immune cells play vital roles in the suppression of tumor growth through inducing apoptosis of cancer cells, a subset of immune cells also promote tumorigenesis by creating an immunosuppressive TME preventing the function of anti-tumor immune cell populations. For instance, macrophages are part of the innate immune response that are activated and skewed towards either a classical (M1) or alternative (M2) phenotype. M1 macrophages function in an anti-tumor manner and are activated in response to IFN-\(\gamma\) releasing pro-inflammatory cytokines, including TNF-\(\alpha\), and express MHC-I & II antigens to facilitate phagocytosis of tumor cells\textsuperscript{16}. In contrast, M2 macrophages are activated in response to IL-4/IL-13 to inhibit the pro-inflammatory response by M1 macrophages and inhibit cytotoxic T-cells through direct cell-cell interaction\textsuperscript{16,17}. In cancer, tumor associated macrophages (TAMs) comprise as much as 50% of the tumor mass and typically possess a M2 expression pattern. Although macrophages in the TME are often described as having a distinct M1 or M2 phenotype, data suggests that macrophage phenotypes exist on a continuum of macrophage polarization between M1 and M2 such that the TME pivots tumor killing M1 macrophages towards tumor-promoting M2 macrophage phenotypes\textsuperscript{18}. M2 macrophages of the TME promote tumor progression.
through suppression of pro-inflammatory cytokines such as IL-12 and IL-23, and the increased expression of the catabolic arginine enzyme, arginase-1\textsuperscript{16}. Arginase-1 metabolizes arginine into ornithine and polyamines, precursors necessary for cellular proliferation thus promoting tumor progression\textsuperscript{19}. In efforts to enhance effector T-cell function in cancer, immune checkpoint blockade therapies targeting the immune checkpoint inhibitors, CTLA-4 and PD-1-PD-L1 are currently being utilized in the treatment of many different cancer types with varying results from complete cure to lack of efficacy in tumors such as melanoma\textsuperscript{20} and prostate cancer\textsuperscript{21}, respectively.

\textit{Endothelial cells}

The endothelial cell population in the TME play key roles in cancer progression. Endothelial cells form new blood vessels during development and during tumor growth. During development, vasculogenesis is the process of establishing new vasculature during embryogenesis from bone marrow endothelial progenitor cells. In comparison, during tumorigenesis, endothelial cells also form new blood vessels, but this process is called neo-angiogenesis and is the generation of new blood vessels from existing vessels to support tumor expansion. In response to signals from the tumor, such as increased production of vascular endothelial growth factor (VEGF), endothelial cells become activated, alter their secretome, proliferate, migrate and form vessels.

Endothelial cell activation leads to the degradation of basement membrane, endothelial cell migration, proliferation, and differentiation leading to the formation of blood vessels that contribute to promote tumor progression and metastasis\textsuperscript{22}. Because of the reliance of endothelial cells on VEGF for activation and angiogenesis, anti-
VEGF/VEGF receptor therapies have been a long-standing area of investigation and the target of drug development to prevent tumor angiogenesis with the hopes that preventing blood supply to tumor cells would inhibit cancer progression. The excitement in targeting angiogenesis was in part due to the notion that this could be a universal approach to treating cancer since it was assumed that endothelial cells were the same regardless of the organ environment they resided. Thus, the ability to attenuate the expansion of blood vessels would ‘starve’ any tumor, regardless of their organ site.

While effective in inhibiting tumor growth *in vitro* and in mouse models, the use of anti-angiogenic therapy showed limited efficacy in clinical trials. The human VEGF antibody, Bevacizumab, was shown to be effective in a limited number of cancer types with improved overall and progression-free survival in colorectal, lung, kidney, and glioblastoma when used in combination with other chemotherapies. Initially, Bevacizumab was thought to show efficacy in treating metastatic disease as well. However, side effects such as hypertension, thromboembolic events, ventricular dysfunction, myocardial infarction, gastrointestinal perforation, and proteinuria\textsuperscript{23} showed that the toxicity of Bevacizumab in combination with chemotherapy did not improve overall survival. Some possible explanations for the lack of efficacy of anti-angiogenic therapies include the assumptions that endothelial cells in the TME were genetically stable and reliant solely upon VEGF for their activation. Further investigation into tumor associated-endothelial cells identified that they may not be genetically stable with one study indicating that tumor associated endothelial cells are aneuploid, harboring an abnormal number of chromosomes\textsuperscript{24}. Studies have also characterized the abnormal function and structure of tumor blood vessels. While normal blood vessels are highly organized and efficient in the circulation of blood, nutrients, and drugs throughout the
body, tumor blood vessels are inefficient conduits possessing disorganized, leaky vasculature and blind-ending sprouts that prevent proper perfusion of the tumor reducing effective drug delivery\textsuperscript{25}. Studies have also demonstrated that some cancers become resistant to anti-VEGF therapy resulting in continued tumor angiogenesis and tumor progression\textsuperscript{26}. Due to the significant level of circulating VEGF within the TME, Bevacizumab treatment at doses that limit adverse side effects is insufficient to effectively sequester circulating tumor-derived VEGF; this potentially allows for endothelial cells to continue functioning using alternative growth factors along with low levels of VEGF.

Aberrant vasculature is a common characteristic of the TME contributing to the inefficacy of anti-angiogenic therapy as well as chemotherapy. Due to the constant demand for nutrients in the TME and the increased VEGF secreted by tumor cells, tumor blood vessels are generated rapidly and are immature resulting in an abnormal vasculature. Normal blood vasculature consists of a mature, tightly organized endothelial cell layer that is surrounded by basement membrane and pericytes such as smooth muscle cells (Figure 1.2). In contrast, tumor vessels possess abnormal vasculature due in part to the rapid proliferation of the endothelial cell population resulting in gaps in the endothelial cell barrier making this newly generated vasculature leaky and fenestrated (Figure 1.2)\textsuperscript{27}. 

6
Leaky vessels also prevent even distribution of basement membrane and inefficient pericyte coverage making these vessels stunted in their development with collapsed lumens\textsuperscript{28}. Due to the poorly constructed blood vessel network in tumors, the distribution of chemotherapy or other targeted therapies for cancer treatment is relatively inefficient.

Dr. Rakesh Jain first described the concept of tumor vascular normalization through sequestering VEGF and other pro-angiogenic proteins permitting maturation of the vasculature by restoring the balance between pro- and anti-angiogenic proteins. The decrease in pro-angiogenic protein such as VEGF suppresses endothelial cell proliferation and migration allowing increased adhesion between endothelial cells and pericyte coverage of blood vessels. It was proposed that tumor vascular normalization leads to longer, more functional vessels with adequate lumens increasing blood flow and providing a window to improve chemotherapeutic efficacy. In support of the concept of tumor vascular normalization, Dickson, \textit{et al}, showed that treatment of a mouse model of neuroblastoma with a single dose of Avastin, a murine anti-VEGF sequestering antibody,
increased vessel maturation, drug penetrance, and trended towards a reduction in tumor growth when used in combination with topotecan\textsuperscript{29}. In addition, Winkler, \textit{et al}, showed that treatment with the VEGF2R antibody DC101, was effective in normalizing the tumor vasculature of human glioblastoma xenografts resulting in a decrease in hypoxic regions, increased pericyte coverage, and reduced basement membrane thickness through increased degradation by matrix metalloproteinases\textsuperscript{30–33}. While many studies have investigated the use of anti-angiogenic therapies to promote tumor vasculature normalization, the limited window and the adverse side effects indicate a need for other approaches to normalize tumor vessels. Chapter 4 will discuss my work investigating alternative approaches to tumor vascular normalization independent of pharmacologic targeting.

\textit{Fibroblasts}

Another major cellular population in the TME is the fibroblast. Fibroblasts are typically quiescent cells that reside in the interstitial spaces of tissues. They become activated in response to a variety of stimuli such as injury or activating signals such as transforming growth factor-\(\beta\) (TGF-\(\beta\))\textsuperscript{34}. In response to injury such as a wound in the skin, fibroblasts enter a reversible activation state where they become contractile and migratory, and gain expression of markers including vimentin, and alpha smooth muscle actin (\(\alpha\)-SMA). Activated fibroblasts deposit extracellular matrix (ECM) components, such as type I and IV collagens and also remodel the surrounding ECM through the
production of matrix metalloproteinases (MMPs) to degrade existing matrix, until the wound is closed and has healed\(^1\). Tumors are often referred to as a wound that is unable to heal\(^35\), due in part to the similarity between activated fibroblasts in wounds and the activated state of fibroblasts in cancer (Figure 1.3).

Cancer associated fibroblasts (CAFs) have a similar phenotype and function as normal fibroblasts but are considered to be constitutively activated. As a result of increased and persistent production of TGF-\(\beta\) and other fibroblast activating factors by cancer cells in the TME, CAFs are unable to return to a quiescent/resting state resulting in their increased contractility, proliferation, migration, altered secretome and ECM remodeling. CAFs remodel the ECM at higher rates as compared to activated normal fibroblasts through the production of collagens as well as increased secretion of MMPs that degrade and reorganize the ECM. CAFs remodel the ECM in a pro-tumorigenic manner by secreting factors including hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and TGF-\(\beta\) which promote migration, invasion, and metastasis of cancer
cells. Gaggiolli et al., showed that squamous carcinoma cells were able to migrate through a matrix layer in a fibroblast dependent manner albeit through direct cell-cell contact.

The identification of a specific CAF marker has been long sought after as both normal fibroblasts and CAFs express α-SMA and vimentin. Studies now agree that the cell surface receptor, fibroblast activation protein (FAP) may be the best marker for CAFs as FAP expression is primarily upregulated on CAFs. FAP is a member of the dipeptidyl peptidase (DPP) family of enzymes, sharing 50% homology with its closest member, DPPIV. FAP monomers become activated through the formation of homodimers or heterodimers with other FAP monomers and DPPIV monomers, respectively (Figure 1.4). FAP remodels components of the ECM in response to injury, and has been shown to increase the expression of α-SMA, collagen type I, and fibronectin. FAP expression is typically low in normal tissue, but is upregulated on CAFs in many different tumor types including pancreatic cancer, breast cancer and lung cancer; its high expression is also associated with poor prognosis of cancer patients.
With the identification of FAP as a relatively specific CAF marker, this has presented a therapeutic option to target fibroblasts in the TME for cancer treatment. The targeting of FAP expressing cells in an effort to suppress tumor growth has become an area of active investigation. Genetic ablation or pharmaceutical inhibition of FAP in vivo inhibited colon cancer tumor growth\(^41\) and lung cancer in mouse models\(^42;\), pharmacological inhibition of FAP with the GluBoroPro dipeptide, PT630, inhibits the enzymatic functions of FAP and DPPIV and efficiently inhibited tumor growth in these models. FAP’s expression within the tumor stroma has also led to the development of chimeric antigen receptor (CAR) – T cells that are designed to target FAP\(^+\) cells. The use of FAP specific CAR-T cells inhibited mesothelioma and lung cancer growth and increased cytotoxicity within tumors in addition to ablating FAP+ cardiac fibroblasts in a mouse model of cardiac fibrosis\(^43,44\).

**Stress and the Tumor Microenvironment**

As a result of the exponential proliferation rate of cancer cells, they sequester resources for themselves triggering various stressors in the TME including hypoxia, acidosis, and nutrient or amino acid deprivation. Due to the disorganized and inefficient vasculature associated with a rapidly developing tumor, a common stress is the restriction of oxygen or hypoxia. In response to hypoxia, stabilization of the transcription factors hypoxia inducible factors (HIFs)\(^45\) occurs in both cancer and normal cells. The oxygen-sensing subunit of HIFs, HIF-1\(\alpha\), dimerizes with HIF-1\(\beta\) to induce the transcription of genes allowing cells to alter their metabolism into an aerobic glycolytic state to continue the production of energy during low oxygen conditions\(^46\). The HIF-1\(\alpha\)
subunit is kept at low levels during normoxia by its ubiquitination and proteasomal degradation by the von Hippel-Lindau protein (pVHL). Specifically, HIF-1α becomes hydroxylated by the oxygen-dependent proly-hydroxylase domain-containing proteins (PHDs) at proline sites facilitating binding by pVHL\textsuperscript{45,47}. HIF-1α expression is upregulated in cancers and in the TME inducing changes in resident cellular populations to support tumor progression, such as the transformation of fibroblasts to CAFs\textsuperscript{45}. As a consequence of increased hypoxia, cells of the TME cannot utilize oxidative phosphorylation to produce energy from glucose. This results in the utilization of anaerobic glycolysis which is an alternative method for cells to produce energy in hypoxic environments, albeit at lower levels. Interestingly, cancer cells were discovered to convert glucose into energy in a less efficient manner regardless of oxygen status, which is termed the Warburg effect\textsuperscript{48}. As a result of inefficient perfusion of the TME, both anaerobic glycolysis and the Warburg effect expel high amounts of lactic acid into the surrounding TME resulting in an acidic microenvironment\textsuperscript{49}. Acidosis of the TME results in metabolic reprogramming of cancer cells and CAFs alike to aid in tumor progression and chemoresistance\textsuperscript{50}. For instance, Hexokinase 2 (HK2) has been shown to be upregulated in CAFs resulting in increased cell cycle progression and suppressed p27 expression\textsuperscript{51}. Despite the acidic and hypoxic conditions of the TME, cancer cells continue to rapidly proliferate sequestering nutrients resulting in nutrient deprivation within the TME. Nutrient deprivation is a hallmark of cancer that forces cancer cells to become scavengers to acquire the nutrients necessary to maintain their proliferation in a stressful environment. Avagliano \textit{et al.} suggest that CAFs in the TME become forced to use the Warburg effect to produce high energy metabolites that cancer cells utilize to maintain their proliferation\textsuperscript{52}. In addition to metabolites, such as pyruvate and ketone
bodies, amino acids are a viable resource that are scarce and preferentially taken up by rapidly proliferating cancer cells. Attempts to target enhanced amino acid consumption has resulted in varying effects. While glutamine overconsumption is a common trait of the TME, targeting the essential amino acid, leucine, can induce apoptosis of human breast cancer cell lines\textsuperscript{53}. To counter the stressful microenvironment, the integrated stress response (ISR) pathway is an evolutionarily conserved pathway that evolved so that cells could survive the effect of stressors ranging from viral infection, misfolded proteins, acidosis and nutrient deprivation.

**The Integrated Stress Response Pathway**

The core of the ISR pathway consists of a class of kinases that are activated exclusively in response to specific stimuli (Figure 1.5). The kinases heme-regulated inhibitor eIF2\(\alpha\) kinase (HRI), double-stranded RNA-dependent protein kinase (PKR), PKR-like ER Kinase (PERK), and general control non-depressible protein 2 (GCN2) all converge to phosphorylate the eukaryotic initiation factor 2 alpha (eIF2\(\alpha\)) subunit resulting in 1) the inhibition of global translation and 2) translation of specific proteins with conserved upstream open reading frames such as activating transcription factor 4 (ATF4). ATF4 transcribes genes generating non-essential amino acids or promoting autophagy. Prolonged stress increases expression of the apoptosis inducing C/EBP homologous protein (CHOP). General non-depressible protein 2 (GCN2), heme-regulated inhibitor (HRI), RNA-dependent protein kinase (PKR), and PKR-like ER (PERK). Adapted from Pakos-Zebrucka, \textit{et al}, EMBO Reports 2016.

![Figure 1.5: Schematic of the Integrated Stress Response Pathways.](image)
factor 2-α (p-εIF2α) subunit. This results in the halting of general protein translation with the exception of genes including activating transcription factor 4 (ATF4). ATF4 transcribes genes that initially promote cell survival under stress including autophagy related gene 5 (ATG5) to promote autophagy. However, when the stress is prolonged, ATF4 transactivates genes that promote cell death including pro-apoptotic genes such as C/EBP homologous protein (CHOP).

The HRI arm of the pathway is activated in response to heme deprivation. In normal conditions, heme binds both the N-terminus and kinase binding domain to keep HRI inactive, but when heme is deprived HRI dimerizes to auto-phosphorylate itself to become activated.54 While the other arms of the ISR are broadly distributed between tissues, HRI is found within erythroid cells and is required for erythrocyte differentiation and regulating globin production.55 In cancer, HRI can be both anti-tumor or tumor promoting as it has been shown to be regulated by the ternary complex inhibitor, N,N'-diarylureas, to induce the phosphorylation of εIF2α and sequential increase in CHOP to induce prostate cancer cell death and inhibit human prostate tumor growth.56 In contrast, its ablation in bortezomib resistant human prostate cancer cells sensitizes them and induces cell death.57

The PKR arm of the pathway is activated in response to double stranded RNA (dsRNA) that is typically associated with viral infection. When activated, PKR dimerizes and auto-phosphorylates itself at threonine 446 to subsequently phosphorylate εIF2α. In infections such as Hepatitis virus C (HCV), PKR levels are upregulated in response to dsRNA as a result of the increased viral proliferation. Due to chronic infection, HCV progresses and develops into liver cirrhosis and eventually hepatocellular carcinoma (HCC) although the molecular mechanisms underlying this progression are not well
characterized. Hiasa et al, showed that PKR expression was increased in HCV-related tumor regions when compared to non-tumor areas in patient samples suggesting a requirement for PKR in HCV-related HCC.

In response to ER stress, the unfolded protein response pathway becomes activated involving the activation of inositol-requiring enzyme (IRE-1), ATF-6α, and the ISR kinase, PERK. ER stress results from the accumulation of misfolded proteins that arise due to mutations, oxidative stress, heat-shock, or dysregulated calcium flux. These misfolded proteins allow for the PERK regulator, glucose regulated protein-78 (GRP78), to dissociate from and release the PERK kinase allowing it to dimerize, auto-phosphorylate, and become activated. In cancer, PERK and the unfolded protein response pathway is required for tumor cell survival in hypoxic regions of tumors. Rouschop et al, showed that colorectal cancer cells survive hypoxia through autophagy in a PERK-dependent manner; they established that colorectal tumors with PERK inhibition reduced hypoxic regions, reduced tumor growth, and showed increased sensitivity to irradiation. The ISR shows redundancy when one arm is targeted. For example in a genetically engineered mouse model of sarcoma with a deletion of GCN2, the level of eIF2α phosphorylation was maintained in Gcn2Δ/Δ tumors due to compensation by PERK.

The final arm of the ISR pathway, GCN2, is activated in response to amino acid deprivation (AAD). In normal conditions, transfer ribonucleic acids (tRNA) are charged by the corresponding amino acid allowing GCN2 to remain inactivated. However, when cells are deprived of amino acids, tRNAs bind to GCN2 promoting a conformational change that permits dimerization, autophosphorylation, and activation of the kinase. In addition to genes related to autophagy and apoptosis, GCN2-mediated activation of
ATF4 induces the production of enzymes that generate non-essential amino acids, such as asparagine, as needed by the cell. However, when the cell is deprived of essential amino acids, ATF4 promotes autophagy to allow for the degradation of cellular organelles to provide essential amino acids necessary for cell survival until the essential amino acid pool is repleted and the pathway is then inactivated. GCN2 is distributed among many tissue types and is required for cellular processes including cell proliferation, autophagy, and angiogenesis in response to AAD. As is common in the TME, amino acids trigger activation of GCN2 in both normal cells and cancer cells. In human cancer cell lines, GCN2 expression was upregulated and shown to be required for production of VEGF and tumorigenesis while deletion of GCN2 reversed this phenotype and reduced tumor growth rates. Because of the double-edged sword that the GCN2-ATF4 pathway presents in cancer, pharmacological approaches to either activate or inactive (Figure 1.6) the GCN2 pathway depending on the cancer type and the target population has been an area of great interest. Activators of GCN2 raise the levels of uncharged tRNAs through sequestering amino acids, such as asparagine by asparaginase, that subsequently bind and activate GCN2. Inhibitors of GCN2 function to

Figure 1.6: Schematic of GCN2 pathway and pharmacological activators and inhibitors of the GCN2 kinase. Pharmacological approaches have been developed to induce the activation or inhibition of the GCN2 kinase to be used in combination with other therapies. Adapted from Pakos-Zebrucka, et al, EMBO Reports 2016.
inhibit the phosphorylation of eIF2α in response to GCN2 activating stimuli, but their mechanism of action is not specific to GCN264.

The majority of work on the ISR has investigated its function in cancer cells, with few studies investigating the role of the ISR in other cellular populations of the TME. The GCN2/ATF4 pathway has been shown to be required for endothelial cell activation and contribute to tumor progression. In response to AAD, GCN2 activation correlates with increased VEGF secretion by tumor cells while interruption of the pathway significantly reduced VEGF levels and the number of blood vessels in tumors63. Endothelial cells have also been found to secrete higher levels of VEGF in vitro and increased tube formation in a GCN2-dependent manner when deprived of the amino acids methionine and cysteine in the context of hypoxia62. GCN2 has also been shown to be critical in the immune populations of the TME. In a glioblastoma model, GCN2 was found to be critical for tumor infiltration by cytotoxic T-cells and was also shown to be pivotal for their survival during tryptophan deprivation.65 The ISR pathway is activated in response to stress stimuli in fibroblasts just as in many other eukaryotic cells. Most studies have focused on the role of the ISR in mouse embryonic fibroblasts (MEFs), while the role of the ISR in adult stromal cells of the TME has not yet been extensively investigated.

In summary, the TME is composed of various cell types that each contribute towards tumor progression both in concert and independent of other cellular populations. Cells in the TME have the ability to function as both pro- and anti-tumor populations thus it is important to understand the signaling pathways regulating these opposing actions. For example, the immune cell population with its surveillance ability to destroy foreign cells, also exhibits tumor promoting actions through their exhaustion, lack of efficacy, and production of factors that alter other immune populations towards a pro-tumor
phenotype. Endothelial cells are also subject to changes within the TME as their rapid expansion in response to increased production of growth factors from cancer cells prevents the development of fully functioning or mature blood vessels resulting in inefficient vasculature. Cancer associated fibroblasts, abundant throughout the TME, are known to promote tumor growth in part due to their increase in FAP expression which results in fibroblast activation and the production and remodeling of ECM proteins that promote tumor growth, migration and metastasis. While the stresses of the TME are experienced by both tumor cells and the surrounding “normal” cells, the utilization of the ISR pathway promotes cell survival and is an attractive therapeutic target. Thus, it is of great interest to understand the role of the ISR in cells in the TME and in fibroblasts.
CHAPTER 2: Tumor Suppressors and the Integrated Stress Response

Introduction

In response to amino acid deprivation (AAD), previous studies on mouse embryonic fibroblasts (MEFs) have shown that MEFs activate the Integrated Stress Response (ISR) pathway to cope with the lack of nutrients until the stress is alleviated. Tumor suppressors such as the most commonly mutated tumor suppressor, p53, have been well studied for their role in cancer cells but less is known about their role in cells in the tumor microenvironment (TME) \(^{66,67}\). Wild-type (WT) p53 typically inhibits cancer progression through a number of different mechanisms including cell cycle arrest, induction of apoptosis and senescence. However, mutant p53 can be pro-tumorigenic through functions that include regulating cancer cell metabolism with gain of function mutant p53 promoting glycolysis and lipid synthesis and tumor growth\(^{68}\). WT p53 has also been shown to have pro-tumor ability in cancer cells in response to AAD. Cancer cells with WT p53 when subjected to AAD undergo cell cycle arrest to promote their survival through the upregulation of the p53-target gene p21; this upregulation and overall cell survival was lost when p53 was ablated in these cells\(^{69,70}\). Interestingly, upregulation of p21 in this context coincided with activation of the ISR as evidenced through phosphorylation of eIF2\(\alpha\) and increased transcription of genes associated with the AAD response, including CHOP, which was reduced when p53 was mutated\(^{70}\). While these studies have primarily investigated p53 and its effects in cancer cells, p53’s role in other cell types in the TME, including stromal and endothelial cells is less well understood.
Genetic screening of CAFs revealed that although somatic mutations of p53 in cancer models were rare, the potential silencing of genes by epigenetic changes in the TME cannot be ruled out\textsuperscript{71,72}. To this end, the silencing of p53 in CAFs has been investigated in tumor models. The effect of stromal cell-derived p53 on tumor growth has been investigated in pancreatic and carcinoma models. These studies revealed an increase in tumor growth when normal fibroblasts were mixed with cancer cells that was enhanced when p53 was deleted or mutated to mimic potential epigenetic silencing\textsuperscript{73,74}. Studies show that the rate of tumor growth in flank tumor models was significantly faster when tumor cells were co-injected with normal fibroblasts and tumor growth was further enhanced when normal p53 was deleted, mutated or altered epigenetically. However, this effect was abrogated when p53\textsuperscript{74} or stromal cell derived factor -1 (SDF-1)\textsuperscript{73} were targeted in fibroblasts. These findings suggest that p53 in the stroma affects tumor growth but the effect of p53 in stromal cells in the TME in response to stress and activation of the ISR, has had limited investigation. In response to leucine deprivation, MEFs with an inactive p53 mutant still showed induction of a p21 variant in a GCN2-dependent manner that was utilized by cells to survive the stress of AAD\textsuperscript{75}. The depletion of p53 and its homologs, p63 and p73, did not prevent the upregulation of p21 suggesting that p53 is not required for p21 induction in MEFs under these conditions. One potential explanation could be the ability of p53 homologs to act on the promoter regions of p53 target genes. It has been previously shown that the p63 isoform is able to bind the promoter region of p21 in human epidermal keratinocytes at different stages of differentiation\textsuperscript{76}. As these studies have provided a potential link between the ISR, cell cycle arrest and p53, my studies have focused on investigating the effects of other tumor suppressors on the ISR pathway in response to stress of the TME and particularly the fibroblast population.
Another tumor suppressor of interest is the *P14* alternate reading frame (*P14ARF*) tumor suppressor, or the mouse homolog, *p19Arf*. *P14ARF*, the second most commonly mutated tumor suppressor in human cancers, is encoded in the *CDKN2A* gene. The role of P19Arf as a regulator of p53 activation has been well characterized *in vivo* and *in vitro*. In normal conditions, p53 is kept at low levels as it is targeted for lysosomal degradation via the E3 ubiquitin-protein ligase mouse double minute 2 homolog (MDM2). However when p53 activation is required in response to a range of cellular stresses including oncogenic stress, ultra-violet or gamma irradiation, or hypoxia, *p19Arf* expression is upregulated and activated, which results in the binding and sequestering of MDM2, translocating it to the nucleolus permitting the stabilization and functional activation of p53 (*Figure 2.1*). While this narrative presents *p19Arf* as a tumor suppressor only in a p53-dependent context, it has also been shown to function in a p53-independent manner. Mice with triple knock-out of *p19Arf*, *p53* and *Mdm2* develop multiple tumors at a frequency greater than that observed in mice lacking both *p53* and *Mdm2* or *p53* alone. This data demonstrates that

*Figure 2.1: p19Arf -p53 canonical pathway*. Under normal conditions, p53 activity is tightly regulated and targeted for degradation by the E3 ubiquitin ligase, MDM2. In response to cellular insults that require p53 activation, the *p19Arf* tumor suppressor sequesters and relocates MDM2 to the nucleus permitting p53 activation and function.
$p19Arf$ can act independently of the Mdm2-p53 axis in tumor suppression playing roles in cell cycle arrest and senescence in tumor cells\textsuperscript{77}.

$P19Arf$ has been shown to induce cellular responses independent of p53 in response to oncogenic stimuli including anti-tumorigenic functions such as apoptosis, cell cycle arrest, and senescence. $P19Arf$ can also induce these functions in normal MEFs. Weber et al., showed that when $p19Arf$ was reintroduced into MEFs lacking p53, MDM2, and $p19Arf$, these cells displayed an ability to induce cell cycle arrest that was independent of the p53 target gene $p21$\textsuperscript{83}. $p14ARF$ has also been shown to induce cell death independent of p53 in human cancer cells. When $P14ARF$ was transfected into p53-deficient human carcinoma cells, not only was there a reduction in viable cell number and an increase in apoptosis markers \textit{in vitro}, but this was also recapitulated \textit{in vivo} as harvested tumors revealed a reduction in proliferating cells and an increase in cleaved caspase-3\textsuperscript{84,85}. While apoptosis and cell cycle arrest are critical for tumor suppression, senescence is another cellular response that is associated with reduced tumor progression. The $p19Arf$ tumor suppressor is required to induce senescence in response to stress as it works in concert with the tumor suppressors $Ink4a$ or $p16$\textsuperscript{85–87}. In addition, $p19Arf$ can also induce autophagy as another cell survival mechanism that has been viewed as double-edged sword in cancer for its ability to not only induce apoptosis as a tumor suppressive response but also its utilization by cancer cells to maintain sufficient nutrients for tumor progression in certain models\textsuperscript{88}. Previous studies have shown that a small mitochondrial isoform of $p19Arf$ (smARF) induces autophagy and apoptosis in a p53-dependent manner in both fibroblasts and cancer cells\textsuperscript{85,89}. It has also been shown to promote tumor progression by promoting autophagy during nutrient deprivation in lymphomas\textsuperscript{85,90}. While these studies have shown the involvement of
*p19Arf* in cellular responses, limited investigation has connected the ISR and the *p19Arf* tumor suppressor. One study showed the ability of ATF4 to suppress *p19Arf* allowing transformed cells to maintain plasticity and colony formation ability, that was lost when *Atf4* was deleted⁷. Due to the gap in knowledge regarding the link between *p19Arf* and the ISR, my work has been investigating the role of *p19Arf* in stromal cells and its effect on the ISR in response to stress in the TME.

As the loss of *p19Arf* in mouse models of cancer has been shown to promote tumor growth and its loss in MEFs leads to immortalization, I hypothesized that the loss of *p19Arf* in the TME enhances tumor growth through increased activation of fibroblasts. My data demonstrates that in addition to increased tumor growth in a *p19Arf-null* mouse host, primary *p19Arf-null* fibroblasts co-injected with cancer cells showed an increased rate of tumor growth. My data also shows that growth of 3-dimensional (3D) tumor organoids or tumoroids, formed in the context of a fibroblast network was enhanced when co-cultured with *p19Arf-null* fibroblasts. My data also shows that fibroblasts upregulate expression of the *p19Arf* tumor suppressor in response to prolonged leucine deprivation. Our data indicate a role for *p19Arf* in fibroblasts to slow tumor growth in mice and *ex vivo* 3D tumoroid development, in addition to our finding that P19⁴⁴*Arf* is upregulated in response to prolonged leucine deprivation in primary fibroblasts.

**Results**

*Loss of p19Arf in the microenvironment increases tumor growth*

To investigate the role of P19⁴⁴*Arf* in the TME, WT or *p19Arf⁴/c* mice were inoculated with syngeneic murine sarcoma (SKPY) or Lewis lung carcinoma cells (LLC) in the flank
and tumor growth was monitored over time. The rate of tumor growth was increased in
$p19Arf^{−/−}$ mice indicating a role for P19Arf in the tumor microenvironment (Figure 2.2A). To
assess the tumor promoting capabilities of $p19Arf^{−/−}$ adult lung fibroblasts (ALFs), we
generated 3D tumoroids with WT or $p19Arf^{−/−}$ ALFs to more closely recapitulate in vivo
tumor growth and to specifically examine the effect of ALFs on tumor growth. WT or
$p19Arf^{−/−}$ ALFs were seeded in basement membrane extract. Lewis lung carcinoma (LLC)
cells were plated on top of the solidified basement membrane and formed 3D tumors. We
show that LLC tumoroids with fibroblasts show an increase in tumor cell numbers as
compared to growth of LLCs alone, and this phenotype is further enhanced when
fibroblasts with deletion of $p19Arf$ were utilized. This increase in tumor cell proliferation in
3D tumoroids was also observed with $p19Arf^{−/−}$ fibroblasts during long-term leucine
deprivation (Figure 2.2B). To investigate whether cancer cells within the tumoroids
promoted fibroblast activation, tumoroids were digested into single cell suspensions and
cells were mounted and stained for proliferation and fibroblast activation using Ki67 and
α-smooth muscle actin (SMA), respectively. My data shows actively proliferating fibroblast
populations within the tumoroid as evidenced by nuclear Ki67 staining and α-SMA
expression that is increased in $p19Arf^{−/−}$ fibroblasts (Figure 2.2C,D,E). These data suggest
that loss of $p19Arf$ in fibroblasts, promotes tumor cell growth in 3D tumoroid assays and
demonstrates cross-talk between tumor cells and fibroblasts. These data further support
the notion that P19Arf functions as a tumor suppressor in fibroblasts in the TME.
**P19Arf is induced in response to amino acid deprivation and activates the Integrated Stress Response pathway**

P19Arf is known to be induced in response to oncogenic stress and DNA damage, stabilizing and activating p53\(^{67,78,79}\). TP53 has also been shown to be upregulated during AAD in breast cancer cells, but the effect of AAD on P19Arf expression and function has not yet been investigated\(^91\). To cope with AAD, cells activate the ISR exclusively through the GCN2 arm of the pathway resulting in phosphorylation of eIF2\(\alpha\) (p-eIF2\(\alpha\)) and nuclear localization of ATF4\(^{54}\). We probed for activation of the ISR in WT and \(p19Arf^{-/-}\) ALFs in response to prolonged leucine deprivation with our data showing increased p-eIF2\(\alpha\) in WT fibroblasts that was even further increased in \(p19Arf^{-/-}\) fibroblasts confirming ISR pathway activation in both WT and \(p19Arf\)-null fibroblasts (**Figure 2.3A**). Activation of the ISR occurs through many different stimuli. To confirm dependence on GCN2 activity in response to AAD, we treated cells with a GCN2 inhibitor (GCN2-IN-1)\(^92\) and probed for p-eIF2\(\alpha\) in response to leucine deprivation. We show increased phosphorylation of eIF2\(\alpha\) in \(p19Arf^{-/-}\) fibroblasts, which is ablated when cells were treated with GCN2-IN-1 during leucine deprivation confirming increased GCN2 activation in response to the amino acid deprivation in \(p19Arf^{-/-}\) ALFs (**Figure 2.3B**). Exposure of WT ALFs to leucine deprivation revealed upregulation of \(p19Arf\) expression at the mRNA and protein level after 24 hours.
that was sustained over 3 days (Figure 2.3C,D). In addition to overall expression levels of $p19\text{Arf}$, its localization within the cell is also indicative of its activity\textsuperscript{33}. In response to leucine deprivation, we show that nuclear localization of P19$\text{Arf}$ increases when leucine deprivation is prolonged (Figure 2.3E). These findings suggest that $p19\text{Arf}$ induction in response to prolonged leucine deprivation contributes to activation of the ISR pathway in primary fibroblasts.
Discussion

During tumor progression, the TME plays a pivotal role in the maintenance and growth of the primary tumor and metastasis of cancer cells. Of the populations that constitute the TME, stromal cells play critical roles in promoting tumor growth and metastatic progression. Tumor cells are thought to activate normal cells such as fibroblasts, in their local microenvironment leading to remodeling of matrix and creating a favorable niche for tumor growth. Although fibroblasts in the TME are thought to be genetically stable, emerging data suggests that epigenetic regulation of gene expression in ‘normal’ cells in the TME may play important roles in tumorigenesis. Thus studies investigating how loss of tumor suppressor genes such as p53 or p19Arf affects fibroblast function in the TME may offer insight into the role of tumor suppressors in cancer associated fibroblasts (CAFs)\(^74\). In this study, we investigated the role of the second most

\[\text{Figure 2.3: Leucine deprivation (LD) induces p19Arf expression in primary murine adult lung fibroblasts (ALF) and activates the integrative stress response pathway. A. Western blot analysis of phospho-eIF2a and total eIF2a expression in wild-type (WT) and p19Arf-null murine ALFs during LD for the indicated days. Graph quantifies intensity of phospho-eIF2a expression relative to Day 0. β-Tubulin used as loading control. B. Western blot analysis of phospho-eIF2 expression in WT and p19Arf\(^{-/-}\) ALFs upon treatment with a GCN2 inhibitor (GCN2-IN-1; A-92 1µM in DMSO) during overnight LD. Actin used as a loading control. Quantification of phospho-eIF2 expression relative to total eIF2a. N=3. C. qPCR for p19Arf mRNA in WT or p19Arf\(^{-/-}\) fibroblasts during LD for the indicated days. D. Western blot for p19Arf protein expression in WT and Arf-null fibroblasts after LD for the indicated days. E. Representative immunofluorescence images for P19Arf subcellular localization on the indicated days after LD in WT or p19Arf\(^{-/-}\) ALFs. Quantification of nuclear p19Arf is shown in graph on right.}\]
commonly mutated tumor suppressor, p19Arf, in adult lung fibroblasts. Here we show that p19Arf is induced in fibroblasts in response to AAD, a common stress in the TME. While the p19Arf tumor suppressor has been shown to be induced in response to hypoxia and DNA damage, its induction in response to leucine deprivation has not yet been demonstrated.

Although our work did not investigate P53-independent functions of p19Arf in fibroblasts, its role in senescence, ribosome biogenesis, and SUMOylation has been well established with p19Arf expression a commonly used marker of senescence. It has also been suggested that increased ribosome biogenesis is a common hallmark of cancer that provides a potentially actionable target for cancer treatment. Many different types of autophagy have been identified including mitochondrial autophagy (mitophagy) and ribosomal autophagy (ribophagy). Considering p19Arf’s role in regulating ribosome biogenesis and autophagy, it would of interest to investigate p19Arf’s tumor suppressive role through ribophagy.

A known regulator of autophagy is the mTOR pathway which primarily regulates cell proliferation and is dependent on the availability of nutrients. In normal conditions, the mTOR pathway, specifically mTOR complex I (mTORC1) regulates mRNA translation, protein turnover, and cellular metabolism. The mTOR pathway is manipulated in cancer, and components of the pathway have been shown to degrade p19Arf to promote proliferation in MEFs. Further studies will investigate the effect p19Arf loss on the mTOR pathway and its regulation on cell processes including proliferation, metabolism, mRNA translation, and protein turnover.
The loss or mutation of the human $p14\text{Arf}$ tumor suppressor in cancer cells has been identified in numerous cancer types. However, the role of the $p14\text{Arf}$ tumor suppressor in the TME has not been investigated and could offer a promising area of investigation to enhance existing therapies. Collectively, our data demonstrate a role for $p19\text{Arf}$ in fibroblast activation and its loss promotes fibroblast survival and activation during nutrient deprivation leading to a pro-tumorigenic phenotype in primary lung fibroblasts and increased tumor growth. Further elucidation of the specific downstream targets of $p19\text{Arf}$ in primary lung fibroblasts will provide insight into the link between loss of $p19\text{Arf}$ and activation of autophagy during nutrient deprivation and may provide new pharmacological targets in the stroma.
CHAPTER 3: \textit{p19Arf} Loss Enhances Fibroblast Survival and Function During Prolonged Leucine Deprivation

Introduction

We have shown in Chapter 2 that the loss of \textit{p19Arf} in the tumor microenvironment (TME) enhances tumor growth rates in murine models. This was also recapitulated in 3D tumoroid colonies when primary fibroblasts are the only other cell type in the local environment. We also showed that the loss of \textit{p19Arf} in these fibroblasts not only promotes tumor cell proliferation in both normal conditions and leucine deprivation (LD), but also increases fibroblast numbers suggesting bi-directional cross-talk between tumor cells and fibroblasts. We investigated the role of \textit{p19Arf} in these fibroblasts and showed induction of \textit{p19Arf} at both the mRNA and protein levels in response to prolonged LD. Increased P19\textsuperscript{Arf} expression also coincided with activation of the ISR pathway and was further enhanced when \textit{p19Arf} was deleted in these fibroblasts. This suggests that the loss of \textit{p19Arf} enhances fibroblast activation and promotes their stability in response to stresses of the TME. To this end, we investigated the function of \textit{p19Arf} in primary adult fibroblasts to determine the survival mechanisms utilized during long-term amino acid deprivation (AAD) upon \textit{p19Arf} loss. Other studies have investigated the role of \textit{p19Arf} in fibroblasts in diseases other than cancer. In aging related studies, \textit{p19Arf} and \textit{p14ARF} are commonly upregulated and required to induce senescence of late passage cells\textsuperscript{86,96}. \textit{P19Arf} ablation in cells revealed the onset of age-related pulmonary fibrosis and enhanced pulmonary function in mice suggesting that \textit{p19Arf} plays a role in fibroblast function in benign disease\textsuperscript{97}. Fibroblast migration has also been shown to require \textit{p19Arf} as Guo \textit{et al.},
showed a decrease in fibroblast motility when $p19Arf$ was deleted that was rescued when
$p19Arf$ was reintroduced\textsuperscript{98}. In addition to fibroblast motility, the ability of fibroblasts to
invade through basement membrane is also a hallmark of cancer that is associated with
metastasis and poor tumor prognosis\textsuperscript{99}. In cancer cells, $p19Arf$ inhibits invasion through
basement membrane in a p53-independent manner\textsuperscript{100}. While these studies have indicated
a role for $p19Arf$ in fibroblast function and cancer cell invasion in normal conditions, it begs
the question of whether stress in the TME alter these functions, and the contribution of
fibroblast-derived $p19Arf$.

The role of $p19Arf$ has been implicated in various fibroblast functions and activation
thus I hypothesized that the increased activation of $p19Arf$-null fibroblasts was due in part
to increased proliferation requiring autophagy for survival during prolonged LD. Our study
has revealed that the increased proliferation of $p19Arf$-null fibroblasts observed under
normal conditions permits increased survival in response to the deprivation of the essential
amino acid, leucine, that is lost when proliferation of $p19Arf$-null fibroblasts is inhibited.
We also show increased activation of $p19Arf$-null fibroblasts as observed through
increased migratory and invasive abilities even during prolonged LD. Finally, we show a
dependence and utilization of autophagy pathways in $p19Arf$-null fibroblasts that promote
cell survival even at baseline but is further upregulated in response to prolonged LD.
Pharmacological inhibition of autophagy attenuates the survival response of $p19Arf$-null
fibroblasts during long-term LD indicating the necessity of this pathway for survival.
Results

Loss of p19Arf promotes fibroblast survival during leucine deprivation

There is little understanding of how cells in the tumor microenvironment respond to chronic stress during tumorigenesis such as AAD. In response to sustained AAD over days, we show that WT ALFs eventually die in contrast to p19Arf\(^{-}\) fibroblasts that continue to proliferate even during long-term LD (Figure 3.1A). The loss of p19Arf in mouse embryonic fibroblasts (MEFs) increases proliferation due in part to the loss of p53 function and loss of cell cycle checkpoints\(^{67,83}\). p19Arf deletion enhances ALF survival during AAD that is due in part to the increased proliferation of p19Arf-null ALFs relative to WT ALFs. EdU uptake confirms the increased proliferation of p19Arf\(^{-}\) ALFs as compared to WT fibroblasts during long-term AAD (Figure 3.1B). To determine whether increased survival of p19Arf\(^{-}\) ALFs was due to increased proliferation, we treated fibroblasts with mitomycin C to inhibit DNA synthesis (Figure 3.1C) and exposed ALFs to long-term LD. Mitomycin C treated ALFs exposed to AAD revealed that p19Arf\(^{-}\) ALFs die during long-term leucine deprivation similar to WT ALFs suggesting that survival of p19Arf-null fibroblasts may be dependent primarily on their increased rates of proliferation (Figure 3.1C). P19Arf activation is necessary to induce apoptosis in a p53-dependent manner\(^{101,102}\). To assess whether deletion of p19Arf in ALFs reduced apoptosis, fibroblasts were stained with Annexin V after AAD. Our data show similar levels of Annexin V staining between LD WT and p19Arf\(^{-}\) ALFs indicating apoptosis in response to AAD was unaffected by p19Arf status (Figure 3.1D). These data suggest that the loss of Arf enhances ALF proliferation and survival during AAD promoting tumor progression.
Figure 3.1: Loss of p19Arf enhances fibroblast proliferation during leucine deprivation (LD). A. Quantification of trypan blue negative WT or p19Arf<sup>−/−</sup> lung fibroblasts on the days indicated after normal (left graph) media or LD-deprived (right graph) media. Fold change is relative to Day 0 cell counts. B. Representative images of EdU uptake (16-hour pulse) of WT or p19Arf<sup>−/−</sup> lung fibroblasts after LD for the indicated days. Bar = 50µm, n=3, * p<. C. Representative images of EdU uptake of WT or p19Arf<sup>−/−</sup> lung fibroblasts during LD and treatment with mitomycin C (Mito C) and graph of viable cell numbers. Fold change is relative to Day 0.
**P19Arf**⁻/⁻ fibroblasts show increased activation during leucine deprivation

Fibroblast activation and migration in response to injury can be modeled in a scratch assay. ALFs are seeded in tissue culture dishes and grown to confluency then denuded with a pipet tip. We show that p19Arf⁻/⁻ fibroblasts migrate more rapidly than WT fibroblasts covering the denuded area in the presence of LD media faster than WT fibroblasts (Figure 3.2A). Similarly p19Arf⁻/⁻ ALFs migrated more rapidly through a transwell in response to serum in LD conditions further indicating that loss of p19Arf enhances fibroblast activation (Figure 3.2B). When cancer associated fibroblasts are activated, they migrate through collagen and other extracellular matrix in the tumor microenvironment by cleaving and reorganizing matrix. Invasion assays with ALFs through a collagen bed revealed increased invasion by p19Arf⁻/⁻ ALFs, that was maintained during LD suggesting more active fibroblasts and an ability to more rapidly modify collagen (Figure 3.2C). In collagen remodeling, ALFs both degrade and deposit collagen providing an opportunity to investigate the role of p19Arf in collagen deposition by primary fibroblasts. Quantification of hydroxyproline serves as a surrogate for collagen production as hydroxyproline is a major component of collagen. Utilizing supernatant collected from fibroblasts during collagen invasion assays, hydroxyproline levels were measured revealing a surprising reduction in collagen production by p19Arf⁻/⁻ ALFs both in normal media and during LD. This data suggests that either collagen production is decreased in the absence of P19Arf or that the loss of p19Arf in ALFs increases collagen degradation, but not collagen deposition in response to AAD (Figure 3.2D). Overall, these results suggest the loss of p19Arf increases fibroblast activity during LD that could enhance tumor progression.
Loss of p19Arf increases autophagic flux in primary fibroblasts during LD

Increased proliferation and long-term survival of p19Arf-null fibroblasts during LD implicates autophagy as a mechanism by which p19Arf−/− ALFs survive in the absence of the essential amino acid leucine. Previous studies have shown that in response to AAD, cells induce autophagy to recycle organelles and obtain nutrients required for survival. To investigate autophagic flux in WT and p19Arf−/− ALFs during LD, we examined expression of the p62 cargo receptor protein, and the dynamic processing of microtubule-associated proteins 1A/1B light chain 3B (LC3) from LC3-I to LC3-II by Western blot. We show increased p62 expression in p19Arf-null ALFs during prolonged AAD as compared to WT ALFs (Figure 3.3A). Similarly, we find an increase in LC3-II levels during AAD in p19Arf-null ALFs at both baseline and in response to AAD (Figure 3.3B). Collectively these data suggest increased autophagy during LD in p19Arf null fibroblast promoting ALF survival. Treatment of ALFs with bafilomycin, a drug that raises the pH of the autophagolysosome to inhibit autophagy, showed an increase in LC3-II levels during leucine deprivation.
confirming the induction of autophagy (Figure 3.3B). To determine the effect of autophagy on ALF survival, cells were subjected to LD and additionally treated with the autophagy inhibitors bafilomycin or hydroxychloroquine. We show that the survival benefit of \( p19Arf^- \) ALFs during LD is lost when autophagy is inhibited with either chloroquine (Figure 3.3C) or bafilomycin (Figure 3.3D) resulting in decreased ALF survival. These data confirm that \( p19Arf^- \) ALF are dependent on autophagy to promote their survival during long-term LD through enhanced autophagic flux.
Figure 3.3: Loss of p19Arf increases autophagy in primary fibroblasts during leucine deprivation (LD). A, B. Western blot analysis of autophagy markers (A) p62 and (B) LC3-I to LC3-II conversion in WT and p19Arf−/− fibroblasts during LD for the indicated days. C, D. Fibroblast cell numbers in the presence of the autophagy inhibitors (C) chloroquine [100nM] or (D) bafilomycin [1nM] during LD for the indicated days. n=3, * p<0.05, ** p<0.01, *** p<0.001.
Discussion

My data demonstrate that the increased proliferation of $p19Arf^{-/-}$ fibroblasts offer a survival advantage over WT fibroblasts in response to prolonged AAD. In support of this, I show that survival of $p19Arf^{-/-}$ ALFs during long-term LD is lost in the presence of the DNA synthesis inhibitor, mitomycin C indicating that increased proliferation is a critical mechanism for survival during LD. Our data also show increased migration of $p19Arf$-null fibroblasts during LD in scratch assays, through transwells and through a collagen bed suggesting that $p19Arf^{-/-}$ ALFs have the ability to promote tumor growth in the TME with limited nutrients.

In response to nutrient deprivation, cells recycle existing cellular components to be used as nutrients to promote survival, a process known as autophagy. Cancer cells often use this mechanism for survival in the nutrient deprived TME, but increased autophagy has also been shown to inhibit tumor growth. We show here that autophagy is increased in $p19Arf$-null ALFs as compared to WT ALFs during LD as evidenced by the conversion of LC3-I to LC3-II and the degradation of p62. Interestingly, we find that $p19Arf^{-/-}$ fibroblasts at baseline contain higher levels of p62 and LC3-II that were even further upregulated when autophagy was inhibited, suggesting $p19Arf^{-/-}$ fibroblasts enhance autophagy to promote their survival. Using two well characterized autophagy inhibitors bafilomycin and hydroxychloroquine, I confirmed the importance of autophagy for survival of $p19Arf$-null fibroblasts during prolonged LD. The decreased survival of $p19Arf$-null ALFs during LD in the presence of bafilomycin or hydroxychloroquine confirms the necessity of autophagy to promote survival under nutrient deprivation conditions. Further studies will be needed to
determine how p19Arf may directly or indirectly regulate autophagy to inhibit fibroblast survival under conditions of nutrient deprivation. Previous studies have shown that inhibition of autophagy in vivo slows tumor growth rates and inhibits tumor progression. It will be of interest to investigate whether restriction of an essential amino acid through diet could improve autophagy-based cancer therapies.
CHAPTER 4: Shear Stress and its Effect on the Tumor Microenvironment

Introduction

In addition to stresses of the tumor microenvironment (TME) associated with the lack of resources due to a rapidly expanding tumor mass, there are other stresses induced by biophysical stimuli such as blood flow resulting in shear stress. Shear stress is defined as the measure of the force of friction from a fluid acting on a body in the path of that fluid\textsuperscript{103}. The endothelial cell monolayer lining blood vessels throughout the body are directly exposed to the shear stress of blood flow. While physiologic shear stress is necessary for normal vascular function, aberrant or pathologic changes that disturb shear stress or cause oscillatory flow can activate endothelial cells altering their behavior\textsuperscript{104}. Studies have shown that these changes can be associated with plaque deposits, atheroma formation and atherosclerotic disease\textsuperscript{103}. Altered vascular flow affects the vascular endothelium at the cellular and molecular level changing gene expression, cytoskeletal rearrangement, and leukocyte adhesion among other things. These changes together with well-defined risk factors including obesity, smoking and hypertension lead to atherosclerosis, a chronic disease with plaque buildup in arteries restricting blood flow that can result in occlusion of the artery if the plaque ruptures\textsuperscript{105}. Studies have shown that similar molecular pathways in endothelial cells play a role in the progression of both atherosclerosis and cancer\textsuperscript{105}. In cancer, shear stress has been implicated as having both tumor promoting and suppressive abilities. Tumor cells are most likely to encounter shear stress during metastatic progression as they travel from the primary tumor to a distal metastatic organ site and are referred to as circulating tumor cells or CTCs. Studies show that CTCs are exposed to variable levels of shear stress during metastatic progression that can promote its proliferation and extravasation.
In contrast, increased shear stress has also been shown to reduce the number of viable CTCs in various tumor models. Although pathologic stimuli can trigger increased shear stress, physiological methods such as aerobic exercise can also increase shear stress by increasing blood flow.

Shear stress induced by moderate exercise is an important mechanism to improve vascular function by stimulating activation of endothelial cells. As tumor vasculature have poor endothelial function leading to leaky vessels, numerous studies have investigated the effects of normalizing tumor vasculature to improve drug delivery in cancer. As briefly mentioned in Chapter 1, the concept of tumor vascular normalization can be observed with the use of antiangiogenic treatments to restore the balance of pro- and antiangiogenic factors in the TME that leads to restoration of abnormal tumor vasculature towards a more normal state with improved tumor blood flow and increased oxygenation. Tumor vascular normalization as a therapeutic strategy to increase drug delivery have generally utilized antiangiogenic treatments to promote normalization. These therapies lower the levels of pro-angiogenic factors such as VEGF, to attenuate the rapid growth and proliferation of endothelial cells leading to ‘pruning’ of the excess endothelial cells causing vascular regression and a more normal vascular bed. However, the use of antiangiogenic agents not only has a limited window for vascular normalization but is also accompanied by a number of adverse side effects. A physiologic approach to increasing shear stress and activating endothelial cells is through aerobic exercise. We previously published that tumor bearing-mice subjected to increased shear stress via treadmill running during chemotherapy showed normalization of tumor vasculature as evidenced by an increase in mature lumens that were more effective in perfusion and circulation of drugs to the tumor bed. This study
also revealed the importance of the calcineurin - nuclear factor of activated T-cells (NFAT) - thrombospondin -1 (TSP-1) pathway in mediating vascular normalization\textsuperscript{116}. More recently, another study demonstrated that TSP1 expression may serve as a biomarker of tumor vasculature normalization\textsuperscript{25,117}. While these studies investigated the effects of chronic exercise on increasing chemotherapeutic efficacy, the effects of acute exercise on enhancing drug delivery and efficacy have not been investigated.

Aerobic exercise interventions can either be chronic, defined as repeated periods of short or longer term exercise, or acute, defined as a single bout of exercise. Based upon the effect of chronic exercise on tumor vascular normalization and increased chemotherapeutic efficacy, I hypothesized that a single session of acute aerobic exercise would not provide sufficient levels of shear stress to activate and remodel vasculature. In our previous study, we showed that chronic exercise not only induced TSP-1 and endothelial nitric oxide synthase (eNOS) expression in the lungs but we also showed that TSP-1 expression was increased in the heart and spleen of these animals. These data confirm increased shear stress throughout the animal. My studies investigating the effect of acute exercise on tumor growth found that administration of drug immediately after a session of acute aerobic exercise, showed a modest suppression of tumor growth as compared to mice treated with drug alone.

Exercise induces adaptations in skeletal muscle through activation of the transcriptional co-activator peroxisome proliferator-activated receptor (PPAR) family of proteins with peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1- α) one of the best characterized family member\textsuperscript{118}. PGC1- α functions in regulating mitochondrial biogenesis and oxidative metabolism and has been studied in a number of disease states including cancer. PGC1- α has been implicated across various
cancer types with increased expression in breast cancer associated with an increase in mitochondrial restoration and metastasis to the bone and lung\textsuperscript{118,119}. It has also been shown to be necessary for drug resistance in colorectal cancer\textsuperscript{120}. PGC1-\(\alpha\) works in concert with the p53 tumor suppressor in promoting cancer cell survival through cell cycle arrest, which was lost when PGC1-\(\alpha\) was deleted or the stress was prolonged\textsuperscript{121}. Since PGC1-\(\alpha\) is essential for exercise-induced upregulation of skeletal muscle VEGF, we were interested in exploring whether this factor effected exercise-induced tumor vascular normalization in our model system. I hypothesized that increased PGC1-\(\alpha\) expression in the skeletal muscles of exercised mice would increase VEGF levels systemically leading to more rapid tumor progression and metastasis to the lungs.

Utilizing PGC1-\(\alpha\) transgenic mice generated by Arany et al.\textsuperscript{122} with overexpression of PGC1-\(\alpha\) in the skeletal muscles, I subjected mice to chronic aerobic exercise and observed no difference in the growth rates between control and transgenic mice. Using an experimental model of spontaneous lung metastases, I examined the incidence of lung metastasis in PGC1-\(\alpha\) transgenic mice and littermate control mice after chronic exercise. My data show no significant difference in lung metastases between exercised control versus transgenic mice. However, my studies do show that chronic aerobic exercise increased metastatic burden in both PGC1-\(\alpha\) transgenic and control mice as compared to non-exercised transgenic or control mice suggesting that long-term exercise in the absence of chemotherapy may promote tumor progression.
Results

*Acute exercise increases chemotherapeutic efficacy attenuating tumor growth*

As shown previously, chronic exercise in tumor bearing mice enhances chemotherapeutic efficacy through the normalization of tumor vasculature through a cellular mechanism that includes activation of TSP-1 and the calcineurin-NFAT pathway in endothelial cells. Since the ability of cancer patients undergoing chemotherapy to maintain a regular exercise program may be limited, we sought to determine if acute aerobic exercise or a single exercise training session would be sufficient to normalize tumor vasculature and increase the efficacy of chemotherapy. Utilizing a murine melanoma xenograft model in syngeneic wild-type (WT) mice, I inoculated mice with melanoma cells and when tumors reached 100 mm$^3$ in volume, mice were treated with Doxorubicin (Dox). The sedentary group were returned to their cages and the experimental group ran on a treadmill for one hour at a moderate intensity of VO$_2$ as previously described$^{25,123}$. These studies showed that acute exercise offered no increased efficacy of Dox treatment as tumors continued to grow even more rapidly in the exercised cohort as compared to the sedentary cohort (Figure 4.1A).
One possible explanation for this increase in tumor growth after an acute exercise intervention in the presence of Dox was that the clearance of Dox occurred more rapidly upon the increase in blood flow from acute aerobic exercise. Next I examined the effect of Dox treatment within 15 minutes after completion of acute aerobic exercise in
melanoma-bearing mice and found that tumor growth was inhibited when chemotherapy was administered after treadmill running as compared to the non-exercised melanoma-bearing cohort (Figure 4.1B). However, when these studies were repeated utilizing a xenograft model of pancreatic ductal adenocarcinoma (PDAC), the administration of Gemcitabine (Gem) either before or after running had no effect on tumor growth (Figure 4.1C). These data indicate that chemotherapy administered after acute exercise may attenuate tumor growth only in specific cancer types. Further, I propose that the increased efficacy of Dox treatment after acute aerobic exercise may be due to the transient increase in shear stress permitting more efficient drug delivery to the tumor.

PGC1-α has no effect on metastatic burden in the lungs after exercise

I next examined how chronic aerobic exercise would effect tumor progression in transgenic mice with PGC1-α overexpression\textsuperscript{124}. PGC1-α transgenic mice and littermate controls were inoculated with syngeneic Lewis lung carcinoma (LLC) cells into their flank and tumors were monitored for growth up to a volume of ~500 mm\textsuperscript{3}. The growth rate of flank tumors were similar between transgenic and control mice (Figure 4.2A). Utilizing an experimental model of spontaneous lung metastases referred to as the injection/resection model\textsuperscript{125}, I resected flank tumors when they reached 500 mm\textsuperscript{3} then both cohorts of mice (PGC1-α transgenic mice and littermate controls) were subjected to chronic aerobic exercise for two weeks at 12 meters per minute for 45 minutes per day and five days a week prior to examining lungs for metastatic disease. After two weeks, mice were euthanized and lungs were harvested, fixed, paraffin embedded and sectioned. Lung sections were stained with hematoxylin and eosin to
identify metastatic lesions but revealed no difference in metastatic incidence, or size of metastatic lesions between the transgenic and control mice cohorts (Figure 4.2B). These data suggest that overexpression of PGC1-α in skeletal muscle has no effect on tumor growth and metastasis and that chronic aerobic exercise had no impact on metastatic progression.

Figure 4.2 Transgenic mice with overexpression of PGC1-α in skeletal muscle showed no difference in lung metastasis as compared to control mice metastasis after chronic aerobic exercise. A. Graph of Lewis lung carcinoma (LLC) flank tumor volume on indicated days. LLC cells were injected into the flank of WT and PGC1-α transgenic mice. Tumor volume was measured by caliper. N=8 (4 sedentary, 4 exercise) mice/cohort. Tumors were resected when tumor volumes were ~500-800mm³. After three days of exercise training, mice were subjected to chronic aerobic exercise 5 times a week at 12m/min for 45 minutes for two weeks at which point lungs were harvested and formalin fixed, paraffin embedded and sectioned. B. Representative images of lung sections from exercised mice stained with H&E. Bar = 50 pixels. Quantification of lung metastases per mouse and average size of lung metastases is shown below. Size of lung metastases was normalized to total lung area. N=27 (6 WT sedentary, 7 WT exercise, 7 PGC1-α sedentary, 7 PGC1-α exercise).
Discussion

My data reveal that the timing of chemotherapy delivery relative to a single bout of acute aerobic exercise is critical to inhibit tumor growth. I showed that tumor growth was attenuated only when Dox was administered immediately after acute aerobic exercise but not before acute exercise. It is possible that the delivery of Dox immediately after acute exercise may be more efficient due to the increased shear stress as a result of aerobic exercise. We have previously shown that when a bolus of Dox was administered before exercise, there was no difference in the amount of drug within the tumor between sedentary and exercised groups. In this current study, I also examined the effect of transgenic overexpression of PGC1-α in muscles fibers on tumor growth and metastatic progression and found no effect on the growth of primary flank tumors as compared to control mice. Similarly, upon investigation of the effect of chronic aerobic exercise on metastatic progression in PGC1-α transgenic mice, I also found no significant difference in metastatic incidence or burden in exercised transgenic versus control mouse cohorts. However, metastatic burden was increased in exercised PGC1-α and exercised control mice as compared to their sedentary counterparts.

My data suggest an additional method to enhance delivery of chemotherapy immediately after an increase in shear stress. Further, our acute exercise regimen in mice would be roughly equivalent to brisk walking on an inclined treadmill for humans, a potentially feasible intervention for cancer patients. It would be of interest to investigate the activation of the calcineurin-NFAT pathway by examining TSP-1 levels in response to acute exercise. While TSP-1 has been implicated in tumor vascular normalization and angiogenesis associated with chronic exercise, it may be altered in acute exercise or may be effected in response to Dox treatment. In addition to the calcineurin pathway and
its activation in response to changes in calcium levels as a consequence of VEGF-mediated activation of endothelial cells, it would also be of interest to investigate the ISR pathway with particular interest in the PERK arm as changes in calcium levels also induces stress in the endoplasmic reticulum. Our studies investigating the role of PGC1-α in tumor progression showed no significant effect of PGC1-α overexpression on tumorigenesis. However, the LLC tumor cells utilized in these mice are an aggressive and rapidly growing tumor line and more subtle effects of PGC1-α overexpression may be lost. Studies should be repeated with an alternate, slower growing tumor model and VEGF levels should be measured in tumor bearing-transgenic mice with and without chronic exercise to determine how VEGF levels are effected. Another limitation to these studies is the fact that transgenic expression of PGC1-α was limited to type-II skeletal muscle fibers, it would be of interest to determine the effects on tumorigenesis if the PGC1-α transgene was conditionally activated in various cell types in the TME such as fibroblasts or endothelial cells or in distal organs that are common sites of metastasis such as the lungs and liver.
CHAPTER 5: Conclusions and Future Directions

Collectively, my thesis work has investigated the role of fibroblasts and endothelial cells in the tumor microenvironment (TME) in response to stress. My studies on fibroblasts demonstrate a role for the p19Arf tumor suppressor in fibroblasts in response to stresses of the TME. The loss of p19Arf in fibroblasts resulted in the increased activity and survival of primary lung fibroblasts during prolonged leucine deprivation (LD). Previous tumor studies have shown that p19Arf is typically inactivated in a reciprocal manner to p53, however, studies have also shown p53-independent functions of p19Arf. While p19Arf-loss in mice leads to tumor development, this has been attributed primarily to impaired apoptosis while studies examining the role of p19Arf during development indicate that it plays a critical role in determining the balance between the rate of proliferation and apoptosis. Published studies investigating the loss of p19Arf in mouse embryonic fibroblasts (MEFs) show that these cells arrest in response to DNA damage and can also bypass senescence during aging. However, there are no studies that have investigated the loss of p19Arf in adult fibroblasts. My work indicates that loss of p19Arf in adult lung fibroblasts are resistant to amino acid deprivation (AAD) primarily due to increased proliferation of p19Arf−/− cells in the absence of p53-mediated activation of cell cycle checkpoints. This rapid proliferation of p19Arf−/− null cells render these cells to be highly dependent on autophagy for survival during LD. This increase in fibroblast survival is tumor promoting in vivo as xenograft tumor models with wild-type (WT) p19Arf show increased tumor growth when transplanted into the flank of p19Arf-/- null mice, with loss of p19Arf in cells in the TME. The novelty of my work has identified a role for p19Arf in the fibroblasts in the TME, in addition to its
upregulation in response to prolonged LD. Future directions include investigating the effects of p19Arf reintroduction into the stroma of the TME on tumor progression, and its role in inhibiting tumor progression in the context of p53 deficiency. Future studies should also include investigating the role of p14ARF in the TME of human cancers.

Our work on endothelial cells in the TME in response to shear stress has identified an alternative approach to tumor vascular normalization to increase the efficacy of chemotherapy. My studies suggest that a single bout of acute exercise may enhance chemotherapeutic efficacy if drugs are administered immediately after a single session of aerobic exercise. My work demonstrates that attenuation in tumor growth rates occurs independent of tumor vasculature normalization and may be attributed to increased blood flow leading to more effective drug delivery to tumor beds. Future studies investigating acute exercise should investigate the mechanism underlying how acute exercise before treatment slows tumor growth and the effect of acute exercise on activation of endothelial signaling pathways.

My studies investigating the effect of increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) in skeletal muscles showed no effect on tumor growth rates or metastatic progression when exposed to chronic exercise. These studies did show that chronic exercise increased metastatic progression in both wild-type and PGC1-α transgenic mice. Although this effect was not specific to overexpression of PGC1-α in skeletal muscles, future studies should investigate the role of PGC1-α overexpression in chemotherapeutic efficacy in other cancer models with and without either chronic or acute aerobic exercise.
**P19Arf is induced in fibroblasts in response to prolonged leucine deprivation**

My work shows that primary lung fibroblasts promote tumor growth in response to stresses of the TME. In this study we showed that the loss of p19Arf in adult lung fibroblasts enhanced tumor growth rates of sarcoma cells with WT p19Arf. A caveat to our mouse studies is that the loss of p19Arf occurred in all cell types including immune cells such as macrophages. Other groups have shown that p19Arf is required to prevent the transition of tumor suppressing type-I macrophages from becoming type-II, tumor promoting macrophages. To address the effect of p19Arf loss specifically in fibroblasts, I utilized a 3D tumoroid model to limit the effect of tumor cell growth to primary lung fibroblasts. I generated 3D tumoroids by resuspending tumor cells and primary adult lung fibroblasts (ALFs) in Basement Membrane Extract (BME) that promote the formation of 3D-tumors with fibroblasts intercalating throughout the tumoroids. Using this system, we showed that the loss of p19Arf in primary lung fibroblasts enhanced overall cell number that was further increased during LD. The increase in fibroblasts was associated with an increase in the number of actively proliferating α-SMA positive fibroblasts. While the deprivation of many essential amino acids is a common stress in the TME due to the rapid proliferation of tumor cells and overall nutrient deprivation in the TME, deprivation of the essential amino acid leucine was utilized in my experimental systems because of its potent effect on suppressing activation of the mTOR pathway during long-term LD and its inhibition of cancer cell survival.

My data reveal the novel finding that prolonged LD induced p19Arf expression and activation of the integrated stress response (ISR) pathway in fibroblasts. ISR activation was even further increased in the absence of p19Arf. Interestingly, I found elevated levels of phosphorylated eIF2-α at baseline in both WT and p19Arf−/− primary
fibroblasts. One potential explanation could be an artefact of tissue culture as fibroblasts are activated in tissue culture due to the stiffness of tissue culture plastic. Studies have shown increased fibroblast activation on stiff substrates with tissue culture plastic log-fold stiffer than the extracellular matrix that fibroblasts are exposed to in vivo. This likely induced stress that was independent of AAD as the phosphorylation of eIF2-α is not exclusive to this stress\textsuperscript{128}. Despite the baseline activation of the ISR in both WT and p19Arf-null fibroblasts, my studies focused on investigating the effect of LD on fibroblast activation and the role of p19Arf.

In this study I show that the loss of p19Arf increased fibroblast function and survival during prolonged LD through increased proliferation that persisted during long-term exposure to stress. I confirmed that the increased proliferation rate of p19Arf-null fibroblast was sufficient to maintain fibroblast survival during long-term LD as the increased proliferation rate was greater than the rate of apoptosis. The treatment of p19Arf-null fibroblasts with mitomycin C (Mito C) to inhibit cellular proliferation revealed the dependence on increased proliferation of p19Arf-null fibroblasts for survival during long-term LD. My data also found that increased fibroblast activation and function continued during prolonged LD. My data show an increase in collagen invasion by p19Arf-null fibroblasts although the number of cells that migrated entirely through the collagen bed was limited. Thus it is possible that the collagen gel prevented a nutrient gradient between cells in a serum-free chamber to the nutrient replenished media. It would be of interest to assess migration of p19Arf-null fibroblasts as compared to WT fibroblasts in the presence of tumor-conditioned media. I utilized a hydroxyproline assay to quantify collagen production by measuring hydroxyproline as a surrogate for collagen levels. I measured hydroxyproline in conditioned media from p19Arf-null and WT
fibroblasts during invasion assays. However, fibroblast invasion in this context does not necessarily require an increased production of collagen but instead the degradation of collagen that is required for cells to migrate through the collagen bed. Thus my data indicate a decrease in hydroxyproline in conditioned media collected from p19Arf-null fibroblast invasion assays as compared to WT fibroblast invasion assay. It would be of interest to compare the rate of collagen deposition by treating fibroblasts with ascorbic acid, which forces the production of collagen by these cells. The loss of p19Arf did not prevent apoptosis as measured by Annexin V during prolonged stress and I saw no differences in cell death between WT and p19Arf fibroblasts. However, possible caveats to this data are that Annexin V is an early marker of apoptosis thus may not be an accurate indication of cell death in these populations and studies that have shown p19Arf can induce cell death through autophagy independent of conventional apoptosis pathways.

Finally, my research investigated the role of autophagy as a survival mechanism by p19Arf-null fibroblasts to cope with prolonged LD. My data shows increased utilization and dependence on autophagy pathways to survive this long-term stress. At baseline, I show increased expression of the two autophagy markers p62 and LC3-II in p19Arf−/− fibroblasts when compared with WT fibroblasts that was maintained when exposed to prolonged LD. We also confirmed the necessity of autophagy through treatment with the autophagy inhibitor bafilomycin preventing lysosomal degradation of LC3-II revealing increased levels in p19Arf−/− fibroblasts at later time points indicating an increase in autophagic flux. Since bafilomycin treatment and probing for LC3-II expression is an efficient readout to indicate differences in autophagic flux, further studies should compare the amount of lysosomal LC3-II between groups through the labelling of LC3.
with the mCherry-GFP marker. It would also be of interest to determine how \( p19Arf \) effects the expression of autophagy-related genes, such as \( ATG5 \), and how their deletion affects \( p19Arf^- \) fibroblast survival during LD. Overall, our data indicate that the \( p19Arf \) tumor suppressor plays a role in the ISR pathway in primary fibroblasts to induce apoptosis during prolonged AAD to inhibit tumor progression.

**Future directions to identify the effects the loss of stromal \( p19Arf \) in vivo**

Moving forward, identifying the role of \( p19Arf \) and the ISR pathway in primary fibroblasts should be further investigated *in vivo* and in human cells in response to prolonged LD. While antibodies specific for mouse phosphorylated GCN2 (P-GCN2) are not available, a human-specific P-GCN2 antibody is commercially available and can be utilized on human fibroblasts that lack \( p14ARF \) that have been exposed to prolonged LD to compare activation of the ISR. It would also be of interest to compare levels of ATF4 in the context of \( p19Arf^- \) in response to stress in the TME. While most studies have studied ATF4 in the context of total protein expression, notably, nuclear ATF4 is the activated form of the protein and is an indication of its activity level in addition to mRNA levels\(^ {130} \). *In vivo*, studies investigating the role \( p19Arf \) in the TME could be further explored by targeting \( p19Arf \) utilizing a tissue specific and inducible model to conditionally delete exon 2 of \( CDKN2A \) to specifically target \( p19Arf \), and not \( p16ink4 \)\(^ {131} \). While my thesis research has focused on the deprivation of leucine to primary fibroblasts, future studies would also investigate not only the other essential and conditional-essential amino acids, but also other stresses of the TME. Previous studies have shown that mice on a leucine-free diet can prevent the growth of breast cancer
xenograft\textsuperscript{53}, thus it would be of interest to utilize a leucine-restricted diet for mice with a fibroblast specific deletion of \textit{p19Arf} and determine its effects on tumor progression. Additionally, certain cancer types are more reliant on autophagy to promote tumor progression, thereby presenting a targetable, therapeutic window. Rangwala \textit{et al.}, have shown in phase I clinical trials that the utilization of hydroxychloroquine in combination with temozolomide, an anti-tumor alkylating agent, in a final dose cohort had a complete response and prolonged stable disease in melanoma patients\textsuperscript{132}. Combining both a leucine restricted diet to create a greater dependence on autophagy, together with hydroxychloroquine and chemotherapy could enhance the effectiveness of cancer treatment.

Future studies should also investigate the role of LD on activation of the mTOR pathway in fibroblasts of the TME and the role of the \textit{p19Arf} tumor suppressor. While a previous study has shown that deletion of \textit{p19Arf} is necessary to maintain activation of the mTOR pathway, it would be of interest to determine if \textit{p19Arf} \textsuperscript{−/−} fibroblasts are reliant on mTOR during stress to determine its potential as a therapeutic target\textsuperscript{95}. In this current study, I primarily investigated overall induction of autophagy, but there are varying types of autophagy that occur within the cell. Other types of autophagy should also be investigated in greater depth considering the localization of the \textit{p19Arf} isoform, \textit{smARF} to the mitochondria. It would be of interest to determine if \textit{smARF} played a role in the regulation and processing of mitophagy in cancer\textsuperscript{133–135} and to also determine if \textit{smARF} plays a role in the process of mitochondrial fission and fusion, and whether it is altered in cancer\textsuperscript{136,137}. Future studies should also investigate the recycling of the ribosome, ribophagy\textsuperscript{138–140}, not only due to its role in cancer progression, but also because of
previous studies that have shown that one of the p53-independent functions of p19Arf including the regulation of ribosome biogenesis\textsuperscript{94,141}.

**Acute exercise provides therapeutic window**

My work has shown an intervention by which a single session of aerobic exercise prior to the administration of the chemotherapy drug doxorubicin (Dox) was sufficient to attenuate melanoma growth rates *in vivo*. Interestingly, the administration of Dox prior to exercise had no effect on tumor growth and even showed an increase in tumor growth rates in mice when compared with their sedentary control counterparts. One reason for this may be the altered rate of metabolism when the drug is administered. Although our group has previously shown that the presence of Dox within the tumor is increased when mice were subject to a chronic exercise regimen, we also showed that Dox levels in the tumor bed were similar in the acute exercise group as compared with our sedentary group when Dox was administered before exercise\textsuperscript{25}. Further studies should also quantify the amount of Dox present within the tumor when administered after exercise. It is possible that the administration of drug prior to exercise led to increased clearance of Dox due to aerobic exercise-mediated increase in shear stress. Further studies should also measure the amount of drug in the liver to compare the clearance rate of Dox. The effects of acute aerobic exercise may be limited to specific drugs as our data did not see any difference when the drug Gemcitabine was administered either before or after exercise in our pancreatic cancer xenograft mouse model. Since the addition of exercise has been prescribed to enhance the quality of life of pancreatic cancer patients, we anticipated that exercise would alter pancreatic tumor growth\textsuperscript{142}. However, the xenograft
model we utilized inoculated syngeneic murine pancreatic ductal adenocarcinoma cells into the flanks of WT mice and this tumor model has limited physiologically relevance to pancreatic cancer patients. Further studies should utilize one of the many well-characterized genetically engineered mouse models of pancreatic cancer that closely recapitulates the clinical course of pancreatic cancer. Future studies should also investigate other tumor types to determine whether aerobic exercise could be beneficial. Additionally, to quantify the extent of shear stress-induced by acute exercise, endothelial nitric oxide synthase can be measured within the tumor and other tissues to determine how one session of acute exercise alters shear stress.

**Future directions to investigate chemotherapeutic efficacy during acute exercise**

Overall, this study has revealed acute aerobic exercise as an approach to potentially increase chemotherapy efficacy *in vivo*. Future studies will investigate activation of the calcineurin-NFAT pathway in endothelial cells to determine whether it is altered in response to acute exercise. Studies to determine the role of the unfolded protein response (UPR) in endothelial cells in response to increased shear stress would also be of great interest. As mentioned in Chapter 1, the UPR pathway is activated in response to changes in calcium levels and thapsigargin is a drug utilized to stress the endoplasmic reticulum (ER) by depleting calcium within cells. Readouts of the ER stress pathway include activation of PKR-like ER kinase (PERK) resulting in activation of the ISR pathway. One caveat to this could be the overlapping stresses inducing activation of the ISR as shear stress may also activate the heme-regulated eIF2α kinase (HRI) arm of the ISR pathway, and even conditional deletion of specific arms of the ISR may lead to
compensation as previous work has shown in the context of glutamine deprivation. While anti-VEGF therapies and chronic exercise provide approaches for tumor vascular normalization and increasing tumor susceptibility to chemotherapy, perhaps the utilization of anti-VEGF therapy combined with acute exercise would also lead to increased chemotherapy efficacy. A caveat to this is that the increased amount of VEGF produced during exercise may be utilized by the tumor to promote growth. While this poses a potential conflict, utilizing low doses of anti-VEGF therapy to sequester VEGF combined with the increased circulation of the chemotherapeutic agent following acute exercise may provide similar benefits to chronic exercise.

**PGC1-α overexpression has no effect on tumor progression**

In collaboration with the Arany lab, I have shown that peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) overexpression in skeletal muscle has no effect on tumor growth rates or metastasis to the lungs of mice during exercise. In this model, the use of transgenic mice that overexpressed PGC1-α in skeletal muscles were compared to WT littermate control mice. Both cohorts of mice were inoculated with LLC tumor cells in their flanks with tumors resected after reaching 500mm³ volume before initiating a chronic aerobic exercise program for two weeks. After this time period, mice were euthanized and examined for lung metastasis with no significant differences observed between experimental and control cohorts. Future studies could alter the experimental model such that primary tumors were resected at smaller volumes or the use of slower growing tumor cells to ensure that tumors were still fully encapsulated at the time of resection. While these experiments indicate that PGC1-
α overexpression in skeletal muscles do not have an effect on tumor growth and metastasis, it would be of interest to compare the effects that overexpression of PGC1-α or other members of the PPAR family in cell types within the TME may have on tumor progression.

**Future directions to investigate the role of PGC1-α on chemotherapeutic efficacy**

While PGC1-α plays a critical role in cellular metabolism, it would be of interest to combine chemotherapeutic treatment of tumor bearing mice with our single session acute exercise model to determine if the enhanced metabolism by these mice allows for increased clearance or penetrance of the drug within a tumor bed. Future studies comparing the effects of chronic or acute exercise on tumor growth and progression on the background of PGC1-α overexpression will be of great interest.

**Clinical Implications**

Expression levels of the p14ARF tumor suppressor has been suggested to serve as a marker of tumor progression in certain cancer models. In this study I've shown that AAD induces expression of p19Arf expression in primary lung fibroblasts. While p14ARF has been reported to function in either a tumor suppressing or promoting manner depending on the context and on the specific cancer type, my work demonstrates that the p19Arf mouse homolog functions in fibroblasts in the TME to suppress tumor growth and that its loss or potential silencing in fibroblasts in the TME enhances tumor growth and fibroblast function and survival through autophagy. My
research suggests that the role of classic tumor suppressors should be further investigated in cellular populations in the TME. I have also shown that acute exercise increases therapeutic efficacy when administered after exercise. This timing of drug delivery, before or after acute exercise, may have important clinical applications as a single bout of brisk walking on a treadmill prior to drug infusion may be a feasible intervention for cancer patients undergoing chemotherapy. Future studies could identify the cancer types that may be susceptible to acute exercise combined with chemotherapy and this approach could be tested in a clinical trial for patients willing and able to undertake acute exercise during chemotherapy.

In conclusion, my thesis work has shown a role for p19Arf in response to AAD in the TME that suppresses tumor growth through regulating fibroblast survival and activation. While the deletion of the P19Arf in the TME may not be physiologically relevant, it is possible that p14ARF expression may be epigenetically silenced and should be investigated. My thesis research has also shown that a single session of acute aerobic exercise followed by chemotherapy treatment was sufficient to suppress tumor growth, which can lead a feasible intervention for cancer patients.
Primary lung fibroblasts isolation and culture

Fibroblasts were cultured in DMEM-F12 + L-glutamine (Gibco) with 10% heat inactivated FBS, L-glutamine, and penicillin-streptomycin. To isolate primary lung fibroblasts, lungs from male and female 3-5-week-old mice were minced into small pieces with scissors then dissociated in Hank’s buffered saline solution (HBSS) containing 5 mg/ml type II collagenase and 0.5 mg/ml deoxyribonuclease I (Worthington, #LS004176 and #LS002139) with shaking in Thermo Scientific MaxQ 5000 floor shaker at 37°C at 250 rotations per minute for 45 minutes. Dissociated lungs were passed through 100μm and 40μm filters to obtain a single cell suspension before resuspending in culture media and plating; fibroblasts were allowed to adhere for 1-2 hours at 37°C before non-adherent cells were washed off. Fibroblast identity was confirmed by immunostaining cultured cells for vimentin (goat, Santa Cruz #sc-7557, 1:100), CD45.2 (biotinylated mouse, BD Pharmingen #553771, 1:100), and CD31 (rat, BD Pharmingen #553370, 1:100), followed by secondary antibody and streptavidin (Alexa Fluor 647 anti-goat IgG, Alexa Fluor 488 anti-rat IgG, Alexa Fluor 555 streptavidin, all 1:100: Invitrogen #A-21447, A-11006, Thermo Fisher #S-21381 respectively); fibroblasts were >99% vimentin-positive, with <5% CD45+ contaminants and no CD31+ cells present.

Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA, 50 mmol/L Tris-HCl pH 8, 150 mmol/L NaCl, 1% Triton-X, 0.5% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail followed by centrifugation at 16,000 x g for 10
minutes, and supernatant was collected. Total protein content was quantified using BCA assay (Thermo Scientific). Protein loading of fibroblast lysates was normalized using the protein standard curve in the Bio-Rad Protein Assay Kit on samples diluted 1:10. Total protein from fibroblast lysates was analyzed by running samples in 4x Laemmli sample buffer (Bio-Rad) on a 4-12% gradient gel (GenScript). SDS-PAGE of lysates was performed using 5-20μg per sample at 100-110 V on 8, 10, or 12% gels depending on the size of the protein in question using the Bio-Rad Mini Protean Tetra Cell system. Proteins were transferred onto PVDF membranes at 100 V for 1 hour using the Bio-Rad Mini Trans-Blot system. Blots were blocked in 5% non-fat dry milk (LabScientific M-0841) or 5% BSA (Roche, 03-116-956-001) in TBS-T (TBS + 0.1% Tween 20) and incubated with primary antibody diluted in blocking buffer at 4°C overnight (p19ARF: 1:500, Novus Biologicals #NB200-174; temperature (p19ARF (1:500; Novus Biologicals ; #NB200-174), ATF4 (1:1000; Cell Signaling Technology; #11815), phospho-eIF2α (Ser51) (1:500; Cell Signaling Technology; #3597) total-eIF2α (1:1,000; Cell Signaling Technology; #9722), LC3B (1:1,000; Cell Signaling Technology; #2775S), p62 (1:1,000; Cell Signaling Technology; #5114), and NBR1 (1:1,000; Cell Signaling Technology; #9891). Anti-Beta tubulin (1: 1,000; # 2128) and α-actin (1:10,000, Sigma #A2668) was used as loading control. Blots were washed in TBS-T, and secondary antibodies (Horseradish peroxidase–conjugated anti-rabbit (1:2,000; Cell Signaling; #7074), anti-mouse (1:2,000; Cell Signaling Technology; #7076), or goat anti-rat (1:2,000; Cell Signaling Technology; #7077) were incubated in blocking buffer for 1-2 h at room temperature then washed with TBS-T. Bands were visualized using enhanced chemiluminescence reagent (100 mM Tris pH 8.6, 0.2 mM p-coumaric acid, 1.25 mM luminol, 2.6 mM).
**Subcutaneous xenografts**

All animal experiments and subcutaneous (s.c.) xenografts were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Six wild-type and 6 p19Arf−/− (3 male and 3 female mice per group) mice between 6 and 8 weeks old (purchased from Jackson Laboratory) had $1.34 \times 10^5$ SKPY cells implanted s.c. in their flanks. Prior to injection, cells were grown in complete media (DMEM containing 10% FBS). Cells were collected, resuspended in ice-cold serum-free DMEM for injection. For co-injection experiments, a total of $3.75 \times 10^5$ cells (lung fibroblasts: LLC 1:1) were resuspended in ice-cold serum-free media and mixed with Basement Membrane Extract (Millipore Sigma, #3533-005-02) at a ratio 1:1. The final volume per injection for each experiment was 200μL. For acute exercise experiments, animals received 300,000 melanoma (B16F10) or pancreatic ductal adenocarcinoma (PDAC 4462) to their subcutaneous flank and tumors were allowed to grow for 10 days. Mice received doxorubicin (2mg/kg) or gemcitabine (15mg/kg) chemotherapies for melanoma or pancreatic cancer models, respectively, before exercise on day 11 via intraperitoneal injections. Tumor bearing mice were exercised for 60 minutes at a pace of 12m/min using the Columbus Instruments Exer 3/6 Animal treadmill and were returned to housing to have tumor volume measured by calipers until the experimental endpoint. For PGC1-α transgenic mice tumor experiments, mice were provided by Dr. Zolt Arany (Perelman School of Medicine, University of Pennsylvania). PGC1-α transgenic and WT mice received $1 \times 10^6$ Lewis Lung Carcinoma (LLC) cells inoculated subcutaneously in their flank and tumor volume monitored using calipers. Tumors were surgically resected when they reach 500-800mm$^3$ in volume. After three days of recovery, mice were exercised for 45 minutes at a pace of 12m/min 5 times a week for two weeks. After the final
exercise session, mice were sacrificed, and lungs were inflated with formalin followed by paraffin embedding. Tumor volumes were recorded at the indicated timepoints using caliper measurements. The formula, \( V = (L)(W^2) (\pi/6) \), was used to calculate tumor volume. Tumors were harvested and flash frozen in OCT compound for further analyses.

**Aerobic Exercise**

Mice were inoculated with 300,000 tumor cells in 200\( \mu \)l PBS subcutaneously in the flank. When tumors reached \(~100 \text{ mm}^3\) (4–7 days post injection) or the indicated volume, mice began treatment. For both B16F10 and PDAC tumor-bearing mice, acute exercise plus chemotherapy groups performed one session of 60 minutes of treadmill running once at 12 m/min. For PDAC tumor-bearing mice, gemcitabine was delivered once by intraperitoneal (IP) injection before or after exercise. For B16F10 tumor-bearing mice, acute exercise plus doxorubicin groups performed one session of 60 minutes of treadmill running at 12 m/min. Mice received 2 mg/kg of doxorubicin by tail vein injection before or after exercise. After the final tumor measurement, mice were euthanized, and tumors were harvested and frozen in OCT.

**Tumoroid Assay**

Tumor cells were resuspended in Basement Membrane Extract (BME) and layered onto a BME bed containing fibroblasts then exposed to complete or leucine deprived media. Three-dimensional tumoroids were imaged every two days for a week, with cell numbers and sizes were quantified. Tumoroids were collected and dissociated to acquire single cell suspension, concentrated via cytopsin and stained for Ki67 and alpha smooth muscle actin (\( \alpha \)-SMA) (1:100, Abcam, #56947). Alexa Fluor 488-donkey-anti-sheep IgG (1:500, Novus Biologicals, #NBP1-75446), Alexa Fluor 594-goat-anti-rabbit IgG.
Fluorescent images were captured with a laser scanning confocal microscope. Confocal imaging was performed on a Leica TCS SP5 and processed using LAS AF software.

**GCN2 Inhibition**

Fibroblasts were treated with GCN2-IN-1 (MedChem Express HY-100877) at a final concentration of 1µM in leucine deprived media overnight and cells harvested for analysis.

**Autophagy Inhibition**

Fibroblasts were exposed to leucine deprived media and exposed to Bafilomycin (Cayman Chemicals #11038) at a final concentration of 1nM in DMSO 2 hours before harvesting lysates and probing for LC3. In survival assays, fibroblasts were treated chloroquine (Sigma-Aldrich # C6628) at a final concentration of 100nM in leucine deprived media for the times indicated.

**Cell Cycle Arrest**

Fibroblasts were treated with Mitomycin C (Sigma Aldrich #M4287-2MG) at a final concentration of 4µg/mL in culture media and incubated for 6 hours before washing with cells replated in complete media. Cell cycle arrest was confirmed by the absence of EdU incorporation.

**Migration Assay**

Cells were grown to confluency in 12-well plates in triplicate. A scratch was generated with a 200-µL tip across each well and pictures taken at the starting timepoint, and at 2-4 hour increments post-scratch until complete scratch closure. The percentage of area that
was “repaired” was measured using ImageJ software and plotted as the average of the triplicates with standard deviation (SD). Experiments were repeated three times.

qRT-PCR

Total RNA was processed and extracted with Trizol reagent (Life Technologies, catalog no. 15596018) and Direct-zol RNA MicroPrep Kit (Zymo Research, #R2060). RT reaction was performed using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, #4387406). qRT-PCR was then performed using SYBR Green Master Mix (Bimake, #B21202) and a ViiA7 Real-Time PCR Instrument (Applied Biosystems). SYBR probes were used to quantify the expression of p19Arf (Forward: 5’ AGA GGA TCT TGA GAG GGC C 3’; Reverse: 5’ GCA GTT CGA ATC TGC ACC G 3’). Normalization was performed using the housekeeping genes 18S (Forward: 5’ CAATTACAGGGCCTCGAAAG 3’; Reverse: 5’AAACGGCTACCACATCCAAG). Samples were performed in triplicate with each experiment repeated twice.

Hydroxyproline assay

Media was collected from transwells during collagen invasion assays 48 hours post-plating. Hydroxyproline levels were measured using the colorimetric Hydroxyproline Assay Kit (Sigma-Aldrich, #MAK0081KT) per manufacturer’s instructions to determine hydroxyproline content as a surrogate for collagen levels.

Immunostaining
Cells were seeded onto sterile round coverslips (12 mm) on parafilm covered 10cm dishes at a density of 12,500 cells per coverslip. Cells were cultured in their respective media at 37°C, 5% CO₂ for the times indicated. After treatment, EdU proliferation assays were performed with Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen, #C10339) according to manufacturer's instructions; fibroblasts were pulsed with 10 mM EdU for 16–18 hours before fixation and staining. Coverslips were mounted face-down onto microscope slides using Vectashield anti-fade mounting medium (Vector Laboratories). Images were acquired with laser scanning microscope Zeiss LSM 510 with 63 × objective lens (Carl Zeiss AG). All microscope parameters were held constant across samples. At least nine different areas were imaged per sample.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 8 software, using unpaired Student two-tailed t test. Data are presented as mean ± SEM of at least three independent experiments unless indicated that standard deviation was used. Statistical significance was defined as ***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s., not significant.


46. Ziello, J. E., Jovin, I. S. & Huang, Y. Hypoxia-Inducible Factor (HIF)-1 regulatory


54. Karolina Pakos-Zebrucka1, 2,†, Izabela Koryga1, 2,†, Katarzyna Mnich1, 2, Mila Ljujic1, 2, Afshin Samali1, 2 & Adrienne M Gorman1, 2. The integrated stress response. *EMBO Rep.* **17**, 1374–1395 (2016).


134. Pimkina, J., Humbey, O., Zilfou, J. T., Jarnik, M. & Murphy, M. E. ARF induces


