Mechanisms and Impact of Hypoxia Regulated Inhibins in Cancer

Ben Allen Horst

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MECHANISMS AND IMPACT OF HYPOXIA REGULATED INHIBINS IN CANCER

by

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Bachelor of Science
Furman University, 2016

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For the Degree of Doctor of Philosophy in
Chemistry
College of Arts and Sciences
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DEDICATION

This work is dedicated to my wife, Margaret, my parents, and my brother for the love and support you gave me throughout this process.
ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Mythreye Karthikeyan for your support and guidance over the last five and a half years. Through my early years as a scientist learning how to conduct experiments and problem solve, a move across the south, a pandemic, paper submission/revision, and a job search you have provided me invaluable direction through each phase, and I know I would not be where I am without you. I would also like to thank my committee members who have been incredibly patient with me even though I know I am not the best communicator.

To all my fellow lab members, past and present, thank you for making going to work an enjoyable experience. Laura, Priyanka, Lauren, and Shrikant thank you for serving as mentors to me in the lab and making my early growth as a scientist possible. Moe, Mehri, Asha, Elizabeth, and Liz thank you for being amazing lab mates, always being there to talk about experiments or life, and keeping the lab running smoothly. Choi and Kevin, thank you for being my best friends, gym buddies, and lunch dates throughout grad school.

Finally, I would like to thank my loved ones. Margaret, thank you for always being there for me through the good days and the bad. For sacrificing work to move to Birmingham with me and once again moving with me to Boston to start a new phase of our lives. To my parents and brother, thank you for your love and support throughout my life. I would not be the man I am today without your constant love and support.
ABSTRACT

Inhibins are heterodimeric ligands within the TGFβ superfamily, comprised of an α-subunit (INHA) and a β-subunit (either INHBA or INHBB) with emergent roles in cancer. Inhibins are biomarkers of disease burden and prognosis in a subset of cancers and utilize the coreceptors betaglycan (TGFBRIII) and endoglin (ENG) for physiological or pathological outcomes. Previously, we found inhibin promotes angiogenesis in ovarian cancer however no mechanism of regulation for inhibin expression in cancer has been established. Hypoxia, a driver of tumor growth and metastasis, regulates angiogenic pathways that are targets for vessel normalization and ovarian cancer management. However, toxicities and resistance to anti-angiogenics can limit their use making identification of new targets vital. Inhibin is a particularly interesting target for anti-angiogenic therapy within ovarian cancer, as expression is abrogated in healthy post-menopausal women when compared to premenopausal women, but markedly elevated in those with ovarian malignancies. Of note, postmenopausal women make up the majority of ovarian cancer diagnoses, with an average age of diagnosis of sixty-two. In these patients, targeting inhibin may not present the toxicities associated with other anti-angiogenic therapies. Here, we look to establish a mechanism of regulation of inhibin in cancer, define inhibin as a therapeutic target, and assess the prognostic value of inhibin and its network of receptors (endoglin and betaglycan) in a pan-cancer analysis. These studies reveal that inhibin, specifically INHA, is regulated by hypoxia through the transcription factor HIF-1. Hypoxia regulated inhibin
promotes tumor growth, endothelial cell invasion and permeability. Targeting inhibin in vivo through knockdown and anti-inhibin strategies robustly reduces permeability in vivo and alters the balance of pro and anti-angiogenic mechanisms resulting in vascular normalization. A pan-cancer, bioinformatics analysis revealed gene alterations and identified cancer cell lines and types that were most dependent and impacted by members of the inhibin network. Expression of these markers was predictive of both survival and response to chemotherapeutics. Using cancer types where inhibin, endoglin and betaglycan were predictive of survival, gene signature analysis was performed to obtain a highly accurate prognostic model and to determine correlated pathways. Ultimately, these findings provide new insights into the therapeutic potential of inhibin as an anti-angiogenic target in ovarian cancer and shed light on the importance of the inhibin-endoglin-betaglycan network in other cancer types as well.
## TABLE OF CONTENTS

Dedication ......................................................................................................................... iii  
Acknowledgements ........................................................................................................ iv  
Abstract .............................................................................................................................. v  
List of Tables ......................................................................................................................... ix  
List of Figures ....................................................................................................................... x  
List of Abbreviations ........................................................................................................... xii  
Chapter 1: Introduction and Background ........................................................................... 1  
  1.1 Structure and Function of Inhibins ............................................................................. 2  
  1.2 Transcriptional Regulation of INHA ......................................................................... 4  
  1.3 Inhibin Signaling Pathway and Mechanism of Action ................................................. 5  
  1.4 Inhibin as a Diagnostic Marker in Cancer ................................................................. 6  
  1.5 Roles for Inhibin in Cancer ....................................................................................... 7  
  1.6 Hypoxia and Angiogenesis in Ovarian Cancer ......................................................... 9  
Chapter 2: Hypoxia induced inhibin promotes tumor growth and vascular permeability in ovarian cancers ................................................................................................. 14  
  2.1 Introduction .............................................................................................................. 15  
  2.2 Materials and Methods ............................................................................................. 17  
  2.3 Expression and secretion of inhibin is regulated by hypoxia in ovarian cancer cell lines ......................................................................................................................... 27  
  2.4 Inhibinα is increased in ovarian cancer spheroids, patients, and tumor xenografts .......................................................... 30
2.5 INHA is a direct HIF-1 target under hypoxia.................................32

2.6 Inhibin promotes hypoxia induced angiogenesis and stimulates endothelial cell migration and vascular permeability...............................35

2.7 Inhibin promotes vascular permeability through increased VE-cadherin trafficking .................................................................38

2.8 Inhibin’s effects on vascular permeability are mediated by ALK1 and CD105/endoglin that form a stable complex at the cell surface in response to inhibin .........................................................39

2.9 Inhibin promotes hypoxia induced tumor growth in vivo through alterations in permeability and angiogenesis ........................................42

2.10 Discussion ...................................................................................46

Chapter 3: A bioinformatic analysis of the inhibin-betaglycan-endoglin/CD105 network reveals prognostic value in multiple solid tumors ........................80

3.1 Introduction ..................................................................................81

3.2 Materials and Methods .................................................................84

3.3 Inhibins and activins are altered in human cancer .........................87

3.4 Effect of inhibins and the coreceptors on patient survival
varies by cancer type........................................................................89

3.5 Inhibins and activins can predict response to chemotherapy in
luminal A breast cancer ..................................................................92

3.6 Gene signatures from inhibins can predict patient outcomes ..........93

3.7 Functional analysis and interpretation of inhibin’s mechanism of action ..........95

3.8 Discussion and Conclusions .........................................................97

References ........................................................................................116

Appendix A: Inhibin expression in HEY tumors and other cancer cell lines ..........160

Appendix B: Permissions to Reprint ..................................................169
LIST OF TABLES

Table 2.1 Key Resource Table ........................................................................................................53

Table 2.2 Lentiviral and siRNA sequences .......................................................................................58

Table 2.3 List of primers ....................................................................................................................59

Table 3.1 p values and Hazard Ratios (HR) from survival curves assessing the impact of
INHA, INHBA, INHBB, TGFBR3, and ENG on patient survival ..............................................105

Table 3.2 p values and Hazard Ratios (HR) from survival curves assessing the
relationship between TGFBR3/ENG and INHA on patient survival ...............................107

Table 3.3 Prognostic performance of each delineated probit model .................................109

Table A.1 Patient information for inhibin ELISA on ascites fluid .....................................166

Table A.2 INHA expression in cancer cell line panel in response to hypoxia ..........168
LIST OF FIGURES

Figure 1.1. Structure of inhibin.................................................................12

Figure 1.2. Inhibin signaling diagram in epithelial and endothelial cells .................13

Figure 2.1. INHA is increased in response to hypoxia in ovarian cancer cells ..........60

Figure 2.2. Inhibin protein is secreted by ovarian cancer in response to hypoxia. ....62

Figure 2.3. Inhibinα is increased in ovarian cancer spheroids, patient samples, and tumor xenografts.................................................................63

Figure 2.4. HIF-1 is required for INHA expression under hypoxia ......................65

Figure 2.5. INHA is a direct target of HIF-1 ..................................................66

Figure 2.6. Inhibin increases hypoxia induced angiogenesis and endothelial cell migration and permeability in vivo and in vitro respectively ........................................68

Figure 2.7. Inhibin increases endothelial cell contractility ...................................70

Figure 2.8. Inhibin promotes VE-cadherin internalization in endothelial cells ........71

Figure 2.9. Inhibin promotes endothelial cell permeability via ALK1 and endoglin ....72

Figure 2.10. Inhibin increases ALK1-endoglin cell surface complexes while reducing ALK4-endoglin complexes .................................................................74

Figure 2.11. Targeting inhibin decreases in vivo subcutaneous growth ...............76

Figure 2.12. Targeting inhibin alters vasculature in subcutaneous xenografts ..........77

Figure 2.13. Inhibin is correlated with CA125 in patient ascites ..........................79

Figure 3.1. Expression and gene alterations of inhibin and activins ....................110

Figure 3.2. Impact of INHA, INHBA, INHBB, TGFBR3, and ENG on patient survival in indicated cancers .................................................................112
Figure 3.3. ROC plots and gene expression of indicated genes for different chemotherapy regimens ........................................................................................................113

Figure 3.4. Gene signatures and expression patterns for cancers where \textit{INHA}, \textit{TGFBR3}, or \textit{ENG} predicted survival outcomes ..................................................................................114

Figure 3.5. Functional analysis of gene signatures between \textit{INHA} and \textit{TGFBR3} and \textit{INHA} and \textit{ENG} ..........................................................................................................................115

Figure A.1. Inhibin protein is detectable in HEY tumors .....................................................................163

Figure A.2. Inhibin expression in patient ascites fluid ..........................................................................165
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTRII</td>
<td>activin type 2 receptor</td>
</tr>
<tr>
<td>ALK1</td>
<td>activin receptor-like kinase 1</td>
</tr>
<tr>
<td>ALK 2</td>
<td>activin receptor-like-kinase 2</td>
</tr>
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<td>ALK 7</td>
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<td>BMP9</td>
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<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ENG/CD105</td>
<td>endoglin</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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FDR.................................................................false discovery rate
FITC.....................................................................fluorescein isothiocyanate
FRAP.................................................................fluorescent recovery after photobleaching
FSH.....................................................................follicle stimulating hormone
Fsk.......................................................................forskolin
GFP......................................................................green fluorescent protein
HAS.................................................................hypoxia ancillary sequence
HIF.....................................................................hypoxia inducible factor
HMEC-1..........................................................human microvascular endothelial cells
HR......................................................................hazard ratio
HRE.................................................................hypoxia response element
KM Plot..........................................................Kaplan Meier Plot
KO.....................................................................knockout
IL-8 .................................................................interleukin 8
LH..................................................................luteinizing hormone
LPS..................................................................lipopolysaccharide
MEEC ..........................................................mouse embryonic endothelial cell
MLC..................................................................myosin light chain
mRNA................................................................messenger RNA
NBF..................................................................neutral buffered formalin
OS....................................................................overall survival
PKA..................................................................protein kinase A
PFS..................................................................progression free survival
qRT-PCR................................................quantitative real-time polymerase chain reaction
RFS .................................................................relapse free survival
ROC ...............................................................receiver operating characteristic
TGFβ.............................................................transforming growth factor beta
TNBC ............................................................triple negative breast cancer
TRC105..........................................................Tracon 105
VEGF ...........................................................vascular endothelial growth factor
CHAPTER 1

INTRODUCTION AND BACKGROUND
1.1. Structure and Function of Inhibins

Inhibins are dimeric members of the TGFβ superfamily originally discovered and named for its ability to suppress FSH secretion and activin signaling\(^1\)\(^-\)\(^3\). Inhibins are heterodimers linked by a disulfide bond made up of an alpha subunit (INHA) and a beta subunit (INHBA or INHBB) giving rise to either inhibin A (INHA/INHBA) or inhibin B (INHA/INHBB)\(^2\)\(^,\)\(^4\)\(^,\)\(^5\) (Figure 1.1A). Inhibin has a common beta subunit with fellow TGFβ member activin however activins are comprised of homodimers of the beta subunits, forming activin A (INHBA/INHBA) or activin B (INHBB/INHBB) (Figure 1.1C). Both alpha and beta subunits of inhibin are synthesized as precursor molecules with large, cleavable pro-domains\(^5\)\(^,\)\(^6\) (Figure 1.1A). The precursor inhibinα subunit contains a pro-domain and αN domain that are both cleaved separately to produce the mature 18kDa αC domain. The beta subunit has a large N-terminal pro-domain that is cleaved to produce the mature 13kDa βA or βB protein (Figure 1.1A). Interestingly, cleavage of the inhibinα subunit is not required for biological activity of inhibin protein as non-cleavable mutants retained ability to inhibit FSH secretion\(^6\).

Inhibin (inhibinα) is primarily expressed and secreted in the gonads, specifically by the granulosa cells in ovarian follicles and the Sertoli cells in the testes, in females and males respectively\(^7\)\(^-\)\(^10\). Expression of inhibinα is also found outside of the gonads including the pituitary\(^10\)\(^,\)\(^11\), prostate\(^12\), and adrenal glands\(^10\)\(^,\)\(^13\), lung\(^11\), liver, kidney, and bone\(^10\) indicating biological roles outside of the reproductive organs. However, circulating inhibin is primarily produced by the gonads as removal of these organs in mice results in undetectable levels of serum inhibins\(^14\). Females produce both inhibin A and inhibin B. Production of either inhibin A or B driven by beta subunit expression with
the dominant follicles and corpus luteum producing inhibin A and the small antral follicles producing inhibin B\textsuperscript{15-17}. In males, however, inhibin B is the only detectable form of inhibin\textsuperscript{18}.

The use of transgenic mice provided much insight into the physiological function of inhibin. \textit{INHA}\textsuperscript{-/-} mice form gonadal tumors by four weeks that secrete increased levels of activin resulting in tumor cachexia/wasting syndrome and death\textsuperscript{19,20}. However, removal of the gonads from these animals still results in lethality as they develop adrenal cortical tumors\textsuperscript{20}. These mice are also infertile and display high levels of FSH indicating roles in reproduction and in the regulation of FSH\textsuperscript{19}. Overexpression of \textit{INHA} (inhibin\textalpha) in mice results in a decrease in ovulating oocytes, corpus lutea, and antral follicles in females, and decreased sperm count in males further reinforcing the role of inhibin in reproductive function\textsuperscript{21,22}.

Inhibin is unique in the TGF\beta family as it is the only endocrine hormone in addition to having paracrine effects. One of inhibin’s primary functions in adults occurs in regulating the hypothalamic-pituitary-gonadal (HPG) axis through a negative feedback loop. FSH and LH produced by the pituitary gland are released to regulate gonadal function. In response to FSH, \textit{INHA} expression is increased and inhibin is secreted from the Sertoli cells and granulosa cells in the gonads\textsuperscript{23}. In regulating the HPG axis, inhibins act in an endocrine manner where inhibins secreted from the gonads travels to the pituitary (specifically the gonadotrophs) to decrease FSH production by antagonizing activin signaling\textsuperscript{24-26}. This mechanism of action effects spermatogenesis, folliculogenesis, menstrual cycles and menopause\textsuperscript{5,27}. 
During folliculogenesis, follicles are recruited from a primordial pool to form a single oocyte which is surrounded by theca cells, granulosa cells, and stromal cells that regulate the hormonal environment. Inhibin levels cycle throughout this process with inhibin B levels peaking during the early follicular and luteal phases while inhibin A peaks during midluteal phase\textsuperscript{14,28}. Inhibin B production during the early follicular phase serves to down-regulate FSH receptor expression and slow oocyte maturation\textsuperscript{29}. Inhibin A secretion by the corpus luteum has been shown to support follicular dominance during the mid-luteal phase\textsuperscript{30}. The rise and fall of inhibin levels during women’s menstrual cycle is marked by a sharp decline upon the onset of menopause. Depletion of the ovarian follicles results in reduced inhibin levels and subsequent increase in FSH levels driving the menopause transition\textsuperscript{31}.

1.2. Transcriptional Regulation of \textit{INHA}

Inhibin expression is modulated by the gonadotropins FSH and LH. FSH increases intracellular cAMP levels by activating G-protein coupled receptors\textsuperscript{32}. Intracellular cAMP accumulation promotes PKA signaling and downstream phosphorylation of the transcription factor CREB (cAMP responsive binding element binding protein) that binds to consensus CRE (cAMP responsive element) on target genes to promote transcriptional activation\textsuperscript{33}. The \textit{INHA} promoter contains a functional CRE that regulates transcription of the inhibin\textalpha subunit\textsuperscript{34}. The transcription factors SF-1 (steroidogenic factor-1) and LRH-1 (liver receptor homolog 1), both members of the NR5A nuclear receptor family, have been shown to act synergistically with CREB and to enhance \textit{INHA} transcription in granulosa cells\textsuperscript{35,36}. These transcription factors utilize the same binding site and switch which one is occupying this site based on cAMP levels and
In adrenal cortical cells, SF-1 can act synergistically with β-catenin to increase INHA transcription in response to Wnt signaling\(^3^7\). Interestingly, the Wilms’ Tumor Gene 1 (WT1) can act synergistically with SF-1 in Sertoli cells to promote INHA transcription but acts to decrease INHA expression in granulosa cells\(^3^8,3^9\). Members of the GATA family of transcription factors have also been shown to cooperate with LRH-1 to promote FSH induced INHA transcription\(^4^0\) as well as cooperation with SMAD3 in response to TGFβ\(^4^1\). Suppression of INHA occurs through the CCAAT/enhancer-binding protein and inducible cAMP early repressor (ICER) which competitively compete with CREB for binding to the CRE on the INHA promoter\(^4^2\).

### 1.3. Inhibin Signaling Pathway and Mechanism of Action

Inhibin’s signaling pathway is still being delineated, as we have made new discoveries in this field in recent years. The most characterized mechanism of action for inhibin occurs through functional antagonism of activin signaling (Figure 1.2A). Activin signals by binding the TGFβ type II receptor, ACTRII, which phosphorylates and activates the type I receptor, ALK4/ALK7, resulting in downstream phosphorylation of intracellular SMAD2/3 proteins\(^4^3\). Inhibin competes for binding with activin to ACTRII through its beta subunit, which is shared with activin, to prevent activation of this signaling cascade\(^4^4-4^7\). Since activin has two beta subunits capable of binding ACTRII, it has a 10-times higher affinity for the receptor than inhibin\(^4^8\). Inhibin is able to overcome this through binding of betaglycan, a type III TGFβ co-receptor, through its alpha subunit which increases inhibins affinity for the type II receptor thus allowing antagonism of activin signaling\(^4^9\) (Figure 1.2A). Betaglycan is crucial to inhibins function, as siRNA
knockdown and immunoneutralization of betaglycan inhibits inhibin’s ability to suppress FSH secretion and activin signaling in gonadotropes\textsuperscript{49-51}. Mutation of the of the betaglycan binding site on inhibin\(\alpha\) significantly reduced both inhibin A and inhibin B ability to repress FSH secretion and activin signaling further cementing the role for betaglycan in inhibin’s biological function\textsuperscript{52,53}. In addition to binding betaglycan, inhibin\(\alpha\) can also bind ALK4 through its N-terminus to antagonize activin signaling\textsuperscript{54}. Free inhibin\(\alpha\) and an inhibin\(\alpha\) N-terminal peptide were able to functionally inhibit activin signaling representing a model where inhibin can antagonize activin as a free alpha subunit\textsuperscript{54-56}.

Recently, we demonstrated paracrine signaling effects of inhibin on endothelial cells\textsuperscript{57}. Inhibin is able to induce SMAD1/5 signaling in endothelial cells, dependent on the type III TGF\(\beta\) co-receptor endoglin and type I receptor ALK1\textsuperscript{57} (Figure 1.2B). Endoglin is expressed in proliferating endothelial cells while ALK1 is also highly expressed in endothelial cells and is used by other superfamily members like BMP9/10 and TGF\(\beta\) for SMAD1/5 activation\textsuperscript{58-61}. We were unable to show that inhibin directly binds to either of these receptors however inhibin did induce stable complex formation between ALK1 and endoglin\textsuperscript{57} (Figure 1.2B). More research is needed to further elucidate the roles these receptors, along with a potential type II receptor, play in inhibin signaling in endothelial cells.

**Chapter 1.4. Inhibin as a diagnostic marker in cancer**

Serum total inhibin levels, consisting of free inhibin\(\alpha\), inhibin A, and inhibin B, are used as a biomarker for ovarian cancers. Ovarian cancers are divided into subtypes depending on the cell type of origin. Epithelial cancers account for between 85-90% of
ovarian cancers with serous being the most common type followed by endometroid, mucinous, and clear cell carcinoma\cite{62}. Germ cell and stromal tumors make up the other subtypes but are much less common than epithelial cancers. Total inhibin levels were originally found to be elevated in granulosa cell tumors, the most common type of stromal ovarian cancer, with inhibin B being later discovered as the main form of inhibin produced by these tumors\cite{63-66}. Serum inhibin levels are elevated in mucinous tumors and represent a good diagnostic marker, but inhibin levels alone are not an effective diagnostic tool for non-mucinous epithelial tumors\cite{66,67}. However, when used in combination with CA-125, another ovarian cancer biomarker, serum inhibin levels accurately detect 95\% of ovarian cancers\cite{66,68}. Inhibin’s utility as a biomarker is limited to post-menopausal women. Pre-menopausal women still express and secrete inhibin in a cyclic manner making it difficult to derive baseline values for comparison\cite{66,69}.

Consistent with findings of elevated serum inhibin levels in epithelial ovarian cancer patients and previous studies\cite{70-72}, we demonstrated increased inhibin\(\alpha\) expression across mucinous, endometroid, and serous ovarian cancer tissues compared to non-cancerous ovarian tissues\cite{57}. Elevated inhibin levels have also been discovered in other cancer types including gastric\cite{73}, pancreatic\cite{74}, hepatocellular carcinomas\cite{75}, prostate\cite{76,77}, and adrenocortical tumors\cite{78,79}, indicating potential roles for inhibin outside ovarian cancer that need to be further explored.

### 1.5. Roles for inhibins in cancer

While inhibins have been used as a diagnostic for ovarian cancer for decades, roles for inhibin in the context of tumor progression are still being discovered. Originally thought to have tumor suppressor functions, as \textit{INHA-/-} mice developed gonadal tumors,
more recent evidence demonstrates inhibin promotes tumorigenesis pointing to complex roles for inhibin in cancer that require further studies\textsuperscript{19,57,80}. We observed significant inhibinα staining in stromal cells of ovarian cancer tissues and tissues with higher inhibinα expression correlated with micro-vessel density, indicating potential roles for inhibins in angiogenesis\textsuperscript{57}. Consistent with this, we found that inhibin promotes angiogenesis in an ALK1/ENG dependent manner. Knockdown of inhibinα reduced tumor burden and ascites fluid formation and conditioned media from inhibinα knockdown cells reduced angiogenesis compared to conditioned media from cells expressing inhibinα\textsuperscript{57}. Recently, our findings about inhibinα expression being more localized in stromal cells of serous, mucinous, and endometrial tumors was confirmed\textsuperscript{80}. However, in clear cell carcinomas, inhibinα distribution was also found in the tumor epithelial cells along with stromal cells. The inhibinα expressing population of tumor cells was found to be less proliferative, more resistant to chemotherapy, more stem-like, and had more angiogenic potential\textsuperscript{80}. Patients that had inhibinα expressing tumor cells had decreased overall survival and disease-free survival indicating inhibinα is an unfavorable prognostic marker\textsuperscript{80}.

In prostate cancer, inhibinα was originally thought to be a tumor suppressor as well. Inhibinα expression was seen in non-malignant and benign prostate tissues however in malignant regions inhibinα expression was suppressed\textsuperscript{81}. Similarly, prostate cancer cell lines did not express inhibinα and did not respond to exogenous treatment with inhibin A\textsuperscript{82}. Malignant prostate cancer tissue showed significant promoter methylation compared to nonmalignant/benign tissue and de-methylation of the \textit{INHA} promoter in prostate cancer cell lines that did not express inhibinα was able to restore expression\textsuperscript{76,83}. 
However, more recent studies suggest a more complex role for inhibinα in prostate cancer as elevated levels were seen in malignant tissues, including expression in the stromal cells, and inhibinα expression was associated with poor prognosis\(^{77,84,85}\). Risbridger et. al hypothesized that inhibinα might be an early tumor suppressor in prostate cancer but promotes tumorigenesis and metastasis as the disease progresses\(^{84}\). This hypothesis was confirmed later as inhibinα overexpression in androgen dependent prostate cancer cells (representing early stage disease) reduced tumor growth while in androgen independent cells (representing late stage metastatic disease) inhibinα promoted tumor growth, metastasis, and lymph angiogenesis\(^{77}\).

The role of inhibin has also been explored in adrenocortical cancers. Studies in \textit{INHA/-/-} mice point toward inhibin being a tumor suppressor. \textit{INHA/-/-} mice that have been castrated develop adrenocortical tumors derived from pluripotent progenitor cells that differentiate into gonadal-like cells driven by changes in activin signaling and LH due to the lack of inhibin\(^{20,86,87}\). However, differing inhibin levels and inhibinα expression patterns in adrenal cortical tumors indicate a more complicated role. Inhibin appears to be elevated in some tissues, decreased in others, or unchanged between normal and malignant\(^{78,88-92}\). As in prostate cancer, aberrant methylation of the \textit{INHA} promoter has been reported and is associated with decreased \textit{IHNA} expression\(^{79}\). Taken together, this evidence points to potential roles for inhibins in adrenocortical cancers that need further exploration.

1.6. Hypoxia and Angiogenesis in Ovarian Cancer

Angiogenesis, the formation of blood vessels from pre-existing ones, is a vital process during growth and development that provides nutrients and oxygen to tissues.
Similarly, in cancer pathology, solid tumor growth and metastasis are also dependent on blood vessels and angiogenesis for vital resources\(^93\). Tumor vasculature is often characterized by inefficient oxygen delivery, leaky vessels, and stiffening of the extracellular matrix (ECM), leading to build-up of ascites fluid and metastasis by allowing cells to penetrate the blood vessels\(^94\)\(^{-}\)\(^96\). Anti-angiogenic therapy, specifically Bevacizumab (an anti-VEGF antibody) in combination with platinum-based chemotherapy, has shown to significantly improve progression free survival and overall survival in ovarian cancer patients\(^97\)\(^{-}\)\(^102\). Clinical data attributes the benefits seen in combination therapy to a decrease in vascular permeability and normalizing of the tumor vasculature improving drug delivery to the tumor\(^95\)\(^,\)\(^103\). However, the efficacy of anti-angiogenic therapy is hindered by toxicities like hypertension and arterial thromboembolism making identification of new targets of the tumor vasculature critical\(^97\)\(^{-}\)\(^102\).

Hypoxia is a fundamental regulator of angiogenesis in cancer, controlling the expression of pro-and anti-angiogenic genes that drive tumor growth, metastasis, and immune evasion\(^104\). Intratumor hypoxia drives changes in the blood vessels that lead to altered permeability, build-up of fluid and ascites in ovarian cancer, and metastasis by facilitating intra/extravasation of tumor cells\(^104\)\(^,\)\(^105\). Staining of hypoxic regions within ovarian cancer patients found 46% of patients were experiencing intratumor hypoxia below 1% \(\text{O}_2\)\(^106\). During ovarian cancer metastasis, tumor cells shed from the ovary to form spheroids to spread through the peritoneum. Malignant ascites formed as a result of leaky vasculature, has been shown to be a hypoxic environment and aids in the spread of the shed spheroids which have also been shown to be hypoxic\(^105\)\(^,\)\(^107\)\(^,\)\(^108\).
Hypoxia regulates angiogenesis through the HIF proteins, a family of transcription factors that promote expression of angiogenic genes\textsuperscript{109}. The active HIF transcription factor is made up of an alpha subunit, most commonly HIF-1\(\alpha\) or HIF-2\(\alpha\), and a shared beta subunit, HIF\(\beta\)\textsuperscript{110}. Under normal oxygen tensions, the alpha subunit is marked for degradation by prolyl hydroxylases that require oxygen to function. Under hypoxic conditions, the prolyl hydroxylases do not function resulting in stabilization of the alpha subunit where it can translocate to the nucleus\textsuperscript{111}. The active HIF transcription factor binds to hypoxia response elements (HRE’s) of downstream target genes to regulate transcription\textsuperscript{112}. In ovarian cancer patients, increased HIF-1\(\alpha\) expression has been shown to be an unfavorable prognostic indicator including correlations with worse overall survival and progression free survival\textsuperscript{113,114}. Elevated HIF-1\(\alpha\) has also been shown to be correlated with resistance to cisplatin in ovarian cancer patients and knockdown of HIF-1\(\alpha\) in cisplatin resistant ovarian cancer cell lines restored sensitivity to cisplatin\textsuperscript{115,116}. 
Figure 1.1. *Structure of inhibin.* A) Inhibin is a hetero dimer comprised of an alpha subunit (*INHA*) and a beta subunit (*INHBA* or *INHBB*) which form inhibin A or inhibin B, respectively. Both alpha and beta subunits are synthesized as precursors, containing cleavable pro-domains. The alpha subunit also has a cleavable \(\alpha N\) domain. The mature form of inhibin A/B are shown below. B) Inhibin can also exist as a free monomer in both precursor form and mature form. C) Activins are formed as homodimers of either *IHNBA* or *INHBB*, forming activin A or activin B.
Figure 1.2. Inhibin signaling diagram in epithelial and endothelial cells. A) In endothelial cells, inhibin can antagonize activin signaling through two different mechanisms. On the left, inhibin binds the type III co-receptor, betaglycan, through the alpha subunit which increases inhibin’s affinity to bind the type II receptor, ACTRII, through the beta subunit. The formation of this complex competes with activin for binding to ACTRII thus preventing downstream SMAD2/3 activation. On the right, inhibin can bind ALK4 through the alpha subunit to prevent recruitment of this receptor by activin thus inhibiting downstream SMAD2/3 activation. B) In endothelial cells, inhibin induces complex formation between the type III coreceptor, endoglin, type I receptor, ALK1, to activate downstream SMAD1/5 signaling.
CHAPTER 2

HYPOXIA INDUCED INHIBIN PROMOTES TUMOR GROWTH AND VASCULAR PERMEABILITY IN OVARIAN CANCERS

1 Horst, B. et al. Hypoxia induced inhibin promotes tumor growth and vascular permeability in ovarian cancers. Accepted article. Commun. Biol.
2.1 Introduction

Changes in angiogenesis are associated with metastasis in most cancers, including ovarian cancers, with significant impact on tumor progression and ascites development in advanced disease\textsuperscript{117,118}. As such, anti-angiogenic therapies have had significant impact in the management of ovarian cancers\textsuperscript{119}. However, their effectiveness can be frequently limited due in part to toxicities and acquired resistance, leading to challenges with long term use and marginal improvements in overall survival\textsuperscript{119}. Discovery of new and safer angiogenic targets is thus critical.

TGF\(_{\beta}\) family members, particularly BMP9 and TGF\(_{\beta}\), are the most examined regulators of angiogenesis but have not been effective as targets for angiogenic therapy due to their pleiotropic functions in cancer and normal physiology\textsuperscript{120,121}. Similar to TGF\(_{\beta}\) and BMP9, activins’ have controversial and context dependent roles in angiogenesis. Specifically, activin A has been shown to increase VEGF induced angiogenesis in some instances\textsuperscript{122} and in others has been demonstrated to inhibit angiogenesis\textsuperscript{123}. Inhibins’ are a distinct and unique member of the TGF\(_{\beta}\) family as the only endocrine hormone and a functional heterodimer of an alpha (\(\alpha\)) subunit (\textit{INHA}) and a beta (\(\alpha\)) activin subunit (\textit{INHBA} or \textit{INHBB}) forming either inhibin A or inhibin B respectively\textsuperscript{5}. Inhibins’ are distinct from activins which are comprised of homodimers of either beta subunit\textsuperscript{5}. Inhibin\(\alpha\) is synthesized as a pro-peptide with a pro-domain, \(\alpha\)N region, and \(\alpha\)C region. The pro-domain and \(\alpha\)N region can be cleaved to produce the mature Inhibin\(\alpha\) subunit comprising the \(\alpha\)C region. Physiological Inhibin\(\alpha\) production by the sertoli cells of the testes, granulosa cells of the ovary, and the adrenal and pituitary glands\textsuperscript{10} is regulated primarily
by FSH and LH\textsuperscript{124,125} via a cAMP-PKA pathway resulting in cAMP response element binding (CREB) to the cAMP response element (CRE) on the \textit{INHA} promoter\textsuperscript{126}.

While inhibin levels (inhibin A and B) cycle across the lifespan of healthy females and dramatically decrease at the onset of menopause\textsuperscript{127}, elevated inhibin\(\alpha\) levels are found in ovarian, gastric, hepatocellular, and prostate cancers\textsuperscript{73,75,77,128}. Total inhibin protein levels comprising free inhibin\(\alpha\), inhibin A and inhibin B are also an established diagnostic marker alone and/or in combination with CA125, for ovarian cancers\textsuperscript{66} and have been proposed as a potential tumor specific target for therapy\textsuperscript{5,57,77,128,129}. Inhibin\(\alpha\) levels are also predictive of survival in multiple cancer types with gene signatures that correlate with \textit{INHA} expression, providing a highly accurate prognostic model for predicting patient outcomes\textsuperscript{129}. However, the mechanism of inhibin expression in cancers have not been delineated.

Hypoxia is a key mediator of angiogenic responses, regulating pro-and anti-angiogenic genes impacting tumor growth, metastasis, and immune evasion\textsuperscript{104} and is driven by the hypoxia inducible factor (HIF) family of transcription factors. Hypoxia induced changes, specifically in tumors, are characterized by inefficient oxygen delivery, leading to leaky vessels, and altered permeability, build-up of fluid and ascites in ovarian cancer, and metastasis by facilitating intra/extravasation of tumor cells\textsuperscript{104,105}. We previously reported decreased ascites accumulation in mice bearing tumor cells with \textit{INHA} knockdown\textsuperscript{57}, indicating a potential role for inhibin in regulating metastasis and vascular functions, a key contributing factor to ascites accumulation. Moreover, inhibin secreted by tumor cells induces angiogenesis via SMAD1/5 signaling in endothelial cells in a paracrine
manner dependent on the type III TGFβ receptor endoglin/CD105 and the type I TGFβ receptor ALK1\(^{57}\).

To precisely delineate inhibin’s significance in cancer and mechanism of action, we now determine the impact of hypoxia, a key mediator of the angiogenic and metastatic response in cancer\(^{104}\), and the contribution of inhibin to the hypoxia adaptive response. We discover that hypoxia in ovarian xenograft tumors, cancer cells, and patient samples leads to an increase in inhibin synthesis in a hypoxia inducible factor (HIF) dependent manner. We find that hypoxia induced tumor growth and vascular permeability \textit{in vivo} is driven by inhibin. Moreover, intervention using an antibody based therapeutic strategy to inhibin can suppress hypoxia driven tumor biology. Mechanistically, inhibin promotes vascular permeability via endoglin and ALK1. Notably, we also describe for the first time using sensitive biophysical methods the nature and stability of the endoglin and ALK1 interaction at the cell surface in response to inhibin. Our findings not just strongly implicate inhibins as part of the hypoxia adaptive response, but also suggest anti-inhibins’ as an alternative or companion to current anti-angiogenic therapies that may not be well tolerated.

2.2 Materials and Methods

\textbf{Cell Lines and Reagents:} Ovarian epithelial carcinoma cell lines were obtained as described in resource Table 2.1 and were from ATCC, the NCI cell line repository through an MTA, or were as indicated. Cell line authentication was performed at the Heflin Center for Genomic Science Core Laboratories at UAB. HMEC-1s were grown per ATCC instructions. COS7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% FBS, 100 U penicillin/streptomycin and L-glutamine. Mouse embryonic endothelial cells (MEEC) WT and ENG -/- were grown as previously described\(^{130}\). Epithelial carcinoma cell
lines HEY, OVCA420, SKOV3 and PA1 were cultured in RPMI-1640 containing L-glutamine, 10% FBS and 100 U of penicillin-streptomycin\textsuperscript{131}. OVCAR-5 and HEK293 were cultured in DMEM containing 10% FBS and 100U of penicillin streptomycin. ID8ip2Luc was a kind gift from Jill Slack-Davis\textsuperscript{132} and cultured in DMEM containing 4% FBS, 100U of penicillin streptomycin, 5μg/mL of insulin, 5μg/mL of transferrin, and 5ng/mL of sodium selenite. All cell lines were maintained at 37°C in a humidified incubator at 5% CO\textsubscript{2}, routinely checked for myco-plasma and experiments were conducted within 3–6 passages depending on the cell line. For hypoxia experiments, a ProOx Model C21 was used and set to 0.2% O\textsubscript{2} and 5% CO\textsubscript{2}. Anti-inhibin PO/23 and R1 antibodies were obtained from Oxford-Brookes university through an MTA and from Biocare Medical. INHA promoter driven luciferase reporter construct was generated through restriction cloning into pGL4.10 luciferase plasmid. Primers were designed to 547 base pairs of the INHA promoter containing the first HRE site with Nhe1 and Xho1 restriction sites on the ends. Insert was amplified from PA1 genomic DNA. Insert was ligated into pGL4.10 plasmid with T4 DNA ligase and INHA promoter region was verified through Sanger sequencing. Additional details on resource is provided in Table 2.1.

**Generation of cell lines:** INHA and ARNT knockdown were generated in HEY cells infected with shRNA lentivirus, followed by selection in 2.5 μg/ml Puromycin and stable cell lines maintained in 1 μg/ml Puromycin. Luc/GFP cell lines were generated using pHIV-Luc-ZsGreen construct. Transient DNA transfections in HEK293 were performed using Lipofectamine 3000. siRNA transfections were performed using RNAiMax. In HEK293 transfections, single siRNA was used while pooled siRNA was used in HEY and OV90 transfections. Lentiviral particles were generated at the Center for Targeted
Therapeutics Core Facility at the University of South Carolina. shRNA and siRNA sequences are listed in Table 2.2.

**RNA Isolation and RT-qPCR:** Total RNA was harvested using Trizol/Chloroform extraction. RNA was transcribed using iScript Reverse Transcription Supermix and iTaq Universal SYBR Green Supermix. Expression data was normalized to RPL13A. qRT-PCR primer sequences are listed in resource Table 2.3.

**ELISA:** Inhibin ELISA’s were performed according to the manufacturer’s instructions for the quantitative measurement specifically of total inhibin protein (does not detect activin), that detects inhibin A (dimer of INHA/INHBA), inhibin B (dimer of INHA/INHB), and free inhibin alpha subunit (INHA), from conditioned media of tumor cells. Cells were grown to 80% confluency in 24 well plates before media was replaced with fresh full serum media. Cells were placed in hypoxia chamber for 24hrs and media was collected and concentrated using Amicon Ultra centrifugal filter.

**IN VITRO ASSAYS**

**In vitro Permeability Assay** was adapted from Martins-Greene. 1x10^5 HMEC-1 cells were plated onto a Matrigel coated 3μM trans-well filter in full serum media. After 24h hours, a second layer of 1x10^5 HMEC-1 was plated on top to obtain a confluent monolayer of cells. After an additional 24hrs, media was replaced with serum free media in the top of the trans-well and either conditioned media (with 2μg of either R1, PO/23, or IgG) or serum free media containing growth factor in the bottom chamber as indicated in legends. FITC-dextran was added to the lower chamber (10μg/ml). At indicated time points 10μL aliquots were taken from the top chamber in triplicate and measured using microplate reader for
FITC-dextran passage. At end point, filters were stained with crystal violet to confirm equal monolayers were achieved.

**Trans-well Migration Assay:** 75 000 HMEC-1 were plated on a fibronectin coated (10μg/mL) 8μM trans-well filter in serum free media. Conditioned media (with 2μg of either R1, PO/23, or IgG) or serum free media containing 1nM inhibin A or VEGF A was used as a chemoattractant in the bottom chamber. After 24hrs, unmigrated cells were scraped off the apical side, migrated cells were fixed in methanol:acetic acid, and nuclei were stained with Hoechst. Three random images were taken per filter using 10X objective on EVOS M7000 microscope. Nuclei were counted using ImageJ.

**Trans-endothelial Migration Assay:** HMEC-1 were grown on 8μm trans-well filters as per permeability assay. HMEC-1 monolayer was treated with 1nM inhibin A or untreated for four hours. After four hours of treatment, 150 000 HEY-LucGFP expressing cells were plated on top of the HMEC-1 monolayer and allowed to invade for 18hrs. Filters were fixed in 4% paraformaldehyde, cells on the apical side of the filter were scraped off, and filters were mounted on glass slides for imaging. Migration of GFP+ cells was visualized using 10x objective on EVOS M7000 microscope. Three random fields were captured per filter and GFP+ cells were counted using ImageJ software. Thresholding, circularity, and size gating were used to exclude unmigrated cells and artifacts.

**Chromatin Immunoprecipitation** protocol was adapted from ABCAM. Briefly, OV-90 or OVCAR-5 cells were grown in 150cm² dishes until 80% confluency was reached. Cells were kept under normoxia or placed in the hypoxia chamber set at 0.2% O₂ for either 12hrs (OVCAR-5) or 24hrs (OV-90). DNA was crosslinked using 0.75% formaldehyde and sheared by sonication to fragment sizes between 100-400bp. DNA was
immunoprecipitated with Dyna-beads and either HIF-1α antibody or Normal Rabbit IgG as a control. DNA was purified using Purelink PCR Purification kit and amplified using RT-qPCR with ChIP primers.

**Luciferase Assay:** HEK293 cells were seeded into 24 well plate and co-transfected with a luciferase reporter containing 547 base pairs of the *INHA* promoter (pGL4.10 *INHA*) and a SV40 (Renilla internal control vector). For HIF-1 overexpression, cells were also co-transfected with pcDNA3-HA-HIF1aP402A/P564A or PCDNA3.1. One day after transfection, cells were left in a normoxia incubator or moved to hypoxia chamber (0.2% O₂) for 24hrs. Luciferase activity was measured using the Dual Luciferase Reporter Assay System by calculating the ratio between luciferase and Renilla and normalized to normoxia or PCDNA3.1 as indicated in legends.

**Immunofluorescence:** HMEC-1 cells were grown to confluence on fibronectin (10μg/mL) and treated with either 1nM inhibin A or VEGF A for 30 minutes in serum free media. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% TritonX-100, followed by blocking with 5% BSA in PBS for 1hr. VE-cadherin was labeled with anti-VE-cadherin antibody overnight at 4°C followed by AlexaFluor 488 secondary antibody. F-actin was stained with rhodamine-phalloidin and nuclei were labeled with DAPI. Immunofluorescence imaging was performed on EVOS M7000 microscope or Nikon A1 confocal microscope. Actin fibers were quantified by measuring anisotropy using the FibrilTool Plugin in ImageJ134.

**VE-cadherin Internalization:** HMEC-1 cells grown to confluence on fibronectin (10μg/mL) coated glass coverslips. Cell surface VE-cadherin was labeled with anti-VE-cadherin antibody at 4°C for 30 minutes, washed with ice-cold PBS, and incubated at 37°C
for 30 minutes with 1nM inhibin A, 1nM VEGF A, or serum free media. After internalization was stimulated with growth factor at 37°C, anti-VE-cadherin antibody on the cell surface was removed with mild acid wash. Internalized VE-cadherin was visualized by immunofluorescence microscopy. Internalized VE-cadherin was quantified using BlobFinder software\textsuperscript{135,136}. Nuclei and cytoplasm were delineated and the number of signals per cell was used to quantify internalized VE-cadherin fluorescence.

**Cell Surface Biotinylation**: Briefly, MEEC WT or /ENG-/ were grown to confluence on gelatin coated dishes. Cell surface proteins were labeled with 2mg/mL Sulfo-NH-SS biotin for 30 min at 4°C. After labeling, cells were treated with 1nM inhibin A or untreated in serum free media for 30 min at 37°C or left at 4°C for cell surface control samples. After treatment, cell surface biotin was removed with 20mM MESNA buffer and internalized biotin labeled protein was isolated with neutravidin resin. Internalized biotin labeled VE-cadherin was detected by Western Blot.

**Epitope-tagged plasmids and transfection of COS7 cells for patch/FRAP studies**

The following plasmids were donated by Prof. G. C. Blobe, Duke University Medical Center: HA-tagged endoglin (endoglin-L) in pDisplay, myc-endoglin generated by PCR incorporation of the myc tag sequence into untagged endoglin in pDisplay and re-cloned in pcDNA3.1, and HA- or myc-tagged ALK1 in pcDNA3.1\textsuperscript{137}. Human ALK4 with C-terminal myc-DDK tags in pCMV6 was obtained from OriGene Technologies (Rockville, MD), and subcloned into pcDNA3.1 by PCR followed by restriction digest and re-ligation. A stop codon was introduced at nucleotide 1516 to delete the C-terminal tags to generate untagged ALK4. This was followed by insertion of N-terminal HA tag by overlapping PCR after nucleotide 72 to generate extracellularly tagged HA-ALK4. All constructs were
verified by sequencing. COS7 cells were transfected using TransIT-LT1 Mir2300 according to manufacturer's instructions. For Patch/FRAP experiments, cells grown on glass coverslips in 6-wells plates were transfected with different combinations of these vectors encoding myc- and/or HA-tagged receptor constructs. The amounts of the vectors (between 0.5 and 1 μg DNA) were adjusted to yield similar cell surface expression levels, determined by quantitative immunofluorescence.

**Fluorescent antibody labeling and IgG-mediated cross-linking for patch/FRAP:** COS7 cells were transfected with various combinations of the above epitope-tagged expression vectors. After 24 h, The cells were serum-starved (1% FBS, 30 min, 37 °C), washed with cold Hank’s balanced salt solution (HBSS containing 20 mM HEPES, pH 7.2) and 2% BSA (HBSS/HEPES/BSA), and blocked with normal goat γ-globulin (200 μg/ml, 30 min, 4 °C). For FRAP studies on singly-expressed receptors, the cells were then labeled successively at 4 °C in HBSS/HEPES/BSA (45 min incubations) with: (i) monovalent murine Fab’ anti myc tag (αmyc) or anti HA tag (αHA; 40 μg/ml), prepared from the respective IgGs as described by us earlier\(^{138}\); (ii) Alexa 546-Fab’ goat anti mouse (GαM; 40 μg/ml), prepared from the respective F(ab’)\(_2\) as described\(^{139}\). For patch/FRAP studies, they were labeled by one of two protocols. Protocol 1 employed successive labeling with: (i) monovalent mouse Fab’ αmyc (40 μg/ml), alone or together with HA.11 rabbit αHA IgG (20 μg/ml) and (ii) Alexa 546-Fab’ GαM (40 μg/ml) alone or together with Alexa 488-IgG goat anti rabbit (GαR; 20 μg/ml). This protocol results in the HA-tagged receptor crosslinked and immobilized by IgGs, whereas the myc-tagged receptor, whose lateral diffusion is then measured by FRAP, is labeled exclusively by monovalent Fab’.

Alternatively, we employed protocol 2 for immobilizing the myc-tagged receptor and
measuring the lateral diffusion of a co-expressed Fab’-labeled HA-tagged receptor: (i) monovalent mouse Fab’ αHA (40 μg/ml) together with chicken IgY αmyc (20 μg/ml) and (ii) Cy3-Fab’ donkey anti mouse (DαM; 40 μg/ml) together with FITC-IgG donkey anti chicken (DαC; 20 μg/ml). In experiments with inhibin A, the ligand was added after starvation along with the normal goat γ-globulin and maintained at the same concentration throughout the labeling steps and FRAP measurements.

**FRAP and patch/FRAP:** COS7 cells co-expressing epitope-tagged receptors labeled fluorescently by anti-tag Fab’ fragments as described above were subjected to FRAP or patch/FRAP experiments as described\textsuperscript{140}. FRAP studies were conducted at 15 °C, replacing samples after 20 min to minimize internalization. An argon-ion laser beam (Innova 70C, Coherent, Santa Clara, CA) was focused through a fluorescence microscope (Axioimager.D1; Carl Zeiss MicroImaging, Jena, Germany) to a Gaussian spot of 0.77 ± 0.03 μm (Planapochromat 63x/1.4 NA oil-immersion objective). After a brief measurement at monitoring intensity (528.7 nm, 1 μW), a 5 mW pulse (20 ms) bleached 60–75% of the fluorescence in the illuminated region, and fluorescence recovery was followed by the monitoring beam. Values of $D$ and $R_f$ were extracted from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process\textsuperscript{140}. Patch/FRAP studies were conducted analogously, except that IgG-mediated cross-linking of epitope-tagged endoglin preceded the measurement\textsuperscript{140}.

**Patient Ascites:** Specimens from patients diagnosed with primary ovarian cancer was collected and banked after informed consent at Duke University Medical Center, with approval for the study from Duke University’s institutional research ethics board. ELISA’s were conducted using ELISA for Total inhibin from Ansh labs (#AL-134).
Public Data Mining: Clinical data and normalized RNA-seq were obtained from cBioportal\textsuperscript{141}. The ovarian serous cystadenocarcinoma (TCGA, PanCancer Atlas) and breast invasive carcinoma (TCGA, PanCancer Atlas) were assessed for \textit{INHA} expression and hypoxia (Buffa or Winter) scores. \textit{INHA} expression was plotted against hypoxia score for each patient for correlation analysis.

\textit{IN VIVO ASSAYS.} All animal studies and mouse procedures were conducted in accordance with ethical procedures after approval by UAB’s IACUC prior to study commencement.

Matrigel Plug Assay: Matrigel plugs were formed using 200µL of Matrigel mixed with 50µL of HEY conditioned media and injected subcutaneously into the underside of BALB/c female mice aged 5–6 weeks. For conditioned media, HEY cells were grown until 80\% confluence in 24 well plate before media was replaced with fresh full serum media. Cells were placed in hypoxia chamber for 24hrs and media was collected and concentrated to 50µL Savant SpeedVac SPD1030. Conditioned media was incubated with 2µg of either R1 or IgG overnight before injection. Plugs were harvested 12 days after injection and hemoglobin content was determined according to Drabkin’s method\textsuperscript{57}.

\textit{In vivo subcutaneous tumor growth and permeability analysis:} 3x10\textsuperscript{6} HEY cells either exposed to normoxia or hypoxia (0.2\% O\textsubscript{2}) for 24hrs were subcutaneously injected into right flank of 6-week-old Ncr Nude mice (Taconic). Tumor volume ((LxW\textsuperscript{2})/2) was calculated by caliper measurements every other day starting at day 10 until harvest at day 30. In animals receiving anti-inhibin treatment, R1 (BioCare) was administered IP at 2mg/kg three times weekly. Da Vinci Green diluent (BioCare) was administered as vehicle.
For measurement of permeability, tumors were harvested between 700-800 mm$^3$. At end point, Rhodamine Dextran 70 000 MW was intravenously injected at 2mg/kg two hours before euthanasia. Tumors were fixed in 10% NBF and sections were analyzed for rhodamine-dextran by immunofluorescence on EVOS M7000. Three sections per tumor were quantified and four images per section were taken. Thresholding was performed in ImageJ and kept constant for all images. ROUT analysis (Q= 10%) was performed to test for outliers.

For tumor hypoxia analysis, tumors were harvested at varying sizes between 200-1400 mm$^3$. Pimonidazole (HydroxyProbe) was injected intravenously at 60mg/kg 1hr before sacrifice. Tumors were fixed in 10% NBF and sections were analyzed for pimonidazole adducts using anti-pimonidazole monoclonal antibody.

**Immunofluorescence on Tissues:** Briefly, formalin fixed, paraffin-embedded tissues from subcutaneous tumors were deparaffinized by sequential washing with xylene, 100% ethanol, 90% ethanol, 70% ethanol and distilled water for 10 min each. Antigen retrieval was performed by boiling tissues in sodium citrate buffer (pH 6.0). Blocking was performed with Background Punisher. Primary antibodies, anti-pimonidazole (1:50) and anti-CD-31 (1:100), were diluted in Da Vinci Green Diluent and incubated overnight at 4°C in a humidified chamber followed by AlexaFluor 594 secondary antibody. Nuclei were stained with DAPI. 10x images were acquired on EVOS M7000 microscope.

Quantitation of CD-31 labeled vessel size and number as well as pimonidazole was performed in ImageJ. Images were converted to binary and thresholding mask was applied equally to all images. For CD-31, objects smaller than 25 pixels were removed as were deemed too small to be vessels. For each image, average vessel size (area) and average
vessel number was measured. Four images per section and two sections per tumor were used for quantitation. For pimonidazole, a 10x stitched image comprising the whole tumor section was used. The total area covered by signal was acquired and divided by total tumor area to calculate the % hypoxic area for each tumor.

**Angiogenesis Proteome Array** was performed according to manufacturer’s instruction (R&D Systems, Table 2.1). Briefly, tissues were homogenized in PBS with 1% TritonX-100 and PI cocktail. 200μg of protein was used per sample (two samples for shControl and shINHA tumors each). Pixel intensity was quantified for each dot using ImageStudio software after background subtraction.

**Statistical Analysis:** All data are representative of three independent experiments, unless otherwise described in legends. Statistical analyses were performed using GraphPad Prism 9, with statistical test chosen based on experimental set up and specifically described in the figure legends. Data are expressed as mean ± SEM. Difference between two groups was assessed using a two-tailed t-test. Multiple group comparisons were carried by the analysis of variance (ANOVA) using One or Two-way ANOVA followed by appropriate post-hoc tests as indicated in Figure legends.

**2.3 Expression and secretion of inhibin is regulated by hypoxia in ovarian cancer cell lines**

We and others have previously demonstrated increased expression of inhibinα mRNA and protein in a broad spectrum of cancers leading to increased angiogenesis in *vitro* and *in vivo* impacting metastasis. Based on the potential role of inhibins’ in cancer angiogenesis, we tested the impact of hypoxia, a key regulator of angiogenesis, on *INHA* expression. The high grade serous ovarian cancer cell lines HEY and OV90 cells
were exposed to varying levels of oxygen (control tissue culture conditions (20%), 10%, 5%, 2.5%, 1%, and 0.2% O₂) for twenty-four hours to evaluate INHA expression and VEGFA expression (as a positive control) by semi-quantitative RT-PCR. INHA expression was significantly elevated in 0.2% O₂ (4.9-times) (Figure 2.1Ai) in both HEY cells and OV90 cells. In OV90 cells, INHA was elevated at 1% (2.7-times) as well, however not significantly (Figure 2.1Ai). A similar pattern was observed for VEGFA expression with significant increases in both HEY and OV90 at 0.2% O₂ (HEY: 3.8-times and OV90: 4.3-times) and at 1% in HEY (2.4-times) (Figure 2.1Aii). HIF-1 stabilization was evaluated by western blotting to confirm an active hypoxic response that was oxygen tension dependent (Figure 2.1Aiii). To further test the impact of hypoxia on INHA expression, a panel of ovarian cancer cell lines representing a broad spectrum of ovarian cancer subtypes, including HEY, OV90, OVCAR5 of high-grade serous origin, PA1 a teratocarcinoma cell line of the ovary, and ID8ip2 a mouse ovarian cell like, were grown for twelve or twenty-four hours under either hypoxic conditions (0.2% O₂) or normoxic control tissue culture conditions (17-21%). We find 3-6 times increase in INHA expression across all four cell lines (HEY: 4-times, OVCAR5: 4.4-times, PA1: 5.28, OV90: 4.8-times, ID8ip2: 4-times, Figure 2.1Bi). All cell lines showed maximum INHA increases after 24hrs of hypoxia growth except for OVCAR5 which increased INHA expression within 12hrs under hypoxia. VEGFA was evaluated side by side as a positive control and representative of the hypoxia response in all four cell lines and was elevated 2-6-times (HEY: 3.5-times, OVCAR5: 3.1-times, PA1: 5.18-times, OV90: 2.5-times, ID8ip2: 2-times, Figure 2.1Bii). The INHA response to hypoxia was also more robust in tumor cells as compared to endothelial cells (HMEC-1) grown under hypoxia (0.2% O₂) for either 12hrs or 24hrs.
(Figure 2.1C) indicating that inhibinα increases in response to hypoxia occur more significantly in tumor cells.

To test if INHA expression remains elevated after re-exposure to oxygen, we first determined how long HIF-1 protein remained stabilized in cells when returned to normoxic conditions (reoxygenation) after 24hr exposure to hypoxia. HIF-1 protein began to decrease 5 minutes after re-exposure to hypoxia (reoxygenation) and went back to baseline at 60 minutes in HEY and OV90 cells (Figure 2.1Di). Since we observed HIF-1 levels return to baseline after 60 minutes, we began our time course for testing INHA expression after re-oxygenation at one hour. In HEY cells, INHA expression was increased four-times upon exposure to hypoxia (Figure 2.1Dii). Upon one hour of reoxygenation, INHA expression decreased significantly in both cell lines and was no longer statistically different from normoxia grown cells (Figure 2.1Dii). Slight elevation in INHA levels remained, particularly in OV90 cells for the duration of the time course (Figure 2.1Dii) that did not however reach statistical significance. Taken together, these data strongly indicate that inhibinα mRNA and protein expression is increased under hypoxia conditions.

INHA translates into the protein inhibinα which can be secreted as a free monomer or can dimerize with INHBA or INHBB to produce dimeric functional inhibin A or inhibin B. Thus, total inhibin ELISA, specific to inhibinα so as to detect all three inhibin forms, was used to test if the changes in INHA mRNA resulted in alterations to secreted protein. We find that conditioned media collected from HEY and OV90 exposed to hypoxia increased total inhibin protein secretion as well, (4.2-times in HEY and 3.8-times OV90, Figure 2.2A). These data suggest that INHA mRNA and functional secreted inhibin protein, is increased by hypoxia.
Since total inhibin protein, reflecting either inhibin A/B and free inhibinα, increased in response to hypoxia (Figure 2.2B), we evaluated mRNA changes in *INHBA* and *INHBB* subunits in HEY and OV90 cells. While *INHA* was increased three to five-times in response to hypoxia (Figure 2.1Bi), *INHBA* and *INHBB* levels were unchanged in the two cell lines evaluated (Figure 2.2B), indicating that changes in inhibin protein levels (Figure 2.2A) were largely related to increases in inhibinα.

### 2.4 Inhibinα is increased in ovarian cancer spheroids, patients, and tumor xenografts

To evaluate other pathologically relevant hypoxic conditions pertinent to ovarian cancer growth and metastasis, we evaluated hypoxia and *INHA* expression in cells grown in spheroids under anchorage independence, an environment that is often hypoxic\(^\text{107}\). PA1 and OVCA420 cells were chosen due to their ability to form spheroids\(^\text{143,144}\). Cells were grown on poly-hema coated plates for either 72hrs (PA1) or 48hrs (OVCA420). Under such anchorage independent conditions (referred to as 3D), where HIF-1α was stabilized (Figure 2.3Ai), *INHA* was increased 7.8-times in PA1 and 4.6-times in OVCA420 when compared to 2D growth conditions in a dish (Figure 2.3Aii).

Previous studies have established that in healthy pre-menopausal women, inhibin levels cycle across the menstrual cycle reaching a peak of 65.6 pg/mL, while in post-menopausal women, total serum inhibin levels are below 5 pg/mL\(^\text{145}\). Ovarian cancer patients are commonly postmenopausal\(^\text{146}\) and tumor tissues can display higher inhibin levels\(^\text{57}\). We thus wanted to assess if the peritoneal ascites fluid of advanced ovarian cancer patients, which has been shown to be a hypoxic environment\(^\text{108}\) and contains disseminated ovarian cancer spheroids\(^\text{105}\), also displays detectable or elevated inhibin levels. To test if
inhibin protein is secreted and detectable in clinical ascites, total inhibin ELISA was performed on a cohort of 25 patient ascites. We find total inhibin levels in the range of 6.7 to 120.53 pg/mL in the ascites fluid indicating the presence of inhibin protein in ascites fluid (Figure 2.3B).

We next evaluated if \textit{INHA} expression was elevated \textit{in vivo} with increasing xenograft tumor size. 5 million HEY cells were subcutaneously implanted and harvested at varying tumor sizes. Tumors greater than 500 mm$^3$ were found to be hypoxic based on pimonidazole staining which has a detection threshold of below 10 mmHg O$_2$, or 1.2% O$_2$ (4.8-times, Figure 2.3Ci,ii)$^{147}$. \textit{INHA} expression was increased 9.8 times in tumors greater than 500 mm$^3$ as compared to tumors less than 500 mm$^3$ (Figure 2.3Di). \textit{INHA} expression was also significantly correlated with tumor size (Figure 2.3Dii). To further examine the potential clinical relevance of inhibin$\alpha$ expression in response to hypoxia, we analyzed the TCGA/PanCancer Atlas patient data set from cBioportal$^{141,148}$ and obtained hypoxia scores from two different hypoxia gene signatures (Buffa and Winter)$^{149,150}$. The signatures consisted of 51 (Buffa) and 99 (Winter) hypoxia related genes from a large meta-analysis of breast and head and neck squamous cell cancer that were independently verified for prognostic value$^{149,150}$. Using these signatures, inhibin$\alpha$ (\textit{INHA}) expression was significantly correlated with both hypoxia Buffa ($r=0.1961$, $p=0.0221$) and Winter hypoxia ($r=0.223$, $p=0.009$) scores in the ovarian cancer data set (Figure 2.3Ei,ii). Analysis of breast cancer data revealed a similar trend as \textit{INHA} expression was significantly correlated ($r=0.2026$, $p=0.0165$) with the Winter hypoxia score (Figure 2.3Eiii). Taken together, these data strongly indicate that inhibin$\alpha$ mRNA and protein expression are increased under hypoxia conditions in ovarian cancer cell lines, xenograft tumors and in patients.
2.5 *INHA* is a direct HIF-1 target under hypoxia

Hypoxia inducible factors (HIFs) are key transcriptional regulators of the hypoxia adaptive response and increase expression of critical pro-angiogenic genes\(^{104}\). To test whether HIF proteins are regulators of *INHA* expression, we first utilized cobalt chloride (CoCl\(_2\)), a well characterized chemical stabilizer of HIF’s\(^{151}\). HIF-1\(\alpha\) was stabilized in PA1 and OVCAR5 cells treated with 100\(\mu\)M of CoCl\(_2\) for either 6, 12, or 24 hrs (Figure 2.4Ai). We find that *INHA* expression was significantly increased; 10-times in OVCAR5 after 12hrs and 11.5-times in PA1 cells after 24hrs of CoCl\(_2\) treatment (Figure 2.4Aii). Maximum increases in *INHA* expression with CoCl\(_2\) occurred at the same time points as exposure to hypoxia (12hrs for OVCAR5 and 24hrs for PA1, Figure 2.1Bi). *VEGFA*, used as a positive control increased 4.8 and 4.3-times at 12hrs and 2.7 and 3.7-times at 24hrs in both OVCAR5 and PA1, respectively (Figure 2.4Aii). To test if *INHA* could be a direct hypoxia target leading to increased inhibin\(\alpha\) expression, we evaluated the effect of reducing the levels of HIF-1\(\beta/ARNT\) which is the binding partner for all HIF’s\(^{152}\). Stable *ARNT* knockdown cells were generated in HEY cells (Methods). We find that control HEY cells increase *INHA* levels 2.8-times under 0.2% hypoxia (Figure 2.4B). However, shRNA *ARNT* lead to a 2.7-times reduction in hypoxia induced increase in *INHA* mRNA levels (Figure 2.4B) indicating direct contributions of HIFs to the regulation of inhibin.

To determine the roles of the HIF-1 and HIF-2 heterodimeric transcriptions factors, that both require ARNT\(^{152}\), in the transcriptional regulation of *INHA* we used siRNA to knockdown the levels of HIF-1\(\alpha\) and HIF-2\(\alpha\) in two ovarian cell lines (OV90 and HEY). Knockdown of HIF-1\(\alpha\) and HIF-2\(\alpha\) using siRNAs to each isoform individually or a combination of siRNAs was confirmed through western blotting (Figure 2.4Ci,ii).
Knockdown of HIF-1α decreased hypoxia induced INHA expression 2.1 times in HEY and 1.9 times in OV90 compared to siScr (siHIF-1α, Figure 2.4Ci,ii). siRNA to HIF-2α did not result in a significant change in hypoxia induced INHA expression compared to control (siHIF-2α, Figure 2.4Ci,ii). To further test that HIF-1 was the required HIF isoform for hypoxia induced INHA expression, we utilized a double knockdown of HIF-1α and HIF-2α. In HEY and OV90 cells the double knockdown resulted in a 2-times and 1.8-times decrease in INHA expression compared to siScr, respectively (siHIF-1,2α, Figure 2.4Ci,ii). These data suggest that increases in INHA under hypoxia were more significantly impacted by HIF-1 as compared to HIF-2.

*In silico*, analysis of the INHA gene, which is located at Chr:2q35 revealed two hypoxia response element (HRE) consensus sites within 2Kb of the promoter, GGCGTGG and CGCGTGG, at -144 and -1789 bp from the transcription start site (TSS) (Figure 2.5A) respectively. These HRE sites conform precisely to the (G/C/T)(A/G)CGTG(G/C) consensus sequence\(^{152}\). Two hypoxia ancillary sequences (HAS) (CAGGG and CACGG) were also found directly flanking the proximal HRE sequence at -169 and -173 bp from the TSS, respectively. One HAS sequence (CACGT) was found flanking the distal HRE sequence at -1761 bp from TSS (Figure 2.5A). A previously well characterized CREB binding site (CRE) is designated for reference (Figure 2.5A).

To test direct interactions between HIF-1 and the INHA promoter, chromatin immunoprecipitation (ChIP) was performed using OVCAR5 and OV90 cells. Primers were designed to amplify the region including the HRE site closest to the transcription start site (HRE1) and chromatin shear size optimized accordingly (Methods). We find that exposure to hypoxia led to a 4-times increase in enrichment of HIF-1 binding to INHA’s HRE site in
OVCAR5 and 3-times in OV90 (Figure 2.5Bi). The second HRE site is GC rich which lead to modest amplification. Despite this, a 2-times increase in HIF-1 enrichment at this site in OV90 cells was observed (Figure 2.5Bii) which was however not statistically significant.

Given the poor enrichment of HIF-1 at the distal promoter site (Figure 2.5Bii), we next evaluated if the proximal promoter was sufficient to increase INHA levels under hypoxia and if this was dependent on HIF-1. To achieve this, we made an INHA promoter driven luciferase reporter construct, containing 547 base pairs of the INHA promoter, containing the first HRE site (Figure 2.5C, Figure 2.5A). The effect of HIF-1 on INHA promoter activity, was evaluated in HEK293 cells exposed to hypoxia (0.2% O2) for 24hrs and compared to cells under normoxia (Figure 2.5Ci), or in the presence or absence of HIF-1 ODD (pcDNA3-HA-HIF1aP402A/P564A) (Figure 2.5Cii) that prevents degradation of the HIF1α subunit. We find that in un-transfected or control vector expressing cells (pcDNA3.1), INHA promoter driven luciferase activity is increased two times in response to hypoxia (Figure 2.5Ci) that was mimicked by stabilization of HIF-1α (HIF-1 ODD) under normoxia conditions (Figure 2.5Ci). We also confirmed the requirement of HIF-1α in HEK293 using either control or HIF1/2α siRNAs. HEK293 were exposed to hypoxia for 24hrs and efficacy of HIF1/2α knockdown was confirmed by immunoblotting (Figure 2.5Di). Notably, siRNA to HIF-1α (siHIF-1α) decreased hypoxia induced INHA expression 1.8-times as compared to scramble controls (siScr; Figure 2.5Dii). However, siRNA to HIF-2α resulted in a smaller (1.25-times) and non-significant reduction in INHA expression compared to siScr when exposed to hypoxia (Figure 2.5Dii). These data point to a central role for HIF-1 in regulating INHA expression under hypoxia.
INHA has been previously reported to be regulated by other factors particularly the cAMP response element binding (CREB) family member in multiple systems\textsuperscript{5}. The CREB family of transcription factors can act downstream of the hypoxia response\textsuperscript{154}. To thus test whether cAMP was involved in regulating INHA expression under hypoxia, we utilized forskolin (Fsk), an activator of cAMP previously shown to induce INHA expression and the PKA inhibitor H89 previously shown to inhibit forskolin induced INHA expression\textsuperscript{155}. Treatment of ID8ip2 cells with Fsk increased INHA expression 5.2-times under hypoxia compared to just 2-times under normoxia (Figure 2.5Ei). This relationship appeared to be additive and not synergistic as addition of the PKA inhibitor, H89, was not able to reduce hypoxia induced INHA expression (Figure 2.5Ei). The effect of blocking PKA signaling under hypoxia was also tested in OV90 cells. Hypoxia increased INHA expression 4.5-times however treatment with H89 did not significantly reduce INHA expression under hypoxia (Figure 2.5Eii). Taken together, these data implicate HIF-1 as being the key transcriptional factor responsible for increase of INHA in hypoxia.

2.6 Inhibin promotes hypoxia induced angiogenesis and stimulates endothelial cell migration and vascular permeability

Hypoxia is a key driver of endothelial cell migration and blood vessel permeability within the tumor leading to alterations in angiogenesis\textsuperscript{142}. To determine the overall contribution of inhibin to hypoxia induced angiogenesis \textit{in vivo}, we utilized an \textit{in vivo} Matrigel plug assay. Conditioned media (CM) from HEY tumor cells exposed to normoxia or hypoxia was used to stimulate angiogenesis into the plugs, and a well-established anti-inhibin\(\alpha\) antibody, R1 (recognizing the junction between the \(\alpha\)N region, and \(\alpha\)C region)\textsuperscript{156} was used to block inhibin in the CM with IgG as a control. We find that CM from hypoxia
grown cells increased hemoglobin in the plugs 2.9-times compared to CM from normoxia
grown cells (Figure 2.6Ai-ii). Anti-inhibinα in the hypoxic CM fully reduced the
hemoglobin content in the plug (2.1-times suppression, Figure 2.6Ai-ii) indicating that
inhibin is required for hypoxia induced blood vessel formation in vivo.

Since blood vessel flow is an indication of endothelial cell functionality\textsuperscript{157}, we
sought to define the specific effects of increased inhibinα on hypoxia induced endothelial
cell biology, specifically endothelial cell chemotaxis and vascular permeability. To
determine the impact on endothelial chemotaxis to hypoxic CM, CM from either hypoxia
(24 hrs, 0.2% O\textsubscript{2}) or normoxia grown OV90 or HEY cells were used as a chemoattractant
to measure migration of human microvascular endothelial cells (HMEC-1; Figure 2.6B).
Two anti-inhibinα antibodies, R1 and a second well established antibody PO23
(recognizing the C-terminus of the αC region)\textsuperscript{156}, were used with IgG controls to test the
effect of blocking/sequestering hypoxia produced inhibinα. We find that CM from hypoxia
grown tumor cells significantly increased migration of endothelial cells (IgG, Figure 2.6B)
and incubation of hypoxic CM with anti-inhibinα R1 significantly suppressed hypoxia
induced endothelial migration (2.1 and 1.6-times for OV90 and HEY conditioned media
respectively, Figure 2.6Bi,ii). Anti-inhibinα PO23 was also able to significantly suppress
CM stimulated endothelial migration (1.5 and 1.75-times for OV90 and HEY CM,
respectively, Figure 2.6Bi-ii). Similar to the effects of hypoxic CM, recombinant inhibin
A was also able to stimulate HMEC-1 migration to similar extents as VEGF A at equimolar
amounts (Figure 2.6Biii).

We next evaluated the effect of CM from hypoxic tumor cells on changes to
permeability across an endothelial monolayer using a trans-well permeability assay that
measures solute (FITC-dextran) flux across endothelial monolayers. Permeability was monitored across a four-hour time course and CM from hypoxic tumor cells was used to induce permeability across the HMEC-1 monolayer. Effect of inhibin in the CM was evaluated either in the presence of anti-inhibinα (PO23 and R1) or IgG control (Figure 2.6Ci-ii). We find that both inhibinα antibodies (R1 and PO23) significantly decreased solute flux induced by hypoxic CM from two tumor cell lines, albeit with moderate differences in the kinetics and time to inhibition (Figure 2.6Ci-ii). Specifically, significant inhibition of permeability was seen beginning at two hours for CM treated with PO23 and three hours for R1. PO23 was moderately more effective than R1 as it effectively reduced permeability within 1 hour (Figure 2.6Ci-ii). Recombinant inhibin was also able to induce endothelial cell permeability to similar extents as LPS (Figure 2.6D), an established permeability inducing factor\textsuperscript{108}. Since perturbations to the endothelial barrier are critical to invasion and extravasation of cancer cells during metastasis\textsuperscript{158}, we tested whether inhibin induced vascular permeability facilitates tumor cell extravasation. To test this, we used a trans-endothelial cell migration assay to mimic the process. HEY tumor cells infected with GFP adenovirus to distinguish them from migrated non-GFP endothelial cells were plated on top of a non-GFP endothelial cell monolayer that was then either pre-treated with 1nM inhibin A for 4 hours or left untreated. We find that HEY GFP tumor cells, were 2.9-times more invasive across the inhibin treated monolayer than untreated conditions (Figure 2.6Eii-iii). All together, these data implicate inhibin as a robust contributor to hypoxia mediated angiogenesis, vascular permeability and thereby tumor cell extravasation across the vascular endothelium.
2.7 Inhibin promotes vascular permeability through increased VE-cadherin trafficking.

Endothelial permeability is regulated through changes in junctional proteins which are maintained through contacts with the actin cytoskeleton\textsuperscript{159}. VE-cadherin is a critical junctional protein involved in regulating endothelial cell permeability\textsuperscript{159}. To delineate the mechanism of inhibin’s effects on vascular permeability, we first evaluated the effect of inhibin on endothelial cell junctions and the actin cytoskeleton through immunofluorescent staining of VE-cadherin and actin (Figure 2.7A). Examination of the actin cytoskeleton revealed significant contractile actin staining, with a significant increase in stress fiber formation after 30 minutes of inhibin A treatment (two times increase, Figure 2.7Ai-ii). VEGF A treatment was used as a comparison that also led to similar changes in actin stress fiber formation (Figure 2.7A). VE-cadherin localization also appeared to be reduced qualitatively at the cell-cell junctions after 30 minutes of inhibin treatment as compared to untreated cells, suggestive of perturbation of the endothelial cell barrier at the level of the cytoskeleton (Figure 2.7A). Loss of VE-cadherin at the cell junctions was also observed in VEGF A treated cells (Figure 2.7A). However, total VE-cadherin levels were unchanged in response to inhibin as evaluated over a time course of 60 minutes (Figure 2.7B) indicating no change in the total pool of VE-cadherin in response to inhibin A. Actin contractility and stress fiber assembly is regulated through phosphorylation of myosin light chain (MLC)\textsuperscript{159}. In accordance, we find that phosphorylation of MLC-2 (Ser19) increased within 5 minutes of inhibin A treatment and was sustained across a 60-minute time course (Figure 2.7Ci,ii).
Based on the qualitative changes in VE-cadherin in response to inhibin A treatment (Figure 2.7A), we tested whether alterations in VE-cadherin at the cell-cell junctions were due to inhibin induced VE-cadherin internalization. To determine this, HMEC-1 membrane localized VE-cadherin was labeled at 4°C with an anti-VE-cadherin antibody recognizing the extra-cellular domain. HMEC-1 cells were washed with acid to remove membrane bound anti-VE-cadherin leaving only any internalized VE-cadherin that may have been labeled at 4°C prior to treatment with inhibin A or VEGF A (Figure 2.8A). Stripping of cell surface VE-cadherin was verified by cell surface immunostaining of VE-cadherin with little to no internalized VE-cadherin detected (Figure 2.8Bi,iii). Cells were then either left untreated or treated for 30 minutes with inhibin A at 37°C and VE-cadherin evaluated by immunofluorescence (Figure 2.8Bii). We find that inhibin A increased the internalized VE-cadherin pool compared to untreated cells 1.4-times (Figure 2.8Civ) and to similar extents as VEGF A (1.6-times, Figure 2.8Civ). These results indicate that inhibin induces rapid changes in the actin cytoskeleton and trafficking of VE-cadherin from the cell junctions of endothelial cells.

2.8 Inhibin’s effects on vascular permeability are mediated by ALK1 and CD105/endoglin that form a stable complex at the cell surface in response to inhibin

Previously, we demonstrated that inhibin’s effects on angiogenesis and endothelial cell signaling were dependent on the TGFβ receptors ALK1 and endoglin57. To evaluate if ALK1 and endoglin are required for inhibin’s influence on vascular permeability, we treated HMEC-1 cells with TRC105, a humanized endoglin monoclonal antibody 160, or with ALK1-Fc, a human chimeric ALK1 protein161. At four hours, treatment with (i)
TRC105 and (ii) ALK1-Fc decreased inhibin A induced permeability by 2.2 and 1.5-times, respectively (Figure 2.9A) indicating both ALK1 and endoglin are required for inhibin’s effects on endothelial cell permeability.

We next evaluated if internalization of VE-cadherin by inhibin was dependent on endoglin using mouse embryonic endothelial cells (MEEC) that are either wild type (WT) or null for endoglin expression51 (Figure 2.9B). Cell surface biotinylation of VE-cadherin was used to quantitatively assess VE-cadherin internalization. Towards this, cell surface proteins were labeled with Sulfo-NH-SS biotin and allowed to internalize for 30 minutes at 37°C in the presence or absence of inhibin followed by stripping of cell surface biotin, immunoprecipitation with neutravidin resin and immunoblotting to detect internalized biotin labeled VE-cadherin (Figure 2.9C). Treatment with inhibin A increased internalized VE-cadherin 1.9-times in MEEC WT compared to control (Figure 2.9Di), similar to extents seen by immunofluorescence in HMEC-1 cells (Figure 2.9B). However, in the absence of endoglin in MEEC ENG/- cells inhibin A did not change the internalized VE-cadherin pool (Figure 2.9Dii). This data indicates that endoglin is essential for inhibins effects on VE-cadherin.

Based on the significant dependency of inhibin’s effects on endothelial cell permeability and VE-cadherin internalization on endoglin and ALK1 respectively (Figure 2.10A,D), we evaluated biophysically, in a sensitive and quantitative manner, the extent of the endoglin-ALK1 interaction in response to inhibin. We utilized a patch/FRAP (fluorescence recovery after photobleaching) methodology to measure interactions between endoglin and ALK1 at the surface of live cells. This method differentiates between stable and transient interactions as described in detail previously162. Herein, one receptor
carrying an extracellular epitope tag is patched and immobilized through cross-linking with a double layer of IgGs. The effects of this immobilization on the lateral diffusion of a co-expressed, differently tagged receptor labeled exclusively with Fab’ fragments are then measured by FRAP (Methods). Stable complex formation between the two co-expressed receptors (complex lifetimes longer than the characteristic FRAP fluorescence recovery time) reduces the mobile fraction \( (R_f) \) of the Fab’-labeled receptor, since bleached Fab’-labeled receptors associated with immobilized receptors do not appreciably dissociate from the immobile patches during the FRAP measurement. On the other hand, transient complexes (short complex lifetimes) would reduce the apparent lateral diffusion coefficient \( (D) \), since each Fab’-labeled receptor molecule can undergo multiple association-dissociation cycles during the FRAP measurement\(^{162}\). For these studies, COS7 cells were transfected with myc-ALK1, HA-endoglin or co-transfected with both, and subjected to patch/FRAP experiments in the absence or presence of 4 nM of inhibin A (Figure 2.10A). Figure 2.10Ai-iii depict representative FRAP curves showing the lateral diffusion of myc-ALK1 (Figure 2.10Ai), IgG-crosslinked and immobilized HA-endoglin (Figure 2.10Aii), and myc-ALK1 co-transfected with HA-endoglin followed by IgG cross-linking of HA-endoglin in the presence of inhibin (Figure 2.10Aiii). Average values derived from multiple independent experiments are shown in \( (R_f) \) in Figure 2.10Av, \( D \) values Figure 2.10ACv). Singly expressed myc-ALK1 had lateral mobility resembling other TGF-\( \beta \) superfamily receptors\(^{140}\), which was insensitive to inhibin treatment Figure 2.10ACi ,iv). Immobilization of HA-endoglin (Figure 2.10Aii,iv) reduced \( R_f \) of myc-ALK1 by about 45%, and the presence of inhibin increased this reduction significantly (from 45% to 70% reduction) (Figure 2.10Aiii,iv). Under all these conditions, the lateral diffusion coefficient
(D) of myc-ALK1 was not significantly affected (Figure 2.10ACv), indicating that endoglin and ALK1 form stable complexes at the plasma membrane which are enhanced and stabilized by inhibin.

Previous studies indicate that inhibinα may bind to ALK4\textsuperscript{54}, an established Type I receptor for the Activin family of proteins\textsuperscript{5}. We thus employed patch/FRAP to determine the interactions between endoglin and ALK4 and to examine whether inhibin A enhanced these interactions. To this end, we expressed HA-ALK4, myc-endoglin or both in COS7 cells, and subjected them to patch/FRAP studies on the lateral diffusion of HA-ALK4 without and with IgG cross-linking of myc-endoglin, and with or without inhibin A. In the absence of inhibin A, endoglin and ALK4 exhibited significant stable interactions, as demonstrated by the reduction in $R_f$ of HA-ALK4 upon immobilization of myc-endoglin (40% reduction in $R_f$, with no effect on the $D$ value) (Figure 2.10Bi-ii). However, in contrast to the observations with endoglin-ALK1 complexes, the interactions between endoglin and ALK4 were weakened in the presence of inhibin A (the reduction in $R_f$ decreased to 20%) (Figure 2.10Bi). Taken together, these results indicate that inhibin shifts the balance of endoglin complexes from interactions with ALK4 to interactions with ALK1, both of which (endoglin and ALK1) are required for inhibin mediated vascular permeability.

2.9 Inhibin promotes hypoxia induced tumor growth \textit{in vivo} through alterations in permeability and angiogenesis

The significance of hypoxia in ovarian cancer is well documented and we previously demonstrated increased ascites accumulation in tumor bearing mice in the presence of inhibin\textsuperscript{57}. To precisely define the contribution of inhibin to hypoxia induced
tumor growth and angiogenesis, we first evaluated the effects of pre-exposure to hypoxia on tumor growth in a subcutaneous model in vivo, a model that allows for quantitative analysis of the vasculature in tumors. HEY pLKO.1 control vector (shControl) cells were pre-exposed to hypoxia (0.2% O2) for 24hrs or kept under normoxia followed by injection into the right flank of Ncr nude mice. Tumors were measured throughout and harvested after 30 days (n=10 mice). HEY cells pre-exposed to hypoxia produced rapid growing tumors compared to those that originated from normoxia grown cells (Figure 2.11A, purple versus black line). In parallel, we utilized two methods to perturb inhibin: 1) shRNA knockdown of INHA in HEY cells (Figure 2.11Bi) and 2) intraperitoneal administration of anti-inhibinα antibody (R1). R1 is a human antibody and consistent with this no overall toxicity was noted in pilot toxicity studies that utilized daily injections of R1 (Figure 2.11C). shINHA cells exposed to hypoxia maintained their knockdown to INHA at the end of the study (Figure 2.11Bii) and produced tumors with significantly slower growth rates than shControl hypoxia tumors (Figure 2.11A, blue versus black lines). In complementary findings, hypoxia exposed tumor cells had significantly reduced tumor growth upon receiving treatment with the R1 antibody when compared to tumors in mice that received vehicle only (Figure 2.11A, red versus blue line, n=6 for R1 treated mice). The group receiving anti-inhibinα (R1) grew at a similar rate as the shINHA hypoxia tumors (Figure 2.11A, red versus blue line). In mice with shINHA tumors, treatment with R1 further reduced tumor growth albeit moderately compared to vehicle shINHA (Figure 2.11A, blue versus green line). These data indicate that perturbation of inhibin through shRNA targeting, and anti-inhibin antibody treatment reduces tumor growth.
Next, we sought to evaluate the effect of hypoxia induced inhibin on angiogenesis within tumor xenografts. To rule out whether the reduction in tumor growth in shINHA cells was due to slower proliferation of tumor cells, growth rate of HEY shINHA and HEY shControl was evaluated in culture under hypoxia for 3 days. No significant change was observed (Figure 2.12A) suggesting that the major effect of inhibin on tumor growth are likely through effects on the tumor vasculature due to the effects of hypoxia regulated inhibin on angiogenesis and vascular permeability in vitro (Figure 2.6).

Next, we sought to determine the effect of shINHA on the angiogenic cytokine profile of the tumors from Figure 2.11A using a proteome array of 55 different human angiogenesis targets. We find that the most up-regulated proteins in control tumors compared to shINHA tumors were a subset of pro-angiogenic cytokines IL8 (2.5-times) and EGF (2.1-times) (Figure 2.12Bi) indicating a proangiogenic profile of the tumor cells in the presence of inhibin. In contrast, the shINHA hypoxia tumors showed increases in proteins including ADAMTS-1 (1.6-times) and Pentraxin-3 (1.3-times), indicating an anti-angiogenic profile in shINHA tumor cells as both have been demonstrated to be anti-angiogenic. Activin A and endoglin were also found to be elevated in shINHA tumors (Figure 2.12Bi). To complement the human tumor array, we analyzed changes in the mouse angiogenic proteome as well to delineate any host differences in response to shControl and shINHA tumor cells. We find that host cells also upregulated significantly more pro-angiogenic proteins, including CXCL16, PIGF-2, and NOV in shControl tumors compared to shINHA tumors (Figure 2.12Bii). Taken together, these data suggest that altering inhibin in the tumors results in a change in the balance of angiogenic factors
leading to a significant reduction in pro-angiogenic factors and slower overall tumor growth.

We thus determined the effect of inhibin on the tumor vasculature and associated permeability changes as a contributing factor to the altered tumor growth in shINHA and antibody treated hypoxia tumors (Figure 2.12A). To this end, HEY shControl or shINHA cells pre-exposed to hypoxia for 24hrs were injected subcutaneously into the right flank of Ncr nude mice (n=4 mice) and tumors in all groups were harvested upon reaching 700-850mm\(^3\) (Figure 2.12Ci) to eliminate any tumor size effects on angiogenesis. These tumors (Figure 2.12Ci) were evaluated for changes in vascular permeability by visualization of a rhodamine-dextran dye that leaks from the blood vessels into the tumors when administered into mice prior to sacrifice. We find that rhodamine-dextran was present at 5.5-times higher levels in shControl tumors compared to shINHA tumors indicating higher vascular permeability within the tumors in the presence of inhibin (Figure 2.12Cii-iii). To further characterize the differences in the vasculature between shControl and shINHA tumors, blood vessels were stained with CD-31 to evaluate vessel number and size (Figure 2.12D). We find an increase in the total number of blood vessels in shControl tumors compared to shINHA tumors (Figure 2.12D i,iii). Quantitation of the size of the vessels revealed significantly smaller vessels in shControl tumors as compared to the shINHA tumors (Figure 2.12Dii,iii). These data together demonstrate that reducing inhibin in the tumor decreases vascular leakiness, alters vessel size and numbers and promotes more normalized vasculature in the tumors.
2.10 Discussion

Hypoxia significantly impacts several aspects of tumor progression by regulating pathways that can be targeted for cancer management, particularly angiogenic mechanisms. We have for the first time identified inhibins’, that are well established biomarkers for ovarian and other cancers and a member of the TGFβ superfamily, to be targets of the hypoxic response. We significantly extended our previous findings\textsuperscript{57} to demonstrate that hypoxia induced tumor growth, angiogenesis and vascular leakiness is accompanied with, and dependent on inhibin levels in cells and tumors, and relevant to the ovarian cancer patient population. In keeping with this, hypoxia induced tumor growth can be suppressed by treatment with a selective inhibin antibody that leads to a shift in the angiogenic balance in tumors. We also provide mechanistic evidence for the involvement of ALK1 and CD105/endoglin in inhibin’s effects on permeability via increased VE-cadherin internalization. Due to the lack of systemic inhibin expression in post-menopausal women, establishing the therapeutic significance of targeting inhibin in this patient population may be particularly beneficial to evade systemic side effects seen with targeting other hypoxia associated angiogenic pathways.

Significant information exists on the cycling levels of inhibins’ in premenopausal women, the decline of inhibin during perimenopause, and as a marker whose decline defines the onset of menopause leading to complete absence of inhibin in normal post-menopausal women\textsuperscript{127}. Contrastingly, several studies have reported elevated levels of Inhibin in a subset of cancers\textsuperscript{73,77,78,128}. Our studies shed light on the potential mechanisms leading to elevated inhibin. We also find that total inhibin is elevated in the ascites fluid of patients with ovarian cancer, a hypoxic environment that aids in dissemination of shed
ovarian cancer spheroids\textsuperscript{105,108} (Figure 2.3). Serum inhibin and CA125 levels are both markers for ovarian cancer\textsuperscript{66,128} and were also positively correlated with each other in this patient ascites (Figure 2.13). Menopause status was unknown in these patients however the median age of the cohort was 62 and only two patients were below 50 years of age (Figure 2.13). \textit{INHA} expression and hypoxia are also correlated through a hypoxia gene score (Figure 2.3). Supporting our hypothesis that inhibin is regulated by hypoxia, we also found that exposure of ovarian cancer cells to hypoxia increased \textit{INHA} expression and inhibin secretion (Figure 2.1, 2.2). Surprisingly, we did not note consistent and statistically significant increases in the activin subunits, \textit{INHBA} or \textit{INHBB} which only appeared to be moderately elevated (Figure 2.2) indicating that increased secretion levels were driven by inhibin$\alpha$. Previous reports indicate activin, specifically \textit{INHBA}, increases in response to hypoxia in endothelial cells\textsuperscript{169}. However, here the increase in \textit{INHA} levels in endothelial cells in response to hypoxia was only moderate as compared to in tumor cells (Figure 2.1) suggesting a potential competition in the tumor microenvironment between activin and inhibin. The inhibin ELISA used here does not detect dimeric or free activin, as it is specific to inhibin$\alpha$ and detects all forms of inhibin, namely inhibin A/B and free alpha subunit. Hence, a hypoxia dependent increase in the alpha subunit (\textit{INHA}), in the absence of a change in activin mRNA levels (Figure 2.2), could potentially shift the dimerization of the beta subunits (\textit{INHBA}/\textit{INHBB}) from activin homodimers to inhibin heterodimers and remains to be determined.

Evidence of HIF-1 dependency was observed when hypoxia exposed cells were re-exposed to oxygen (re-oxygenation). HIF-1 levels returned to near baseline levels after one hour which corresponded with \textit{INHA} expression decreasing to levels not significantly
different than baseline normoxic levels (Figure 2.1Dii). We did observe elevated, although non-significant, INHA in OV90 after 1hr of reoxygenation which could be attributed to mRNA turnover mechanisms. Mechanistically, we find through knockdown studies and ChIP studies that INHA expression is likely regulated through the HIF-1 transcription factor binding directly to the INHA promoter (Figure 2.4, 2.5). Our findings on a hypoxia response in a pathological condition as seen here, is consistent with a previous report demonstrating that FSH can drive INHA expression in granulosa cells dependent on HIF-1 in what appeared to be in an indirect manner\textsuperscript{170}. Intriguingly, evidence for INHA regulation by hypoxia, specifically dependent on HIF isoforms, has been demonstrated in cytotrophoblasts\textsuperscript{171}. Here we present detailed evidence of regulation by HIF-1, with HIF1 interacting at INHA’s promoter under hypoxia (Figure 2.5). cAMP and PKA can be activated in response to hypoxia as well. However, the PKA inhibitor, H89, was not able to reduce hypoxia induced INHA expression indicating that cAMP may not be involved in the hypoxia transcriptional regulation of INHA (Figure 2.5). This does not preclude a role for cAMP-PKA in the regulation of INHA as it is well established that the cAMP-PKA signaling axis enhances tumorigenesis in ovarian cancer\textsuperscript{172}. As the effect of forskolin was additive on INHA expression, cAMP and PKA could represent an alternative or additive mechanism of regulation of INHA in ovarian cancer.

In prostate and adrenocortical cancers reports of both increased and decreased inhibinα levels have been reported\textsuperscript{76,77,79}. In adrenocortical tumors with lower INHA levels, methylation of the INHA promoter was reported to occur at the CpG island within the proximal HRE site that we identified, suggesting potential roles for epigenetic regulation of INHA as well\textsuperscript{79}. HIF transcription factors have reduced binding to methylated hypoxia
response elements\textsuperscript{173}. To this end, it is possible that not all cell lines will increase inhibin expression in response to hypoxia. If this is the case, methylation of \textit{INHA}’s promoter may play a role making further understanding of the regulation of \textit{INHA} expression, particularly in patients necessary in the future.

Previously we also demonstrated inhibin’s effects broadly on angiogenesis\textsuperscript{57}. Here, we sought to define more precisely the outcomes of inhibin’s effects on angiogenesis, specifically in the context of hypoxia. Using recombinant inhibin and antibodies to the alpha subunit of inhibin, we find novel roles for inhibin as a permeability inducing factor with implications for tumor cell extravasation (Figure 2.6). Inhibin induced permeability was dependent on ALK1 and endoglin (Figure 2.9). The VE-cadherin dependent mechanism of permeability observed by us (Figure 2.7, 2.8) is consistent with prior findings on the effects of other TGF\(\beta\) family members’ roles in promoting vascular permeability, specifically BMP6\textsuperscript{136}. BMP6 induced vascular permeability was mediated through the Type 1 receptor ALK2\textsuperscript{136}, whereas we expect the Type 1 receptor ALK1 to be more critical for inhibin induced vascular permeability. Interestingly, inhibin strongly increased the stable interaction between ALK1 and endoglin (Figure 2.10), in line with our observation that both endoglin and ALK1 are required for permeability, and endoglin being critical for VE-cadherin internalization (Figure 2.9). These findings have broad implications for other TGF\(\beta\) family members that may regulate permeability dependent on Type 1 receptors. The patch/FRAP studies (Figure 2.10) support our current and previous findings\textsuperscript{57}. Although there are some reports suggesting that inhibin can bind to ALK4\textsuperscript{54}, our findings show that inhibin does not enhance endoglin-ALK4 complex formation but rather weakens it (Figure 2.10). We have previously demonstrated that endothelial cells
such as HMEC-1 express very little ALK4 compared to ALK1\textsuperscript{57}, supporting the idea that inhibin acts in endothelial cells preferentially via ALK1 in line with a potential physiological relevance of inhibin-mediated increase in endoglin-ALK1 interactions. However, these findings in endothelial cells do not contradict the current understanding of inhibin’s function in non-endothelial cells, which may express more ALK4 than ALK1. These findings also do not allow us to conclude whether the ALK1-endoglin complex, which is enhanced by the binding of inhibin, is signaling or kinase competent, as non-signaling receptor complexes may exist and impact signaling in an indirect manner. Such complexes were previously reported in the context of activin and ALK2\textsuperscript{174,175} and need further examination for inhibins.

Targeting inhibin through shRNA knockdown and antibody treatment was found to be an effective anti-angiogenic strategy leading to reduced vascular permeability increased blood vessel size but fewer number of vessels and a likely more normalized vasculature (Figure 2.12). Interestingly, in our analysis of the angiogenic proteome of HEY tumors, permeability promoting cytokines, EGF, IL-8, and DPP4 were significantly lower in sh\textit{INHA} tumors which were less permeable as compared to shControl tumors (Figure 2.12). Interestingly, the sh\textit{INHA} tumor cells produced more activin and endoglin compared to shControl tumors (Figure 2.12). Increased activin fits the profile of the sh\textit{INHA} tumors expressing more anti-angiogenic proteins as activin has been shown to inhibit angiogenesis\textsuperscript{123} which could also be a result of decreased inhibin\alpha leading to a shift in the balance to increased dimerization of INHBA/B and thereby activin levels. Similarly, increases in tumor cell endoglin levels in sh\textit{INHA} tumors \textit{in vivo} may reflect compensatory responses to changes in inhibin expression consistent with recent reports on endoglin
expression changes in ovarian cancers\textsuperscript{176}. Whether these changes impact metastasis and angiogenesis and are directly related to changes in inhibin levels in patients remains to be examined. Which of these altered proteins contributes the most to the either pro or anti-angiogenic tumor microenvironment remains to be determined as we unravel new roles for inhibins. In the mouse host cells where inhibin is likely to interact with endoglin from the endothelia to affect angiogenesis, endoglin levels were slightly higher in shControl receiving hosts that had more vessels compared to sh\textit{INHA} (Figure 2.12). These findings also suggest that blocking inhibin could shift the balance between pro and anti-angiogenic genes.

We also demonstrate for the first time that anti-inhibin in a therapeutic regimen can reduce tumor growth \textit{in vivo} (Figure 2.11). The subcutaneous model utilized does not induce ascites formation, unlike the intraperitoneal model used previously, where mice with sh\textit{INHA} tumors produced less ascites than those with shControl tumors\textsuperscript{57}. However, this model was chosen as it better allows for evaluation of the vasculature \textit{in vivo} and short exposure to hypoxia leads to increased tumor growth as seen here (Figure 2.11) and as seen in other models as well\textsuperscript{177,178}. Our findings that inhibin is elevated in patient ascites (Figure 2.3) supports the idea that inhibin may promote ascites formation, likely through increased vascular permeability. The effectiveness of anti-angiogenic therapies is attributed to increased vascular normalization resulting in reduced intra-tumoral hypoxia, perfused and functional vessels that improve delivery of other chemotherapeutics and enhanced immune response\textsuperscript{179}. Further studies utilizing intraperitoneal or intrabursal models that present additional steps of disease progression and metastasis are warranted to evaluate hypoxia and anti- inhibin approaches therein. Resistance to current anti-angiogenic therapies is also
common and inhibin A levels have been reported to be increased in patients non-responsive to anti-angiogenic therapy\(^{180}\) (combination of TRC105 and Bevacizumab) indicating inhibin as a potential alternative mechanism of angiogenesis in tumors resistant to other anti-angiogenic therapies. Further studies exploring the impact of anti-inhibin therapy on the effectiveness of chemotherapeutics and anti-tumor immune response as well is most certainly warranted.

In conclusion, our study shows that targeting inhibin is an effective anti-angiogenic strategy. We demonstrate for the first time a contextual mechanism for the regulation of inhibin directly driven by hypoxia and HIF-1 and fully define inhibin’s contributions to hypoxia induced angiogenesis. Based on our findings and the previously known physiological functions of inhibin, we speculate that targeting inhibin may have potential improved therapeutic value in post-menopausal cancers including a significant percentage of ovarian cancers.
Table 2.1. Key Resource Table

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**Plasmids, siRNA, Lentivirus**

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**Other**

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| 8μM trans-well filter | Greiner Bio-One | #662630 |
| PureLink PCR Purification Kit | Invitrogen | #K310002 |
| Amicon Ultra Centrifugal Filter | EMD Millipore | UFC501024 |
| Proteome Profiler Human Angiogenesis Array Kit | R&D Systems | #ARY007 |
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Table 2.2. Lentiviral and siRNA sequences

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</table>
Figure 2.1. \textit{INHA} is increased in response to hypoxia in ovarian cancer cells. A) Relative qRT-PCR analysis of (i) \textit{INHA} and (ii) \textit{VEGFA} mRNA expression normalized to levels in 20\% O_2 in HEY and OV90 cells exposed to indicated oxygen concentration for 24hrs. Mean±SEM, (n=3). *, p<.05; **, p<.01; ***, p<.001; ****, p<.0001. One-way ANOVA followed by Tukey’s multiple comparison. (iii) Western blot of HIF-1\(\alpha\)
stabilization at indicated oxygen concentrations. B) Relative qRT-PCR analysis of (i) INHA and (ii) VEGFA mRNA expression normalized to corresponding levels in normoxia in indicated cells grown under hypoxia (0.2%) or normoxia (17-21%) for 24hrs except for OVCAR5 (12hrs). Mean±SEM, n of independent trials for PA1=3, OVCAR5 n=7, HEY n=3, OV90 n=3, *, p<.05; **, p<.01, unpaired t-test. (iii) Western blot of HIF-1α stabilization in indicated cell lines. C) Relative qRT-PCR analysis of INHA mRNA expression normalized to corresponding levels in normoxia in HMEC-1 cells grown under hypoxia (0.2%) or normoxia (17-21%) for either 12 or 24hrs. Mean±SEM, n=3 for 12hrs and 2 for 24hrs. n.s., not significant, unpaired t-test. D) (i) Western blot of HIF-1α levels in HEY and OV90 following exposure to hypoxia (0.2% O₂) for 24hrs and after indicated re-oxygenation times. (ii) Relative qRT-PCR analysis of INHA expression in HEY and OV90 cells following exposure to hypoxia (0.2% O₂) and indicated re-oxygenation time normalized to corresponding levels in normoxia. Mean±SEM, (n=3). *, p<.05; **, p<.01, One-way ANOVA followed by Tukey’s multiple comparison.
Figure 2.2. Inhibin protein is secreted by ovarian cancer in response to hypoxia. A) Total inhibin ELISA (inhibin A/B, inhibinα) of conditioned media collected from OV90 and HEY cells grown in normoxia or after 24hr exposure to hypoxia (0.2% O₂). Mean±SEM, (n=3). **, p<.01, unpaired t-test. B) Relative qRT-PCR analysis of INHBA and INHBB mRNA expression normalized to corresponding levels in normoxia in OV90 or HEY cells grown under hypoxia (0.2%) or normoxia (17-21%) for 24hrs. Mean±SEM, n=3. N.s., not significant, unpaired t-test.
Figure 2.3. Inhibinα is increased in ovarian cancer spheroids, patient samples, and tumor xenografts. A) (i) Western blotting of HIF-1α protein from indicated cells grown in either 2D or under anchorage independence (3D) conditions, vinculin is loading control and (ii) relative qRT-PCR of INHA mRNA expression in OVCA420 and PA1 after 48hrs
(OVCA420) or 72hrs (PA1) of growth under anchorage independence (3D). Mean±SEM, n=3. **, p<.01; ***, p<.001, unpaired t-test. B) Total inhibin ELISA of ascites fluid from 25 ovarian cancer patients sorted by stage. C) (i) Representative images of pimonidazole staining in HEY xenograft tumors used for calculation of hypoxic area. Scale bar: 500µm. (ii) Percent hypoxic area in tumors of indicated size range determined by quantitation of pimonidazole staining in tumors (i). Graph represents average hypoxic area of all HEY xenograft tumors sorted by size as <500mm³ or >500mm³. Mean±SEM, n=4 for <500mm³ and n=7 for >500mm³. *, p<.05, unpaired t-test. D) (i) Relative qRT-PCR of INHA expression in tumors from indicated sizes of HEY cells implanted subcutaneously. Mean±SEM, n=8. ***, p<.001, unpaired t-test. (ii) Correlation analysis of INHA expression and HEY subcutaneous tumor volume (mm³). (E) Correlation analysis between INHA expression and either (i) Buffa or (ii) Winter hypoxia scores from TCGA OVCA (i-ii) or breast (iii) cancer patient data sets from cBioportal measured by RNA-Seq. Correlation analysis was performed by Pearson correlation.
Figure 2.4. HIF-1 is required for INHA expression under hypoxia. A) (i) Western blot of HIF-1α at indicated time points after treatment with 100μM CoCl₂. (ii) Relative qRT-PCR analysis of INHA and VEGF mRNA in OVCAR5 and PA1 cells after indicated time of treatment with 100μM of CoCl₂ normalized to untreated. Mean±SEM, (n=2). *, p<.05; **, p<.01, One-way ANOVA followed by Tukey’s multiple comparison. B) Relative qRT-PCR analysis of INHA and ARNT mRNA in HEY shControl or shARNT cell lines after exposure to hypoxia (0.2% O₂) for 24hrs normalized to corresponding shControl normoxia levels. Mean±SEM, (n=4). n.s.,not significant; *, p<.05; **, p<.01, unpaired t-test. C) Representative western blot (above) and relative qRT-PCR analysis of INHA expression (below) from (i) HEY or (ii) OV90 cells transfected with either siScr, siHIF-1α, siHIF-2α, or a combination of siHIF-1/2α and exposed to hypoxia (0.2% O₂) for 24hrs. Mean±SEM, (n=3) *, p<.05; ***, p<.001; ****, p<.0001, One-way ANOVA followed by Tukeys multiple comparison test.
Figure 2.5. *INHA is a direct target of HIF-1* A) Schematic of INHA promoter containing location of hypoxia response elements (HRE), hypoxia ancillary sequences (HAS), cAMP response element (CREB), and ChIP primers. Schematic is drawn to scale with appropriate scale information below. B) Relative qRT-PCR analysis using primers that amplify the (i) proximal HRE region in the INHA promoter or (ii) distal HRE region after chromatin immunoprecipitation (ChIP) of HIF-1α in OVCAR5 and OV90 cells. ChIP qRT-PCR results were quantified as normalized enrichment over IgG and normalized to normoxia. Mean±SEM, OVCAR5 (n=3), OV90 (n=2). n.s., not significant; *, p<.05; **, p<.01, Two-way ANOVA followed by Fishers LSD test. C) Luciferase activity of HEK293 cells transfected with the INHA promoter driven luciferase reporter construct (pGL4.10) and a SV-40 renilla control vector. Cells were either (i) exposed to hypoxia (0.2% O₂) or (ii) cotransfected with HIF-1α overexpression plasmid (HIF-1 ODD) and luciferase activity measured and normalized to either normoxia in (i) or PCDNA3.1 in (ii). Mean±SEM, n=3 (Hypoxia), n=2 (HIF-1 ODD) *, p<.05; **, p<.01, unpaired t-test. D) (i) Representative western blot and (ii) relative qRT-PCR analysis of INHA expression from HEK293 cells transfected with either siScr, siHIF-1α or siHIF-2α and exposed to hypoxia (0.2% O₂) for 24hrs. Mean±SEM, (n=4). *, p<.05; ***, p<.001; ****, p<.0001, One-way ANOVA followed by Tukeys multiple comparison test E) (i) Relative qRT-PCR analysis of INHA mRNA from ID8ip2 cells treated with either 10μM H89 or 20μM forskolin (Fsk), grown under normoxia or hypoxia 0.2% O₂ for 24hrs and normalized to normoxia. Mean±SEM, n=3. (ii) Relative qRT-PCR of INHA mRNA from OV90 cells treated with 10μM H89 and grown under normoxia or hypoxia (0.2% O₂) for 24hrs and normalized to normoxia. Mean±SEM, n=3.
Figure 2.6. Inhibin increases hypoxia induced angiogenesis and endothelial cell migration and permeability in vivo and in vitro respectively. A) (i) Hemoglobin content
in Matrigel plugs collected 12 days after subcutaneous injection of HEY conditioned media collected from cells exposed to normoxia or hypoxia for 24hrs and mixed with either 2μg of IgG or of anti-inhibin R1 antibody. Mean±SEM, n=6 plugs per condition. n.s., not significant; ***, p<.001, One-way ANOVA followed by Tukey’s multiple comparison test.

(ii) Representative images of Matrigel plugs from (i) Scale bar: 2mm. B) Quantitation of HMEC-1 migration through fibronectin coated 8μm trans-well filter (i-ii) towards conditioned media from OV90 or HEY cells exposed to hypoxia (0.2% O₂) with either 2μg of R1 or PO23 anti-inhibin antibody or IgG as a control, or towards (iii) serum free media containing 1nM inhibin A or 1nM VEGF A. Nuclei from three representative fields per filter were counted. Mean±SD. **, p<.01; ***p<.001; ****, p<.0001, One-way ANOVA followed by Tukey’s multiple comparison. C-D) Quantitation of endothelial cell permeability by measuring FITC-dextran changes across a HMEC-1 monolayer treated with (i-ii) conditioned media from (i) OV90 or (ii) HEY cells exposed to hypoxia (0.2% O₂) with either 2μg of R1 or PO23 anti-inhibin antibody or IgG as a control, or (D) treated with 1nM inhibin A or 10μg/mL LPS. Mean±SEM *, p<.05; ***p<.001; ****, p<.0001, One-way ANOVA followed by Tukey’s multiple comparison. E) HEY trans-endothelial migration (TEM) across HMEC-1 monolayer either treated with inhibin A for 4 hrs or untreated. (i) Representative transmigrated GFP positive HEY cells and (ii) quantitation of transmigration (n=3). *, p<.05, unpaired t-test. Scale bar: 100μm.
Figure 2.7. Inhibin increases endothelial cell contractility. A) (i) Representative immunofluorescence images of F-actin (red) or VE-Cadherin (green) from HMEC-1 cells grown to confluence on fibronectin coated coverslips and treated with either 1nM inhibin A or 1nM VEGF A for 30 minutes. (ii) Quantitation of actin stress fibers from (i) using ImageJ Fibril tool plugin (Methods). ***p<.001; ****, p<.0001, unpaired t-test. Scalebar: 25μm. B) HMEC-1 grown to confluence were treated with 1nM Inhibin A for indicated time. Lysates were immunoblotted for VE-cadherin and β-Actin. C) (i) Western blot analysis of pMLC-2 from HMEC-1 cells upon 1nM inhibin A treatment for indicated times. (ii) Quantitation of pMLC-2 changes in (i).
Figure 2.8. Inhibin promotes VE-cadherin internalization in endothelial cells. A) Schematic of VE-cadherin internalization (Methods). B) (i) Representative immunofluorescent images of (upper panel) cell surface labeled VE-cadherin at 4°C detected by labeling with an extracellular domain anti-VE-cadherin antibody. (Lower panel) efficiency of stripping of extracellular labeled VE-cadherin with a mild acid. (ii) Internalized VE-Cadherin at 37°C detected with a FITC-secondary antibody in either untreated or cells treated with 1nM inhibin A or 1nM VEGF A after acid wash. Green arrows represent internalized VE-cadherin. Red, actin. Blue, DAPI. Quantitation of internalized VE-Cadherin at (iii) T₀ or (iv) T₃₀ by Blobfinder ImageJ Plugin (Methods). *, p<.05, unpaired t-test. Scalebar: 25μm.
Figure 2.9. Inhibin promotes endothelial cell permeability via ALK1 and endoglin. A) Quantitation of endothelial cell permeability by measuring FITC-dextran changes across a HMEC-1 monolayer treated with 1nM inhibin A in the presence or absence of (i) 100μg/mL TRC-105 or (ii) 10 ng/mL ALK1-Fc. FITC-dextran diffusion across the HMEC-1 monolayer is presented. Mean±SD, n=4 for i and n=3 for ii. n.s., not significant; ***, p<.001; ****, p<.0001. B) Relative qRT-PCR analysis of ENG mRNA expression in MEEC WT or MEEC ENG-/-.

C) Schematic of internalization of VE-cadherin measured by cell surface biotinylation. Biotin labeling of cell surface proteins was performed on MEEC WT or MEEC ENG-/-.

Internalization was induced by treatment with 1nm Inhibin A for 30 minutes at 37°C followed by stripping of cell surface biotin. Internalized VE-
cadherin was detected by IP with neutravidin resin and D) (i) immunoblotting with anti-VE-cadherin and (ii) quantitated as internalized VE-cadherin over input VE-cadherin normalized to 37°C control. Mean±SD, n=2. n.s., not significant; *, p<.05, unpaired t-test.
Figure 2.10. Inhibin increases ALK1-endoglin cell surface complexes while reducing ALK4-endoglin complexes. A-B) Patch/FRAP studies on the effect of Inhibin A on endoglin-ALK1 (A) and endoglin-ALK4 (B) complex formation. COS7 cells were transfected with myc-ALK1 and HA-endoglin (A) or with (each vector myc-ALK4 and HA-endoglin (B) (each vector alone, or together). C) After 24 h, singly transfected cells were labeled for FRAP by anti-tag Fab’ followed by fluorescent secondary Fab’ (Methods) and subjected to FRAP studies. For patch/FRAP, cells were subjected to protocol 1 of IgG-mediated patching/cross-linking (CL) (Methods), resulting in HA-endoglin patched and labeled by Alexa 488-GαR IgG (designated “CL: IgG αHA”), whereas myc-ALK1 is labeled by monovalent Fab’ (with secondary Alexa 546-GαM Fab’). In control experiments without HA-endoglin CL, the IgG labeling of the HA tag was replaced by exclusive Fab’ labeling. Where indicated, inhibin A (4 nM) was added during the fluorescent labeling step and maintained throughout the measurement. Representative FRAP curves are depicted in
panels (i-iii), showing the lateral diffusion of singly-expressed myc-ALK1 (i), singly expressed HA-endoglin immobilized by IgG CL (ii) and of myc-ALK1 in the presence of co-expressed and IgG-crosslinked HA-endoglin in the presence of inhibin A (iii). Panels (iv-v) depict average $R_f$ (iv) and $D$ values (v) of multiple experiments. Bars represent Mean ± SEM values, with the number of measurements (each conducted on a different cell) shown in each bar. Some of these numbers are lower in the $D$ values panels, since only $R_f$ can be extracted from FRAP curves yielding less than 20% recovery. Asterisks indicate significant differences between the $R_f$ values of the pairs indicated by brackets (****, $p < 1 \times 10^{-15}$; ***, $p = 1 \times 10^{-9}$; one-way ANOVA followed by Bonferroni post-hoc test). B) Cells were labeled for patch/FRAP using protocol 2 (Methods), leading to immobilization (CL) of the myc-endoglin and Fab’ labeling of HA-ALK4, whose lateral diffusion was then measured by FRAP. (i) Average $R_f$ values. (ii) Average $D$ values. Bars are mean ± SEM with number of measurements (n) depicted in each bar. Asterisks indicate significant differences between the $R_f$ values of the pairs indicated by brackets (****, $p < 1 \times 10^{-15}$; **, $p = 5.6 \times 10^{-3}$; one-way ANOVA followed by Bonferroni post-hoc test). No significant differences were found between $D$ values following myc-endoglin immobilization.
Figure 2.11. Targeting inhibin decreases \textit{in vivo} subcutaneous growth. A) Growth curves of subcutaneously implanted HEY shControl or sh\textit{INHA} tumors exposed to either normoxia or hypoxia (0.2% O\textsubscript{2}) 24hrs prior to injection. 10mg/kg R1 antibody or vehicle control was intraperitoneally injected three times a week. Mean±SEM, n=10 for vehicle and n=6 for R1 receiving groups. **, p<.01; ****, p<.0001, Two-way ANOVA followed by Tukey’s multiple comparison test. B) Relative qRT-PCR analysis of \textit{INHA} mRNA expression in HEY shControl and sh\textit{INHA} cells (i) prior to subcutaneous injection and (ii) at end point after harvest normalized to shControl. Mean±SEM, n=2. C) Weight of Ncr nude mice across two-week time course of anti-inhibin R1) or IgG antibody delivery. Injections were given every other day starting at 5mg/kg and escalated to 10mg/kg after 7 days.
Figure 2.12. Targeting inhibin alters vasculature in subcutaneous xenografts. A) Growth curves of HEY shControl or shINHA after indicated time under hypoxia (0.2% O2). Data is normalized to growth under normoxia after 72hrs. Mean±SEM, n=2. n.s., not significant, unpaired t-test. B) Fold change of proteins most altered in shControl and shINHA tumors (Fig 7A) using the (i) human or (ii) mouse angiogenesis proteome array. (n=2 tumors per group). C) (i) Average tumor volume of HEY shControl or shINHA subcutaneous tumors used for analysis of vasculature and permeability in ii and iii. Mean±SEM, n=4. (ii) Quantitation of extravasated rhodamine-dextran (red) shown as signal per 10x field from tumors in Fig 7Ci (Methods). Mean±SD. n=12 fields from 4 tumors. ***, p<.001, unpaired t-test. (iii) Representative images of rhodamine-dextran (red) extravasation into either shControl or shINHA subcutaneous tumors from C.i Scalebar:100μm D) (i-ii) Quantitation of average (i) vessel number and (ii) size in a 10x field using ImageJ (Methods). Mean±SD. n=8 which represents averages of 8 fields in 4 tumors from C.i. (iii) Representative images of CD-31 (red) stained blood vessels in HEY
shControl and shINHA subcutaneous tumors from Fig 7Ci. Scalebar: 100μm, insets scalebar: 20μm. *, p<.05; **, p<.01, unpaired t-test.
Figure 2.13. Inhibin is correlated with CA125 in patient ascites. A) Correlation analysis of total inhibin (pg/mL) and CA125 (pg/mL) in patient ascites. B) Median age of patients used for patient ascites ELISA analysis.
CHAPTER 3

A BIOINFORMATIC ANALYSIS OF THE INHIBIN-BETAGLYCAN-ENDOGLIN/CD105 NETWORK REVEALS PROGNOSTIC VALUE IN MULTIPLE SOLID TUMORS

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3.1 Introduction

Inhibins and activins are dimeric polypeptide members of the TGF-β superfamily, discovered initially as regulators of follicle-stimulating hormone (FSH) \(^{1,5,181-187}\). Activins are homodimers utilizing different isoforms of the monomeric \(\beta_A\) or \(\beta_B\) subunits located on different chromosomes \(^{3,188,189}\). Inhibin is a heterodimer of an \(\alpha\) subunit (\(INHA\)) and a \(\beta\) subunit (either \(\beta_A, INHBA\), or \(\beta_B, INHBB\)). Thus, the inhibin naming reflects the \(\beta\) subunit in the heterodimer: inhibin A (\(\alpha/\beta_A\)) and inhibin B (\(\alpha/\beta_B\)), respectively \(^{2,4,5,190,191}\).

Activins, signal primarily through the transcriptional proteins SMAD2/3, much like TGF-β \(^{43,192}\). Initial receptor binding of activin occurs via type II serine-threonine kinase receptors (ActRII or ActRIIB). These then recruit and phosphorylate type I serine-threonine kinase receptors (ActRIB/Alk4 or ActRIC/Alk7) leading to subsequent phosphorylation of SMAD2/3\(^{5,43,45,193-195}\). In multiple tissues, activin signaling is antagonized by inhibin \(^{49}\). Thus, the biological and pathological function of activin is directly impacted by the relative levels of the mature \(\alpha\) subunit. Inhibins, however, have a much lower affinity for the type II receptors compared to activins themselves. The affinity can be greatly enhanced by the presence of the Type III TGFβ receptor, betaglycan (\(TGFBR3\)), which binds inhibin’s \(\alpha\) subunit with high affinity \(^{5,45,49,54}\). Thus, the most established mechanism of antagonism by inhibin, is via its ability to competitively recruit ActRII preventing activin induced downstream signaling in a betaglycan-dependent manner \(^{5,45,49,54}\). This competition model does not allow for direct inhibin signaling. However, conflicting reports on the presence of a separate high affinity inhibin receptor \(^{48,196}\), recently discovered interactions of the \(\alpha\) subunit with the Type I receptor Alk4 \(^{54}\), and our recent findings on the requirement of the alternate Type III TGF-β co-receptor
endoglin (ENG/CD105) for inhibin responsiveness in endothelial cell function suggest complex roles for inhibins themselves.

Betaglycan and endoglin, are both coreceptors of the TGF-β superfamily with broad structural similarities, including glycosylation in the extracellular domain (ECD), a short cytoplasmic domain and common intracellular interacting partners. Sequence analysis of betaglycan and endoglin reveal the highest shared homology in the transmembrane (73%) and cytoplasmic domains (61%), with the most substantial difference being in the ECD sequence that impacts ligand binding. Both betaglycan and endoglin knockouts (KOs) are lethal during embryonic development due to heart and liver defects and defective vascular development, respectively, highlighting the shared physiological importance of these coreceptors. In contrast to the above-described similarities, betaglycan is more widely expressed in epithelial cells, while endoglin is predominantly expressed in proliferating endothelial cells.

In cancer, betaglycan and endoglin impact disease progression by regulating cell migration, invasion, proliferation, differentiation, and angiogenesis in multiple cancer models. Betaglycan can act as a tumor suppressor in many cancer types and its expression is lost in several primary cancers. However, elevated levels of betaglycan have also been reported in colon, triple-negative breast cancers and lymphomas, with a role in promoting bone metastasis in prostate cancer, indicating contextual roles for betaglycan in tumor progression. Endoglin is crucial to angiogenesis, and increased endoglin and tumor micro-vessel density is correlated with decreased survival in multiple cancers. Evidence in ovarian cancer also suggests that endoglin expression may impact metastasis. Inhibins have been robustly implicated in cancer, and...
much like other TGF-β members may have dichotomous, context-dependent effects. Inhibins are early tumor suppressors, as the **INHA** mice form spontaneous gonadal and granulosa cell tumors. However, elevated levels of inhibins in multiple cancers are widely reported. Active roles for inhibins in promoting late stage tumorigenesis, in part via effects on angiogenesis, have also been reported in both prostate cancer and more recently in ovarian cancer.

Inhibins have been widely used as a diagnostic marker for a subset of cancers and both betaglycan and endoglin have been evaluated as therapeutic strategies in cancer. TRC-105, a monoclonal antibody against endoglin, was tested in twenty-four clinical trials. Current data also suggest benefits of combining anti-endoglin along with checkpoint inhibitors. Similarly, a peptide domain of betaglycan called p144 and soluble betaglycan have been tested in multiple cancer types as an anti-TGF-β treatment strategy that decreases tumor growth, angiogenesis, metastasis, and augments immunotherapy.

Prior and emerging studies reveal the dichotomous functions of inhibin’s on cancer depending on either betaglycan or endoglin. Hence, further characterization of the relationship between inhibins-betaglycan-endoglin is vital. This study seeks to provide such prescient information by evaluating the significance, impact, and predictive value of this specific network (**INHA**, **INHBA**, **INHBB**, **TGFBR3**, and **ENG**) by utilizing publicly available genomic and transcriptomic databases.
3.2 Materials and Methods

Public databases data mining

Clinical data, gene expression alterations, and normalized expression data of RNA-seq were obtained from cBioPortal\textsuperscript{141,148}. All available studies were assessed for copy number alterations (CNA) and a subset of cancer for mRNA data (Breast Invasive Carcinoma, Glioblastoma, Lower-grade glioma, Cervical Squamous Cell Carcinoma, Stomach Adenocarcinoma, Head and Neck Squamous Cell Carcinoma, Kidney Renal Clear Cell and Renal Papillary Cell Carcinomas, Liver Hepatocellular Carcinoma, Lung Adenocarcinoma, Ovarian Serous Cystadenocarcinoma, Prostate Adenocarcinoma, Uterine Corpus Endometrial Carcinoma). The results shown here are partly based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga. Survival data was generated from either KM Plotter\textsuperscript{266} or cBioportal (i.e., brain cancers). KM Plotter data for breast, ovarian, lung, and gastric cancer the survival analysis was derived using available gene chip data sets. All others were derived using the RNA-Seq Pan-cancer data sets. The Affymetrix Probe IDs used in gene chip analysis in KM Plotter were: \textit{INHA} (210141\_s\_at), \textit{INHBA} (204926\_at), \textit{INHBB} (205258\_at), \textit{TGFBR3} (204731\_at), and \textit{ENG} (201808\_s\_at). Brain cancer data was generated from TCGA Pan Cancer Atlas 2018 dataset for glioblastoma and low-grade glioma. Overall survival (OS) was assessed for all cancer types except ovarian cancer (progression-free survival, PFS) and breast cancer (relapse-free survival, RFS). Gene expression was split into high and low using the median expression. Log-rank statistics were used to calculate the p-value and Hazard ratio (HR).
**Analysis of gene predictiveness to pharmacological treatment**

Gene predictive information on treatment regiments was obtained from ROC Plotter ([http://www.rocplot.org/](http://www.rocplot.org/))\(^{267}\). Gene expression for the analyzed genes was compared using the Mann-Whitney U test. Receiver Operating Characteristic (ROC) plots and significance was also computed. ROC curves were compared using Area Under the Curve (AUC) values, and values above 0.6 with a significant p value were considered acceptable\(^{267}\). ROC plot assessment was performed in all pre-established categories in ROC Plotter (i.e., breast and ovarian cancers, and glioblastoma). In breast cancer, subtypes (i.e., luminal A, luminal B, triple-negative, HER2\(^+\)) were also analyzed separately. Genes of interest were analyzed for complete pathological response in different pharmacological treatments. All available treatment options were investigated including, taxane, anthracycline, platin and temozolomide. Outliers were set to be removed in this analysis and only genes with a false discovery rate (FDR) below 5% were considered.

**Clustering strategies for genes signatures**

From the normalized expression data from RNA-seq studies, the Spearman’s ρ coefficient was obtained for INHA, TGFB3, and ENG. These data were clustered through a Euclidean clustering algorithm using Perseus 1.6.5.0 (MaxQuant). Clusters containing high and low correlations sets were isolated and compared in a pair-wise fashion. The derived genes obtained were checked for protein interaction in BioGRID (thebiogrid.org)\(^{268}\), and later included in pathway analysis, as described in section 2.5. All plots were performed in GraphPad Prism 8.0.
Gene signature modeling for prognostics

Gene signature modeling was performed using binary probit regression for each set of cancer types related to INHA, TGFBR3, ENG (S5 Table), and their respective outcomes (i.e., positive, 1; or negative, 0). The regression was iterated for presenting only significant elements in the following model:

\[
\Pr(Y = 1 | x_1, \ldots, x_k) = \Phi \left( \beta_0 + \sum_{i=1}^{k} \beta_i x_i \right)
\]

in which \(x_i\) are RNA-seq V2 RSEM expression data for each gene, \(\beta_i\) are obtained coefficients from this regression, \(\Phi\) is the cumulative normal distribution function. Probability values closer to 1 indicate a positive outcome, while values close to 0, indicate a negative outcome. Postestimation of specificity and sensitivity was also implemented. All regression studies were performed in Stata/SE 16.0.

Pathway assessment

For pathway analysis, DAVID Bioinformatics Resources 6.8 was used to acquire compiled data from the KEGG Pathway Database. Genes for the analysis were annotated to map to human pathways. The significant outputs were then assessed for the percentage of genes from analyzed sets and their relevance. To compare pathways between two sets, a pathway significance value ratio (-\(\log_{10} R\)), in which \(R\) is the ratio, was analyzed. Only pathways with an FDR value below 5% were considered.

Gene dependency analysis

Gene dependency of INHA, TGFBR3, INHBA, INHBB, and ENG was analyzed using the DepMap portal (www.depmap.org). Gene expression from Expression Public20Q1 (accessed between March and April 2020) were compared to the cell line
database from CRISPR (Avana) Public20Q1 and Combined RNAi (Broad, Novartis, Marcotte). Gene effect values of less than or equal to -0.5 were used to select dependent genes. To analyze gene co-dependency, Expression Public20Q1 was compared to all CRISPR and RNAi databases. A gene was considered dependent when correlations between datasets displayed similar trends. Each dependent gene-set was compared between \textit{INHA}, \textit{TGFBR3}, \textit{INHBA}, \textit{INHBB}, and \textit{ENG} to count duplicates. The number of dependent genes were plotted as a Venn diagram.

3.3 Inhibins and activins are altered in human cancer

We and others reported previously diverse roles for members of the inhibin/activin family in cancer \textsuperscript{5,57,271-274}. Our and prior mechanistic studies in cancer indicate a strong dependency of inhibin function on betaglycan and endoglin \textsuperscript{50,53,54,57,275,276}. To begin to evaluate the impact of this relationship more broadly in cancers we analyzed gene alterations including mutations, amplifications, and deletions for the genes encoding inhibin/activin subunits (Figure 3.1A) \textit{INHA}, \textit{INHBA}, \textit{INHBB}, and the key coreceptors — \textit{TGFBR3}, and \textit{ENG} in all public datasets available in cBioPortal (Figure 3.1B). While \textit{INHBC} and \textit{INHBE} are activin subunits, these were excluded from the analysis as they have not been demonstrated to form heterodimers with \textit{INHA}\textsuperscript{277}.

Percentage of patients from the whole cohort that possessed any of the alterations either by themselves or concomitantly was analyzed. We find that melanoma (16.26%), endometrial (13.16%), esophagogastric (10.85%), and lung (10.69%) cancers revealed the highest alterations for the genes. The alterations for the genes varied, with \textit{INHBA} and \textit{TGFBR3} exhibiting higher rates of alterations (0–5.65\% and 0.17–3.91\% respectively) that
also varied by cancer type. The range for *INHA*, *INHBB*, and *ENG* was found to be between 0–2.38%, 0–2.62%, and 0–3.23% respectively (Figure 3.1B).

In comparing expression levels of each of the genes in the same TCGA datasets as in Figure 3.1B, we find that overall, *ENG* is the most highly expressed gene (Figure 3.1C) with variance among different cancer types (e.g., lower-grade glioma and cervical vs. renal clear cell and lung adenocarcinoma, p < 0.0001) and subtypes (e.g., luminal A vs. luminal B breast cancers, p < 0.0001). Interestingly, *TGFBR3* expression differed most notably between glioblastoma and lower-grade gliomas (p < 0.0001). Breast cancers exhibited higher expression as compared to ovarian and endometrial (p < 0.0001) cancers. *INHBB* in contrast was mostly expressed in renal clear cell and hepatocellular carcinomas, which differs from renal papillary cell carcinoma and cervical cancer (p < 0.0001). Both *INHBA* and *INHA* were the least expressed as compared to the others (Figure 3.1C). Exceptions were head and neck and esophagogastric cancers that had high expression of *INHBA* and lung adenocarcinoma and renal clear cell carcinoma that had high expression of *INHA*.

While the above analysis examined patient tumors, we next examined cell lines as a way to delineate model systems for future studies. For these analyses, we used the DepMap project (www.depmap.org) which is a comprehensive library of human genes that have been either knocked down or knocked out through CRISPR technology in 1,776 human cell lines representing multiple cancer types. Dependency scores representing the probability of queried gene dependency for each cell line and thereby cancer type is obtained. Here, we find that the ligand encoding gene *INHA* displayed higher dependency than the activin subunit isoforms *INHBA* or *INHBB* or either receptors *TGFBR3* or *ENG* (Figure 3.1D). Notably, esophageal, gastric, and ovarian cancers had the
highest dependency results for \(INHA\) (\(\geq 14\%)\) consistent with the alterations seen in Figure 3.1C. Within these cancers, \(INHBA\) exhibited higher dependency values in ovarian cancer (6%) albeit not as high as \(INHA\). Besides \(INHBB\) in myeloma (6%), no other notable dependency relationships were observed.

In an attempt to identify genes most impacted by alteration to each of the individual genes, we examined how RNAi and CRISPR interventions would affect their correlation to specific genes. Those similarly affected by these techniques were found to be dependent on the investigated set of genes. We find that \(ENG\) exhibited the highest number of dependent genes (Figure 3.1E, \(n = 71\)). Interestingly, only a total of 5 genes were commonly dependent between \(INHA\) and the other genes (Figure 3.1E, MAX with \(INHBA\) and \(GRPEL1, SF3B4, ESR1\), and \(TFAP2C\) with \(INHBB\)). \(INHBA\) on the other hand had several common dependent genes most notably 13 genes were common with \(ENG\) dependency (e.g., \(VCL, TLN1\), and \(LYPD3\)).

### 3.4 Effect of inhibins and the coreceptors on patient survival varies by cancer type

Since alterations in expression of inhibin, activin, \(TGFBR3\) and \(ENG\) exist in human cancers and prior studies have implicated each of these in patient outcomes \(^{57,128,220,227,271,282-285}\); we conducted a comprehensive analysis of each of these genes on overall survival (OS), progression-free survival (PFS), or relapse free survival (RFS) in a broad panel of cancers. The goal here was to identify the patients and cancer types most impacted by changes in gene expression. Analysis was conducted using datasets in KM Plotter (summarized in Table 3.1) \(^{266}\). For ovarian cancer data sets, only p53 mutated ovarian cancers were included. Patients in KM plotter with p53 mutation status known
showed 83% were mutated, cBioportal data sets showed 82.5% frequency of p53 mutation, and it has been reported that over 90% of ovarian cancers present p53 mutations. We find that not all cancers are equally impacted. Of note, we find that in both breast and ovarian cancers all five genes were either positive predictors of survival or non-predictive except INHBB in breast (HR = 1.06, p = 0.034) and INHBA in ovarian (HR = 1.16, p = 0.047) (Figure 3.2). However, in p53 mutated cancers, INHA was a strong negative predictor of survival for both breast and ovarian cancers (HR=1.99, p = 0.0056 and HR = 1.55, p = 0.0039, respectively), along with ENG in ovarian cancer (HR = 1.36, p = 0.0098, Figure 3.2). Additionally, in lung cancers, INHA and ENG differed from TGFBR3, as INHA (HR=1.26, p = 0.00029) and ENG (HR = 1.20, p = 0.0056) were both negative predictors of survival while TGFBR3 (HR = 0.65, p = 3.4E-7) was a strong positive predictor of survival (Figure 3.2). Specifically, we find that INHA and ENG are robust predictors of poor survival in lung adenocarcinomas but not in squamous cell carcinomas (Figure 3.2). Gastric cancers represent another robust cancer type where all five genes were negatively correlated with survival (Figure 3.2). Since HER2 expression is a frequent abnormality in gastric cancer, we examined if there were any differences in survival associated with HER2 expression. All five genes in both HER2+ and HER2- gastric cancers, except INHBA in HER2- gastric cancers, were negatively correlated with survival (Figure 3.2). In kidney cancers, INHA was a negative predictor of survival in both renal clear cell and renal papillary cell carcinoma (Figure 3.2), consistent with prior findings. TGFBR3 was a strong positive predictor of survival in both renal clear cell carcinoma (HR = 0.46, p = 2.1E-7) and renal papillary cell carcinoma (HR = 0.53, p = 0.042, Figure 3.2). ENG (HR = 0.51, p = 8.6E-6) was a positive predictor of survival in renal clear cell carcinoma but not
significantly associated with survival in renal papillary cell carcinoma (Figure 3.2). Finally, in brain cancers, \textit{INHA} was a negative predictor of survival in glioblastoma but a positive predictor in low-grade gliomas (Figure 3.2). Of note, \textit{ENG} appeared to have a lower range of HR values compared to \textit{INHA} and \textit{TGFBRIII}. \textit{INHBA} and \textit{INBBB} were not as significantly correlated with survival as \textit{INHA}, \textit{ENG}, and \textit{TGFBRIII}. \textit{INHBA} was significantly correlated with 8 cancer types while \textit{INHBB} was significantly correlated with 9. \textit{INHBA} and \textit{INHBB} showed similar correlations with survival in gastric cancers, specifically \textit{HER2}+, and renal papillary cell carcinoma (Figure 3.2). \textit{INHBA} and \textit{INHBB} showed opposing effects however in liver cancer where \textit{INHBA} (HR = 0.62, p = 0.0086) was a strong positive predictor but \textit{INHBB} (HR = 1.52, p = 0.025) was a potent negative predictor (Figure 3.2).

Since inhibin’s biological functions have been shown to be dependent on the coreceptors \textit{TGFBRIII} and \textit{ENG} \cite{50,53,54,57,275,276}, we examined the impact of \textit{INHA} based on the expression levels of each of the co-receptor (Table 3.2). In this analysis, we find that when separating patients into high or low expressing \textit{TGFBRIII} or \textit{ENG} groups (Table 3.2) in p53 mutated breast cancers, where \textit{INHA} is a negative predictor of survival in all patients (Figure 3.2), \textit{INHA} was only a predictor of poor survival in patients with low \textit{TGFBRIII} (HR = 2.29, p = 0.015) or low \textit{ENG} (HR = 2.24, p = 0.035). Interestingly, this trend was also repeated in renal clear cell carcinoma, where \textit{INHA} was only a predictor of survival in \textit{TGFBRIII} low (HR = 2.75, p = 9.0E-06) and \textit{ENG} low (HR = 2.6, p = 2.5E-06, Table 3.2). In contrast to breast and renal clear cell cancers where \textit{TGFBRIII} and \textit{ENG} both impacted the effect of \textit{INHA} on survival, \textit{TGFBRIII} levels did not change \textit{INHA}’s impact on p53 mutated serous ovarian cancers (Table 3.2). In \textit{ENG} high p53 mutated serous ovarian
cancer patients, INHA had a more significant negative prediction outcome (HR = 2.12, p = 1.8E-6) compared to ENG low (HR = 0.8, p = 0.18, Table 3.2). Similar outcomes were observed in lung adenocarcinomas with respect to TGFBR3, where INHA remained a strong negative predictor of survival in patients regardless of TGFBR3 expression levels (Table 3.2). However, INHA remained a robust negative predictor of survival in lung adenocarcinomas patients expressing low ENG (HR = 2.12, p = 0.00041) but was not significant in ENG high expressing patients (HR = 1.25, p = 0.14) (Table 3.2). Together, these findings suggest that INHA expression as a predictive tool for survival is influenced by the coreceptors ENG and TGFBR3 in renal clear cell, lung, and p53 mutated breast and ovarian cancers. INHA is dependent on these coreceptors in all breast and ovarian cancers.

### 3.5 Inhibins and activins can predict response to chemotherapy in luminal A breast cancer

We next evaluated the pathological response based classification for each of the genes using the receiver operating characteristic (ROC) plotter (www.rocplot.org) to validate and rank INHA, INHBA/B, TGFBR3 and ENG as predictive biomarker candidates. In a ROC analysis, an area under the curve (AUC) value of 1 is a perfect biomarker and an AUC of 0.5 corresponds to no correlation at all. We first entered all genes to allow for FDR calculation for each gene at FDR cutoff of 5%. We next examined individual genes and find that in luminal A breast cancers ENG, TGFBR3, INHA, and INHBA, were better performing as compared to INHBB particularly for taxane or anthracycline based chemotherapy regimens. ROC plots for the two regimens are displayed in Figure 3.3A.

Both ENG and TGFBR3 were predictive in other cancer types as well (see supplemental data for Chapter 3 citation). Specifically, while ENG performed better in
taxane treatments in HER2+ breast cancer subtype, TGFBR3 performed better for taxane regimens in triple-negative breast cancer (TNBC) and serous ovarian cancer. Interestingly, examining expression (Figure 3.3B) revealed that in the same luminal A breast cancers INHA, ENG and INHBA are less expressed in responders to pharmacological treatment while TGFBR3 is more expressed in these responder groups (Figure 3.3B). Similar trends for TGFBR3 expression were seen in TNBC and serous ovarian cancer groups where TGFBR3 was more expressed in the responders’ group for taxane regimens. ENG was also more expressed in HER2+ breast cancer patients who respond to taxane therapy, which was opposite to the luminal A subtype expression levels (Figure 3.3B). Full data for the ROC curve assessment is available in supplemental data for Chapter 3 citation. In summary INHA, INHBA, TGFBR3, and ENG display clear discrepant profiles of expression among responders and non-responders to both anthracycline and taxane chemotherapy for distinct breast cancer subtypes, specifically luminal A, and serous ovarian cancer. These genes also harbor a possible predictive value to indicate responsiveness to these therapy regimens. Moreover, ENG expression could also differentiate luminal A and HER2+ breast cancers response to taxane therapy. INHBB on the other hand had no predictive value in the assessed cancer types.

3.6 Gene signatures from inhibins can predict patient survival outcomes

INHA, TGFBR3, and ENG impact patient outcomes more broadly and more significantly that INHBB and INHBA. There is also direct functional dependency of TGFBR3 and ENG to inhibin rather than activin. We thus examined signatures associated with either a negative or positive outcome for each of the three genes. Cancer types that presented different survival predictions for INHA, TGFBR3, or ENG were
assessed (Figure 3.4A), and cancer types in which each gene would have a similar patient outcome (i.e., positive overall survival outcome vs. negative overall survival outcome) were separated into groups (e.g., INHA positive outcome vs. negative outcome, Figure 3.4A).

Spearman’s ρ coefficient was calculated for all RNA-seq gene data provided in each of these datasets, and values were clustered, and genes that were either positively and negatively correlated with each individual INHA, TGFBR3 or ENG genes were identified (data located in citation). The top correlated genes from the positive outcome set were then pairwise compared to genes that had lower correlations in the negative outcome set, and vice-versa to obtain a subset of common genes. Examples include TGFB2 and HOXA1 where genes correlated to INHA in the negative outcome set, and OGG1 and STAP2 in the positive outcome group. For TGFBR3, AP1M1 and RILPL1 correlated in the negative outcome context, while FZD5 and MYCN in the positive one. No gene signatures were obtained for ENG. As indicated in section 3.2, the HR value range was the smallest for ENG in the assessed cancer types, which limits the differential gene signature analysis. All these genes also had their mRNA expression assessed in the respective cancer sets, contrasted, and evaluated for difference in expression (Figure 3.4B). Except for 22 genes from sets in which INHA or TGFBR3 had distinct predictions of survival (e.g., CHSY1, LDLR, PPARG, MIA2, TOX3) all others exhibited significant alterations in gene expression (Figure 3.4B).

The altered genes from Figure 3.4B whose difference in expression was significant, were assessed for protein interactions and these iterated for pathway analysis using BioGRID (thebiogrid.org, Figure 3.4C). We find that INHA gene sets were associated
with either PD-L1 expression and PD-1 checkpoint, Rap1 signaling pathways in patients with positive outcomes or cell cycle regulation in patients with negative outcomes. TGFBR3 associated genes on the other hand, relied on VEGF and MAPK signaling pathways for patients with positive outcome and IL-17, p53, or even Wnt signaling pathways in the negative outcome scenario. Detailed descriptions of analyzed genes and pathways are compiled in supplemental data for this manuscript (see Chapter 3 citation).

To determine if the genes associated with INHA and TGFBR3 had true prognostic value, a Probit regression model was applied to the normalized mRNA expression of the genes identified above. The regressions were analyzed for the cancers from Figure 3.2A and Figure 3.2D which had clear outcomes for either INHA, or TGBFR3. The final coefficients and entry genes are also provided in supplemental data for this manuscript (see Chapter 3 citation). We find that the INHA model had 43 genes as dependent elements, and the TGFBR3 model had 37 genes. However, the most suitable model obtained from these sets is the TGFBR3 model, which has a high goodness of fit p-value (p = 0.9494), sensitivity (98.42%), specificity (91.56%), and accuracy (96.70%, Table 3.3).

These analyses reveal that a differential signature obtained from INHA, along with one of its main binding receptors (i.e., TGBFR3) are able to faithfully predict a patient’s outcomes in a wide spectrum of cancer types (e.g., kidney, lung, head and neck, breast, liver, ovarian, stomach, endometrial).

3.7 Functional analysis and interpretation of inhibin’s mechanism of action

Prior functional studies indicate a dependency on ENG and TGFBR3 for inhibin responsiveness. To test if these biological observations hold in patient datasets, we
performed supervised clustering using Euclidean algorithm of genes correlating with either INHA, ENG or TGFBR3 using the RNA-seq data for cancer types with the most significant impact as determined in Figure 3.5A. Only the most enriched transcripts that were either positively or negatively correlated transcripts are shown in Figure 3.5A. Most enriched genes from these clusters were then compared amongst each other in all pairwise combinations for similarities (e.g., positively correlated to INHA vs. negatively correlated to TGFBR3, and so on, Figure 3.5B).

We find that INHA and TGFBR3 comparison rendered 1,430 genes, in which 24.6% were exclusive to INHA (e.g., DLL3, GPC2, TAZ, TERT, XYLTL2) 37.7% to TGFBR3 (e.g., CCL2, CCR4, EGFR, GLCE, IL10RA, IL7R, ITGA1, ITGA2, JAK1, JAK2, SRGN, SULF1, TGFBR2), and 13.1% were positively correlated to both (e.g., CSPG4, COL4A3, FGF18, NOTCH4, SMAD9). When INHA was assessed with respect to ENG we find 1,773 genes of which, 11.2% were exclusive to INHA (e.g., GDF9, PVT1), 21.3% to ENG (e.g., CCL2, GPC6, IL10, IL10RA, IL7R, INHBA, ITGA1, ITGB2, JAK1, SRGN, SULF1, TGFBR1, TGFBR2) and 10.0% were highly correlated to both (e.g., CSPG4, DLL1, FGF18, FZD2, NOTCH4). Lastly, the comparison between ENG and TGFBR3 returned 1,938 genes. However, very few were exclusive to either TGFBR3 (2.84%) or ENG (0.16%), revealing a high functional resemblance between both of these receptors, as most of the profiled genes correlated to both of them (48.5%, e.g., ADAM9, -23, ADAMTS1, -2, -5, -8, -9, CCL2, CSF1R, DLL4, ESR1, FGF1, FGF2, FGF18, GLI1, -2, -3, GPC6, IL10RA, ITGA1, ITGA5, JAK1, MMP2, SDC3, SRGN, SULF1, TGFBR3, TGFBR2, TNC, TWIST2, XYLTL1, ZEB1) or none of them.
We next used each gene set from the cross-comparisons in Figure 3.5B to identify pathways using KEGG \(^{292}\). Unique pathways with an FDR below 5\% were identified for the comparisons and are presented in Figure 3.5C. Although several common pathways were present between groups, such as PI3K-Akt and Ras signaling pathways (see supplemental data in Chapter 3 manuscript citation), some unique pathways were present as well. \(ENG\), for instance, was more exclusively related to cytokine-cytokine receptor interaction and natural killer mediated cytotoxicity (Figure 3.5C), while \(TGFBR3\) was more exclusively related to proteoglycans interaction and chemokine signaling. While cell cycle and DNA replication were not directly associated with \(ENG\) and \(TGFBR3\), Rap1 signaling, and Extracellular matrix (ECM)-receptor interactions were both impacted by \(ENG\) and \(TGFBR3\) (Figure 3.5C). However, no independent pathway could be pinpointed to \(INHA\) alone, revealing dependency on either \(TGFBR3\) or \(ENG\). These studies indicate that inhibin’s effects may vary depending on whether \(ENG\) is more highly expressed as compared to \(TGFBR3\) with significant relevance to defining mechanism and impact of changes in components of this pathway.

3.8 Discussion and Conclusions

This study aimed to evaluate comprehensively the influence of the inhibin-activin network in cancer. Our findings provide significant new information on the specific cancers impacted by the genes investigated here, \(INHA, INHBA, INHBB, ENG\) and \(TGFBR3\), and shed light on potential functional dependencies. Additional gene signature analysis reveals that \(INHA\), along with one of its main receptors (i.e., \(TGFBR3\)) faithfully predicts patient outcomes in a wide spectrum of cancer types.
TGFβ-1 is a representative member of the TGF-β family that has been significantly investigated previously. However, less information exists about the precise impact and role of other members like inhibins and activins. Our findings that INHA is significantly associated with survival in sixteen of the twenty cancers analyzed, correlating positively with survival in five cancers and negatively in ten (Figure 3.2), highlight INHA’s differential role as a tumor suppressor or promoter depending on the specific cancer type. In highly angiogenic tumors like renal clear cell carcinoma and glioblastoma, we found INHA expression to be a significant negative predictor of survival. INHA’s role in promoting tumorigenesis in these cancer types may occur through its effects on angiogenesis as has been previously reported for a subset of ovarian and prostate cancer warranting further investigation. In Luminal A breast cancers, we observed that increased INHA expression was associated with unresponsiveness to chemotherapy (Figure 3.3) while in survival data it was a positive predictor of survival (Figure 3.2). This apparent contradiction can perhaps be explained by the fact that data in KM Plotter contains information on patients that have undergone a wide array of treatments. Likely, INHA is predictive of response to some treatments but not others. In both breast and ovarian cancers, INHA was a negative predictor of survival in patients that had p53 mutations indicating a potential dependency of INHA functions on the p53 status. INHA expression alterations have been observed in p53 mutated adrenocortical tumors and INHA was suggested to be a contributing factor to tumorigenesis in these cancers. One of the most characterized transcriptional activators of INHA is GATA4, which can also regulate p53 in cancer and could contribute to the different survival outcomes observed for INHA in p53 mutated
cancers versus wild-type p53 cancers \(^{298,299}\). **INHA**’s link to functional outcomes in the background on p53 mutations remains to be fully elucidated.

Between the TGF-\(\beta\) family co-receptors (*ENG* and *TGFBR3*) implicated in cancer progression and inhibin function, *ENG* was more expressed (Figure 3.1C), particularly in lung adenocarcinoma and gastric cancers, corresponding with *ENG* being a strong negative predictor of survival (Figure 3.1C and Figure 3.2). These findings are consistent with prior experimental findings as well \(^{300,301}\). In p53 mutated cancers, *ENG* remained a negative predictor. ROC Plotter analysis revealed decreased *ENG* expression to be associated with response to anthracycline therapy in Luminal A breast cancer patients (Figure 3.3). However, a previous study showed that positive *ENG* expression was associated with increased survival in breast cancer patients who had undergone anthracycline treatment \(^{302}\). While Isacke and colleagues did not report a specific subtype in their analyzed cohort \(^{302}\), we obtained significant results for Luminal A breast cancer, specifically. Moreover, an additional study performed in acute myeloid leukemia showed an inverse relationship to that of Isacke et al., consistent with our results in Luminal A breast cancer \(^{302,303}\). We also found *ENG* to be a predictive of response to taxane therapy regimens. An inverse relationship between *ENG* expression was observed in responders for Luminal A and \(HER2^+\) breast cancer, with responders expressing high *ENG* in \(HER2^+\) breast cancers but low levels of *ENG* in Luminal A cancers (Figure 3.3). As Luminal A breast cancer is \(HER2^-\), *ENG* could be affected by HER2 status in these cancer types. In our analysis, expression data was only obtained for Luminal A breast cancers not \(HER2^+\) so differences in expression between the two were not analyzed.
Consistent with \textit{TGFB}R\textsubscript{3}’s role as a tumor suppressor in many cancers, we found it to be a significant positive predictor of survival in all but two cancers (i.e., endometrial and all gastric subtypes, Figure 3.2). Increased \textit{TGFB}R\textsubscript{3} was predictive of response in all treatments and cancers we examined (Figure 3.3), further bolstering \textit{TGFB}R\textsubscript{3}’s role as a negative regulator of tumor progression. Specifically, Bhola et al. (2013)\textsuperscript{304} showed increased levels of \textit{TGFB}R\textsubscript{3} in response to taxane in a small cohort (n = 17) of breast cancer patients; however, response to therapy was not analyzed. \textit{TGFB}R\textsubscript{3} has been shown to act as a tumor suppressor in renal clear cell carcinoma\textsuperscript{282} and non-small cell lung cancers\textsuperscript{261} which was also confirmed here (Figure 3.2). We were also able to expand \textit{TGFB}R\textsubscript{3}’s role in renal cancer to papillary carcinomas as well (Figure 3.2).

Expression of \textit{ENG} and \textit{TGFB}R\textsubscript{3} was not significantly different between wild-type and p53 mutated cancers indicating p53 likely does not impact expression itself. Whether protein secretion of these coreceptors is altered in these cancers is currently unknown, and cannot be ruled out, as previous studies have shown increased endoglin folding and maturation in p53 mutation settings\textsuperscript{305}. \textit{TGFB}R\textsubscript{3} also undergoes N-linked glycosylation, so a similar scenario to endoglin is possible. Alterations in protein maturation could explain the differential patient outcomes observed between wild-type and p53 mutated cancers, when assessing for \textit{ENG} and \textit{TGFB}R\textsubscript{3}, despite changes in expression not being observed.

\textit{INHA}’s dependency on each coreceptor examined in survival analysis revealed distinct signatures between different cancer types (Table 3.2). Prior studies indicate a requirement for \textit{ENG} in inhibin responsiveness and functions\textsuperscript{57}, which was borne out in patient survival data here (Table 3.2). However, a few outliers exist such as p53 mutated breast and renal clear cell carcinoma where \textit{INHA} was not always dependent on increased
ENG and TGFBR3 expression. We found INHA to only be a negative predictor of survival in patients expressing low ENG indicating INHA might act independent of coreceptor in these cancer types. The role of other receptors involved in mediating INHA’s effects in these cancer types remains to be determined.

Betaglycan and endoglin are co-receptors for TGFβ-1,2,3 and have been shown to regulate signaling for isoforms of BMP, Wnt and FGF\(^{306-308}\). However, both endoglin and betaglycan are dispensable for response to the above-mentioned growth factors, playing primarily modulatory functions. Given that TGF-β’s BMPs, Wnt, and FGF can act as both tumor suppressors and promoters in a cancer and context dependent manner, and our analysis indicating that ENG and TGFBR3 are both strong predictors of survival on their own (Figure 3.2) it is likely that ENG and TGFBR3 expression levels impact signaling sensitivity and thereby patient outcomes in the context of those signaling ligands.

In contrast to the above listed growth factors, Inhibins are reported to have functional consequences that dependent primarily on betaglycan or endoglin\(^{49,57}\) consistent with the ability of the gene signatures (Figure 3.4) dependent on TGFBR3 and ENG to distinguish patients’ outcomes. Some notable elements of this signature have been verified previously and even proposed as cancer biomarkers. For example, EPHA2 overexpression has been associated with decreased patient survival and promotes drug resistance, increased invasion, and epithelial to mesenchymal transition (EMT)\(^{309-312}\). HOXA1, a lncRNA overexpressed in cancers such as breast, melanoma, and oral carcinomas, drives metastasis and tamoxifen resistance\(^{313-315}\). For TGFBR3 specifically, three genes revealed high discrimination between positive and negative outcomes: UGT1A9 and GLYATL1 were 25- and 35-fold more expressed in positive outcomes and P2RX3 was 11.5-fold more
expressed in negative outcomes. Of interest, UGT1A9 is a UDP-glucuronosyltransferase (UGT) whose activity has been implicated in drug resistance by affecting the bioactivity of the drug $^{316,317}$. We speculate that as a proteoglycan, increased \textit{TGFBR3} could compete for UDP-glucuronate acid (GlcA) and UDP-xylose, both key elements for UGT1A9 activity, thereby potentially disrupting UGT associated resistance mechanisms and increasing the efficacy of chemotherapy. We also narrowed down which pathways differentiated patient outcomes for either \textit{INHA} or \textit{TGFBR3}. For positive outcomes, we found that \textit{INHA} was associated with PD-L1, Ras, and Rap1 signaling pathways. In adverse outcomes, \textit{INHA} was associated with Hippo, Wnt, and cell cycle pathways. Wnt has been shown to regulate \textit{INHA} transcription in rat adrenal cortex and could increase \textit{INHA} expression in certain tumors to promote tumorigenesis $^{37}$. Recent evidence indicates increased PD-L1 in dendritic cells in INHA$^{-/-}$ mice $^{318}$. We speculate that increased \textit{INHA} in tumors may inhibit PD-L1 expression perhaps via antagonistic effect on other TGF-\textit{\beta} members, increasing anti-tumor immune responses.

There are currently no other cancer prognostic models based on our three assessed genes. The selected prognostic model showed high accuracy (96.7%) with 98.42% sensitivity and 91.56% specificity (Table 3.3). Moreover, most prognostic cancer models are directed to either a specific cancer type (e.g., breast, prostate) or a cancer stage (e.g., lymph node metastases, phases). Our model includes at least ten tumor types, is in the top two for sensitivity, and among the second quartile of specificities on assessment of 48 prognostic cancer models $^{319-322}$. Thus, the \textit{INHA-TGFBR3-ENG} signature has pan-cancer prognostic value. Interestingly, there were very few SMAD and canonical TGF-\textit{\beta} associated pathway members that were part of the probit analysis (S5 Table). However,
several genes associated with non-SMAD TGF-β signaling were included, such as MAP2K6, FZD5, and PHLPP1 which are associated with MAPK, Wnt, and Akt signaling pathways respectively. Much of TGF-β’s functions in EMT, invasion and metastasis have been associated with non-SMAD pathways which are more likely to involve the coreceptors TGFBR3 and ENG. Hence it was not surprising that such non-SMAD pathways were predominant in the INHA-TGFBR3-ENG analysis.

Clustering analysis for genes correlated with INHA, TGFBR3, or ENG in cancers (Figure 3.5) revealed ENG and TGFBR3 had very few genes correlated exclusively to one or the other. As both receptors share similar structures and interact with common ligands, this is not unexpected. Similarly, since ENG and TGFBR3 had significant common gene associations this resulted in common pathways. For instance, a strong correlation with ECM-receptors and Rap1 signaling was observed. ENG has been shown to bind leukocyte integrins, promoting invasion, and ECM remodeling during fibrosis. TGFBR3 has been shown to regulate integrin localization and adhesion to ECM. ENG alone was associated with natural killer cell-mediated cytotoxicity consistent with previous findings showing anti-endoglin therapy augmented immune response in tumors by increasing NK cells, CD4+ and CD8+ T lymphocytes.

In conclusion, our pan-cancer analysis of the inhibin-activin network reveals a prognostic signature capable of accurately predicting patient outcome. Gene signatures from our analysis reveal robust relationships between INHA, ENG, and TGFBR3 and other established cancer biomarkers. Survival analysis implicated members of the inhibin-activin network in cancers previously unstudied as well as corroborated previous findings. Further
analysis of the role of the inhibin-activin network in cancer and relationship to other cancer associated genes, as well as validation as predictive biomarkers to chemotherapy is needed.
Table 3.1 p values and Hazard Ratios (HR) from survival curves assessing the impact of \textit{INHA}, \textit{INHBA}, \textit{INHBB}, \textit{TGFBR3}, and \textit{ENG} on patient survival. Survival curves were generated in KM Plotter for all cancer types. Survival curves represent overall survival, progression free survival (marked with *), or relapse free survival (marked with #).

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**Table 3.2 p values and Hazard Ratios (HR) from survival curves assessing the relationship between TGFBR3/ENG and INHA on patient survival.** Survival curves were generated in KM Plotter for all cancer types. Survival curves represent overall survival, progression free survival (marked with *), or relapse free survival (marked with #) for patients expressing high or low mRNA (split by median) of the indicated gene.

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<th>Subtype</th>
<th>Variable</th>
<th>INHA</th>
<th>TGFBR3</th>
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<th>ENG</th>
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<th>INHA in Low ENG Patients</th>
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107
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**Table 3.3 Prognostic performance of each delineated probit model.**

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<th><strong>TGFB3 model</strong></th>
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<td>Renal Clear Cell;</td>
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<td>BC lum A;</td>
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<td>Lung adenocarcinoma;</td>
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<tr>
<td>BC lum B;</td>
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<td>Renal Papillary Cell;</td>
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<tr>
<td>Low grade glioma</td>
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<td>Head and Neck;</td>
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<tr>
<td></td>
<td></td>
<td>BC lum A;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver;</td>
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<td></td>
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<tr>
<td>Stomach;</td>
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<td>BC p53 mut;</td>
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<tr>
<td>Lung adenocarcinoma;</td>
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<td>Renal Papillary Cell;</td>
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<td>Glioblastoma.</td>
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<td>Specificity</td>
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<td>Sensitivity</td>
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<td>False negative ratio</td>
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<td>Accuracy</td>
<td><strong>92.25%</strong></td>
<td><strong>96.70%</strong></td>
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Figure 3.1. Expression and gene alterations of inhibin and activins. A) Genes encoding INHA, INHBA, and INHBB produce monomeric α and β subunits. These subunits combine to form either homo or heterodimers representing mature inhibin A, inhibin B, activin A, and...
activin B. B) TCGA base analysis of gene alteration frequencies of INHA, INHBA, INHBB, TGBFR3, and ENG. C) Analysis of gene expression levels, also from TCGA sets, of the same genes as in (B) in a subset of cancer types and subtypes. Analysis included 16 studies and 6258 samples. D) DepMap analysis of cell line dependency from indicated cancer types on each of the genes in (B). E) Venn diagram illustrating the number of common dependent genes for each gene in (B). All numeric data are available in S1 Table. Abbreviations – CNS: Central Nervous System; LumA: Luminal A; LumB: Luminal B; mut: mutated; WT: wild-type.
Figure 3.2. Impact of INHA, INHBA, INHBB, TGFBR3, and ENG on patient survival in indicated cancers. A-E) Forest Plot with Hazard Ratios (HR) of indicated genes generated from KM Plotter or data from cBioportal. Black dots represent HR that are not statistically significant (p > 0.05) and red dots represent HR that are statistically significant (p < 0.05). F-H) Representative Kaplan Meier curves for INHA, TGFBR3, and ENG. Event-free survival in indicated cancers using median to separate expression (lighter shade indicates bottom patients expressing bottom 50% and darker shade top 50%). Survival curves represent OS for all cancers except breast cancer (RFS) and ovarian cancer (PFS). Top plots show cancer types where the gene is a negative predictor of survival, and bottom plots show cancer types where the gene is a negative predictor.
Figure 3.3. ROC plots and gene expression of indicated genes for different chemotherapy regimens. A) ROC curves, in which performance ability was verified (i.e., AUC > 0.6), were plotted for ENG, INHA, INHBA, and TGFB3. B) Gene expression for each investigated gene between responders and non-responders for the assessed pharmacological treatments. The sample sizes for each group were the following: ANT LUMA, n = 474; TAX LUMA, n = 375; TAX HER2, n = 143; TAX TNBC, n = 290; TAX OVCA, n = 851. Abbreviations: ANT: anthracycline; TAX: taxane; LUMA: luminal A; TNBC: triple-negative breast cancer; OVCA: serous ovarian cancer.
Figure 3.4. Gene signatures and expression patterns for cancers where INHA, TGFBR3, or ENG predicted survival outcomes. A) Cancer types in which either INHA TGFBR3 or ENG had either positive (+) or negative (-) survival outcomes had their RNA-seq gene data correlation clustered for either low or high degree of correlations to each INHA, TGFBR3 or ENG as indicated. B) mRNA abundance of a subset of common genes obtained from pairwise comparisons of the top correlated genes from the positive outcome with the genes that had lower correlations in the negative outcome set, and vice-versa. mRNA expression was assessed in each cancer set. * p < 0.05 ** p < 0.01 *** p < 0.001 **** p < 0.0001 (A) Pathway analysis after BioGRID assessment of the significant genes from (B), ranked with a ratio of significance between sets from the positive and negative outcomes for each gene.
Figure 3.5. Functional analysis of gene signatures between INHA and TGFBR3 and INHA and ENG. A) Supervised clustering of correlations of RNA-seq data between INHA, TGFBR3, and ENG was performed to obtain sets of positive and negatively correlated genes for each set. B) Common genes that were found in each group of correlated genes (e.g., negative correlation to INHA vs. positive to TGFBR3 and all combinations) is presented. C) KEGG pathway analysis for groups of genes correlated with the indicated combination. Unique pathways with an FDR below 0.05 were identified for the comparisons and are presented.


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Cancer Stat Facts: Ovarian Cancer


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APPENDIX A

INHIBIN EXPRESSION IN HEY TUMORS AND OTHER CANCER CELL LINES
Appendix A Results and Discussion

This appendix includes supportive data for inhibin protein expression in HEY tumors and patient ascites, and INHA expression regulated by hypoxia across a cell line panel.

**Figure A.1.A.** Schematic of the assembly of monomeric inhibinα (left) and dimeric inhibin protein (right) as detected in HEY tumors. The monomeric inhibinα protein is produced as a pro-peptide containing three domains: pro-domain (~9kDa), αN (~23kDa), and αC (~20kDa). As described in the introduction (1.1), these domains can be cleaved to form the mature αC peptide however non-cleaved forms of inhibinα are secreted and bioactive. Unprocessed dimeric inhibin can also be secreted, as shown on the right, which contains the noncleaved inhibinα subunits linked by a di-sulfide bond to the noncleaved beta subunit made up of a pro-domain (~32kDa) and a beta domain (~13kDa).

**Figure A.2.B.** To better understand what forms of inhibin protein were being produced by tumors, inhibin from HEY tumors in Figure 2.11A was detected through western blotting using the R1 anti-inhibinα antibody. Using lysates from HEY PLK01 and shINHA tumors, we confirmed that we did have an effective knockdown of INHA. HEY tumors produced both unprocessed ProαN/αC (~55kDa) protein as well as partially processed ProαC (~27kDa). No fully processed inhibinα was detected which was not surprising as it is typically cleared from circulation in 3-6 minutes.329

**Figure A.2.C.** In Figure A.2.B, lysates were run on a reducing gel meaning dimeric forms of inhibin protein were not detectable, as alpha and beta subunits are linked by a disulfide bond. To determine if the inhibinα protein detected in the tumors was dimeric or free inhibinα, I ran HEY PLK01 tumor lysates and recombinant inhibin A on both reducing (left) and non-reducing gels (right). Under reducing conditions (i), recombinant inhibin A
ran as the mature $\alpha$C form (~15kDa) and the tumor lysates ran as Pro$\alpha$N/$\alpha$C (~55kDa) and Pro$\alpha$C (~27kDa). However, under non-reducing conditions, recombinant inhibin A ran as the mature heterodimer $\alpha$C/$\beta$ (~33kDa). Tumor lysates appeared as Pro$\alpha$N/$\alpha$C (~55kDa), indicating this form is secreted as free inhibin$\alpha$, and as Pro$\alpha$N$\alpha$C/Pro$\beta_{AB}/\beta_{AB}$, meaning some dimeric inhibin is being produced as well. Since the inhibin$\alpha$ subunit is common to both inhibin A and inhibin B, I was unable to differentiate between the two but could be determined with a specific beta subunit antibody. Taken together, this data shows that both HEY tumors produce both unprocessed forms of free inhibin$\alpha$ and dimeric inhibin protein.
Figure A.1. Inhibin protein is detectable in HEY tumors. A) Schematic showing various pre-processed and processed forms and sizes of monomeric inhibinα and dimeric inhibin protein. B) (i) Western blot analysis of HEY PLK01 and shINHA tumor lysates using anti-inhibinα R1 antibody. 40µg of tumor lysate was run per lane and R1 antibody was used at 2µg/mL dilution. (ii) Quantitation of western blot in (i) shown as each form of inhibinα detected normalized to GAPDH. Mean±SEM, n=6, * p<.05, unpaired t-test. C) Western blot of lysates from HEY tumors and recombinant inhibin A ran under (i) reducing or (ii) non-reducing conditions and immunoblotted with anti-inhibinα R1 antibody. 40µg of tumor lysate run per lane or 5ng of recombinant inhibin A and R1 antibody was used at 2µg/mL dilution.
Figure A.2. In Chapter 2 (Figure 2.3B), total inhibin ELISA was performed on a cohort of patient ascites fluid (AF) at Duke. Here, I performed the same ELISA on a separate, smaller cohort of patient ascites samples obtained from Penn State. In Table A.1, the histology and stage corresponding to each ascites sample is also shown. AF10, which was from a patient with a granulosa cell tumor, had the highest inhibin levels that was followed by AF8 and AF17 which were from high grade serous patients. These three samples had elevated levels of inhibin compared to the cohort in Chapter 2 while the remaining samples in this cohort had similar levels to those shown previously. Taken together, this data shows for the first time that inhibin levels are elevated in the ascites fluid of ovarian cancer patients.
Figure A.2. Inhibin expression in patient ascites fluid. Total inhibin ELISA of ascites fluid from 7 ovarian cancer patients using the Ansh Total inhibin ELISA kit. (AF: ascites fluid).
Table A.1. Patient information for inhibin ELISA on ascites fluid. Histology and data for corresponding ascites fluid samples shown in Figure A.2.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Histology</th>
<th>Stage</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>AF8</td>
<td>High Grade Serous</td>
<td>IIIC</td>
<td></td>
</tr>
<tr>
<td>AF10</td>
<td>Granulosa Cell Tumor</td>
<td>1A</td>
<td></td>
</tr>
<tr>
<td>AF14</td>
<td>Adenocarcinoma</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AF15</td>
<td>Adenocarcinoma</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AF17</td>
<td>High Grade Serous</td>
<td>2C</td>
<td></td>
</tr>
<tr>
<td>AF18</td>
<td>High Grade Serous</td>
<td>4</td>
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</tr>
<tr>
<td>AF19</td>
<td>Unknown</td>
<td>Unknown</td>
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Table A.2. As described in Chapter 2 (Figure 2.1), a panel of ovarian cancer cell lines was tested to see INHA expression changes in response to hypoxia but only a select few were included. Here, I have listed the entire panel of cell lines that I tested which include human ovarian cell lines, mouse ovarian cell lines, a human prostate cell line, and a mouse triple negative breast cell line. The table below details the name, species or origin, cancer subtype and the extent and whether the cell line increased INHA in response to hypoxia or CoCl$_2$. Not all cell lines increase INHA in response to hypoxia indicating potential for another mechanism of regulation. As mentioned in the Chapter 2 discussion, in adrenocortical tumors, aberrant methylation has been reported within the proximal HRE site of the INHA promoter$^{79}$. It is possible that there are different methylation patterns within the promoters of the cell lines tested which prevent transcriptional regulation of INHA by hypoxia. Future studies are required to further delineate how INHA is regulated in cancer.
Table A.2. *INHA* expression in cancer cell line panel in response to hypoxia. Cell lines tested for *INHA* expression in response to hypoxia. *INHA* expression was measured by qRT-PCR after exposure to hypoxia for 24hrs and normalized to normoxia.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Species</th>
<th>Type</th>
<th>Hypoxia Responsive</th>
<th>Normalized increase in <em>INHA</em> expression</th>
<th>CoCl$_2$ Responsive</th>
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<tbody>
<tr>
<td>PA1</td>
<td>Human</td>
<td>Teratocarcinoma</td>
<td>Yes</td>
<td>5.28-times</td>
<td>Yes</td>
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<tr>
<td>OVCAR5</td>
<td>Human</td>
<td>High Grade Serous</td>
<td>Yes</td>
<td>4.4-times</td>
<td>Yes</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Human</td>
<td>Serous cystadenocarcinoma</td>
<td>No</td>
<td>3.9-times/not significant</td>
<td>Yes</td>
</tr>
<tr>
<td>OV-90</td>
<td>Human</td>
<td>High Grade Serous</td>
<td>Yes</td>
<td>4.8-times</td>
<td>Not Tested</td>
</tr>
<tr>
<td>HEY</td>
<td>Human</td>
<td>High Grade Serous</td>
<td>Yes</td>
<td>4-times</td>
<td>Not tested</td>
</tr>
<tr>
<td>HEY T30</td>
<td>Human</td>
<td>High Grade Serous (Taxol resistant)</td>
<td>Yes</td>
<td>3.5-times</td>
<td>Not tested</td>
</tr>
<tr>
<td>HEYA8</td>
<td>Human</td>
<td>High grade serous</td>
<td>No</td>
<td>1.8-times/not significant</td>
<td>Not tested</td>
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<tr>
<td>OVCAR3</td>
<td>Human</td>
<td>High grade serous</td>
<td>No</td>
<td>1.7-times/not significant</td>
<td>No</td>
</tr>
<tr>
<td>OVCAR433</td>
<td>Human</td>
<td>Serous adenocarcinoma</td>
<td>No</td>
<td>1.1-times/not significant</td>
<td>Not tested</td>
</tr>
<tr>
<td>OVCA420</td>
<td>Human</td>
<td>Serous adenocarcinoma</td>
<td>No</td>
<td>0.67-times/significant</td>
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<tr>
<td>ES-2</td>
<td>Human</td>
<td>Clear cell carcinoma</td>
<td>No</td>
<td>1.01-times/not significant</td>
<td>Not tested</td>
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<tr>
<td>A2780</td>
<td>Human</td>
<td>Ovarian Endometroid</td>
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<tr>
<td>HEK293</td>
<td>Human</td>
<td>Human Embryonic Kidney</td>
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<td>6.4-times</td>
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<tr>
<td>PC3</td>
<td>Human</td>
<td>Prostate</td>
<td>No</td>
<td>2-times/not significant</td>
<td>Not Tested</td>
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<tr>
<td>4T1</td>
<td>Mouse</td>
<td>Triple Negative Breast Cancer</td>
<td>No</td>
<td>4.9-times/not significant</td>
<td>Not Tested</td>
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<tr>
<td>ID8 Ashwini</td>
<td>Mouse</td>
<td>Ovarian Surface Epithelium</td>
<td>No</td>
<td>1.05-times/not significant</td>
<td>Not tested</td>
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<tr>
<td>ID8 p53/-</td>
<td>Mouse</td>
<td>Ovarian Surface Epithelium</td>
<td>No</td>
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<td>Not tested</td>
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<tr>
<td>ID8 EMD</td>
<td>Mouse</td>
<td>Ovarian Surface Epithelium</td>
<td>Yes</td>
<td>4.1-times</td>
<td>Not Tested</td>
</tr>
<tr>
<td>ID8ip2Luc</td>
<td>Mouse</td>
<td>Ovarian Surface Epithelium</td>
<td>Yes</td>
<td>4-times</td>
<td>No</td>
</tr>
</tbody>
</table>
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