Fall 12-16-2016

Altered Levels of SOX2, and Its Associated Protein Musashi2, Disrupt Critical Cell Functions in Cancer and Embryonic Stem Cells

Erin L. Wuebben
University of Nebraska Medical Center

ALTERED LEVELS OF SOX2, AND ITS ASSOCIATED PROTEIN MUSASHI2, DISRUPT CRITICAL CELL FUNCTIONS IN CANCER AND EMBRYONIC STEM CELLS

BY

ERIN LYNN WUEBBEN

A DISSERTATION

PRESENTED TO THE FACULTY OF THE GRADUATE COLLEGE AT THE
UNIVERSITY OF NEBRASKA MEDICAL CENTER IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

CANCER RESEARCH GRADUATE PROGRAM
EPPLEY INSTITUTE FOR CANCER RESEARCH AND ALLIED DISEASES

UNDER THE SUPERVISION OF PROFESSOR ANGIE RIZZINO
UNIVERSITY OF NEBRASKA MEDICAL CENTER
OMAHA, NEBRASKA

OCTOBER 2016

ACKNOWLEDGEMENTS

My life has been filled with wonder at the beauty and complexity of the world surrounding me. This wonder blossomed under the education and guidance of phenomenal teachers and professors like Mrs. Marsha Stewart at Norfolk Catholic High School and Dr. Lawrence Harshman at the University of Nebraska – Lincoln. However, this wonder grew into true scientific inquiry under the supervision of my Ph.D. advisor Dr. Angie Rizzino. Angie has encouraged my growth as a scientist through daily discussions of recent cancer breakthroughs, experimental design, and effective communication through writing and presenting. However, beyond my development as a scientist, I believe the mentorship I have received from Angie has made me a better person by always challenging me to explore things for myself, to enhance my critical thinking, and to always remember that people are the reason we study this disease; to help save or improve the lives of people living with cancer. For these things, I am extremely grateful.

This philosophy has been further expanded by the people of Eppley Institute and the Cancer Research Graduate Program. Faculty members of my supervisory committee, Drs. Tony Hollingsworth, Xu Luo, and Michel Oullette, as well as Dr. Amar Natarajan's service on my comprehensive committee, have been a great source of knowledge and suggestions for furthering my graduate studies. Furthermore, Dr. Joyce Solheim has been a constant source of advice and listening ear for me and others, always keeping her door open, literally and figuratively, for support during times of uncertainty. Among the many people of Eppley, I want to extend my thanks to all the graduate students of the Eppley Institute for fostering a community of collaboration and fount of memories I'll treasure for years to come.

I must also acknowledge several former and current students and laboratory members who have helped me throughout my studies. Initially, I was taken under the wings of Dr. Sunil

Mallanna who meticulously started the path for my first publication as he prepared his own dissertation. Additionally, Michelle Desler and Dr. Phillip Wilder have been crucial to the steady success of the Rizzino laboratory through not only keeping the laboratory running smoothly, but also through their patience in trouble shooting any problems that arise. Most importantly, I have to thank the former, senior students in Angie's laboratory, Dr. Jesse Cox and Dr. Briana Ormsbee Golden, who carefully taught me so many protocols while also offering their thoughtful support in overcoming experimental roadblocks. Their patience and perseverance instilled in me a fortitude I hope to continue throughout my career. The additional support of several students outside of the laboratory made the ups and downs of graduate study so much more bearable. Dr. Jenna Preusker, Dr. Jennifer Kopanic, Dr. Dana Tabor, Dr. Kristi Anderson, Rachel Utter, Maria Steele, and Corey Suelter often provided the much needed happy distraction to balance the frenzy that is graduate work. Their unwavering support while balancing their own projects was so selfless and kind, and will be forever appreciated.

Finally, I must extend my deepest gratitude to my family, especially the two people who have supported me the longest, Allen and Terri Wuebben. As my parents, they have always reminded me that I'm capable of anything I set my mind to and have always been there to pick me up when I stumble. Their faith in me has given me the confidence to be independent and pursue my dreams. I have been extremely blessed to have them as my parents and as friends.

Altered levels of SOX2, and its associated protein Musashi2, disrupt critical cell functions in cancer and embryonic stem cells

Erin Lynn Wuebben, Ph.D.

University of Nebraska, 2016

Advisor: Angie Rizzino, Ph.D.

The transcription factor SOX2 is widely recognized for its critical roles during mammalian embryogenesis. SOX2 has also been examined in cancer; and it has been implicated in the growth, tumorigenicity, drug resistance, or metastasis of over 20 different cancers, including cancers of the brain and pancreas. Thus, we hypothesized that SOX2 is a major player in cancer and may be a potential therapeutic target; however, the effects of SOX2 on the many facets of human cancer have only begun to be explored. Recently, efforts to understand the mechanisms by which SOX2 mediates its effects have explored SOX2 protein-protein interaction landscapes in a number of cellular systems. Previous studies in our laboratory identified proteins, like the RNA-binding protein Musashi2 (Msi2), which interact with SOX2 in multiple cell types, including embryonic stem cells and brain tumor cells. We hypothesized that proteins that interact with SOX2 in multiple cell types are likely to be necessary for the continued growth and function of these cells. The studies presented in this dissertation demonstrate that ESC require Msi2 to maintain self-renewal and pluripotency; and that MSI2 is also required to support the growth and survival of DAOY, U87, and U118 brain tumor cell lines. This dissertation also examined the roles of SOX2 in pancreatic adenocarcinoma (PDAC). Multiple PDAC cell lines were engineered for either inducible overexpression of SOX2 or inducible knockdown of SOX2. Through *in vitro* growth and tumorigenicity studies with these inducible PDAC cell lines, we determined that SOX2 functions as a biphasic molecular rheostat in PDAC. Furthermore, we determined that inducible elevation of SOX2 in PDAC cells reduces the growth inhibitory effects of MEK and

AKT inhibitors, while the inducible knockdown of SOX2 enhanced growth inhibition in the presence of these inhibitors. Altogether, the work presented in this dissertation extends and strengthens our knowledge of SOX2 and its function as a master regulator in multiple cell types, and provides useful platforms for the continued study of these highly deadly malignancies.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1: Introduction

CHAPTER 2: Materials and Methods

CHAPTER 3: Musashi2 is required for the self-renewal and pluripotency of embryonic stem cells

CHAPTER 6: Conclusions & Future Directions

ABBREVIATIONS

ABC	ATP-binding cassette
AKTi	AKT inhibitor
AML	acute myeloid leukemia
BMP4	bone morphogenic protein
ChIP-Chip	chromatin immunoprecipitation coupled to microarray (chip) analysis
ChIP-seq	chromatin immunoprecipitation couple to high-throughput sequencing
ChIRP	chromatin isolation via RNA purification
CML	chronic myelogenous leukemia
CRISPR	clustered regularly interspaced short palindromic repeats
DMEM	Dulbecco's Modified Eagle's Medium
Dox	Doxycycline
DUB	deubiquitinating enzyme
EGFP	enhanced green fluorescence protein
ESC	embryonic stem cells
FBS	fetal bovine serum
G418	neomycin
GBM:	glioblastoma
GFP	green fluorescence protein
H&E	hematoxylin and eosin
HMG	high mobility group
HSC	hematopoietic stem cells
i-KdSOX2-L3.6	inducible knockdown of SOX2 L3.6 PDAC cells
i-KdSOX2-T3M4	inducible knockdown of SOX2 T3M4 PDAC cells
i-Msi2.1-D3 ESC	inducible (flag-tagged) Msi2 isoform 1 D3 ESC
i-Msi2.2-D3 ESC	inducible (flag-tagged) Msi2 isoform 2 D3 ESC

i-OSKM-ESC	inducible Oct4, (flag-strep-tagged)Sox2, Klf4, c-Myc ESC
i-SOX2-BxPC3	inducible SOX2 T3M4 BxPC3 cells
i-SOX2-HPAF-II	inducible SOX2 T3M4 HPAF-II cells
i-SOX2-T3M4	inducible SOX2 T3M4 PDAC cells
iPS	induced pluripotent stem cells
Kb:	kilobase
LIF	leukemia inhibitory factor
lncRNA	long noncoding RNA
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MB	medulloblastoma
MEG3	maternally expressed gene 3
MEKi	MEK inhibitor
miR	microRNA
Msi1	Musashi1
Msi2	Musashi2
NLS	nuclear localization sequence
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic adenocarcinoma
RA	retinoic acid
Rb	retinoblastoma
RFP	red fluorescent protein
RMST	rhabdomyosarcoma 2-associated transcript
rtTA	reverse tet transactivator
RT-qPCR	quantitative Real-Time polymerase chain reaction

shRNA	small hairpin RNA
siRNA	small interfering RNA
SMA	smooth muscle actin
SOX2OT	SOX2 overlapping transcript
SRR	Sox2 regulatory region
TIC	tumor-initiating cells
TUNA	Tcl1 Upstream neuron-associated
Ube2s	ubiquitin-conjugating enzyme E2S
USP	ubiquitin specific peptidase
UTR	untranslated region

CHAPTER 1: Introduction

1.1 Introduction

The transcription factor SOX2 is widely recognized for its critical roles during mammalian embryogenesis. Although Sox2 was first shown to regulate the transcription of FGF4 in mouse embryonal carcinoma cells [1], its importance was firmly established with the discovery that knocking out both alleles of SOX2 results in embryonic lethality in mice. SOX2 null embryos reach the blastocyst stage, but do not survive after implantation [2]. Shortly thereafter, knocking down Sox2 in mouse embryonic stem cells (ESC) was shown to disrupt their self-renewal and induce differentiation [3]. One year later interest in Sox2 rose dramatically with the paradigm shifting discovery by Takahashi and Yamanaka demonstrating conversion of mouse embryonic fibroblasts into induced pluripotent stem (iPS) cells by the introduction of Sox2 along with Oct4, Klf4, and cMyc [4].

The excitement surrounding the key roles of SOX2 in ESC and iPS cells, which are themselves tumorigenic, soon led to the search for SOX2 in cancer. Within a few years following the discovery of iPS cells, numerous reports of SOX2 expression in human cancer had already appeared. This soon turned into an avalanche of studies examining SOX2 in human cancer. The search terms SOX2 and cancer generate over 1600 hits in the PubMed database since 2006. During that period, SOX2 has been implicated in growth, tumorigenicity, drug resistance, and metastasis in over 20 different cancers, including cancers of the ovary, lung, skin, brain, breast, prostate, and pancreas. In the majority of these cancers, SOX2 has been reported to have increased expression or gene amplification in tumor tissue; however, the effects of SOX2 on tumorigenicity, prognosis, and drug resistance in human cancer have only begun to be explored. Nonetheless, it is evident from the impressive body of work published thus far that SOX2 is a major player in cancer and a potential therapeutic target.

1.2 Sox2 Gene Structure and Regulation

1.2.1 Transcriptional Regulation of Sox2

The transcriptional regulation of *Sox2* has been extensively studied. However, it is evident that there is much more to learn about how this gene is regulated at the transcriptional level. Sox2 is a member of the SRY-related gene family, each member of which contains a well-conserved high mobility group domain (HMG box, 79 amino acids), which mediates its binding to DNA. The *Sox2* gene, a single exon, in mammals, as well as birds, is located within a gene desert (a large genomic region largely devoid of other protein coding genes). Analysis of a 200 kilobase (kb) region of the chicken gene that surrounds the *Sox2* single exon identified at least 27 distinct enhancers that are transcriptionally active for the regulation of *Sox2* during neuro-sensory development in the chicken [5]. Eleven of the enhancers are distributed fairly evenly over a 97 kb region located upstream of the coding region of the *Sox2* gene, and 16 enhancers are fairly evenly distributed over a 110 kb region downstream of the coding region of the *Sox2* gene. The large majority of the enhancers identified in the chicken genome are located in regions that are conserved in mammals. Thus, it is likely that the mammalian Sox2 gene is also transcriptionally regulated by a large number of distinct distal enhancers during different stages of development. However, far more work will be needed to define the regulatory regions of mammalian *Sox2* gene that are active in specific cell types. As discussed below, only three enhancers have been identified as functionally active in mammalian cells, one of which is located ~100 kb downstream of the *Sox2* gene.

In mammalian cells, transcriptional regulation of the *Sox2* gene, including the enhancers that drive *Sox2* expression, has been primarily studied in mouse ESC. In addition to the basal promoter of the *Sox2* gene [6], early studies identified two enhancers, Sox2 regulatory region 1 (*SRR1*) and *SRR2,* which influence the activity of the *Sox2* promoter. *SRR1* is located ~ 4kb upstream of the *Sox2* transcription start site; whereas, *SRR2* is located ~2.5 kb downstream of the

3' end of the *Sox2* coding region [7]. Although *SRR1* has been shown to be active in promoter/reporter gene constructs expressed in ESC, its impact on the expression of *Sox2* in ESC is minimal when *SRR1* is deleted from the endogenous *Sox2* gene [8]. However, deleting a region -5.7 to -3.3 kb upstream of the *Sox2* transcription start site, which contains *SRR1*, abolished expression of *SOX2* in telencephalic neural stem cells and precursors during murine development [9, 10]. *SRR2* is not only active in mouse ESC; it has been used to isolate human iPS cells [11]. For these studies, the *SRR2* enhancer was multimerized (4 tandem repeats) and inserted into a lentiviral vectors which drives the expression of enhanced green fluorescence protein (EGFP) via a minimal promoter only when *SRR2* is active. Subsequently, and discussed further below, this lentiviral vector which drives EGFP was shown to be active in breast cancer cells [12]; and isolation of the subset of EGFP-expressing breast tumor cells were shown to exhibit enhanced tumorigenic potential, but only when NOD/SCID mice were engrafted with a large number of cells [13].

Several studies have examined the transcriptional machinery that regulates the activity of SRR2. The sequence of *SRR2* contains adjacent *HMG* and *POU* motifs (referred to as an *HMG/POU* cassette) that have been shown to be essential for the activity of *SRR2* in *ESC* and bind Sox2 and Oct4 in ESC [7]. These studies led to the conclusion that Sox2 in combination with Oct4 contributes to the transcription of *Sox2* in ESC. However, this may not be the only role of *SRR2* in the transcription of *Sox2*. In fact, several recent studies lead us to suggest that a major role of *SRR2* may be to repress, not activate, *Sox2* transcription, especially during differentiation. First, as in the case of *SRR1*, deletion of *SRR2* from the endogenous *Sox2* gene did not significantly reduce Sox2 expression in ESC [8]. Even more suggestive of a repressive role for *SRR2* is the finding that *SRR2* is able to bind transcriptional repressors, such as p21, $p27^{Kip1}$, and the p130/E2F4-SIN3A repressor complex, in neural stem cells and iPS cells undergoing differentiation [14, 15]. Consistent with these findings, *Sox2* mRNA is elevated in *Rb* (p105) null and *p130* (retinoblastoma family member) null mouse embryonic fibroblasts, and it is elevated in

the pituitary tissue of *Rb* heterozygous mice [16]. Moreover, in pituitary tumors, loss of *Rb* or *p130* has been linked to a defect in the repression of Sox2 expression [16]. Given the roles of p21, $p27^{Kip1}$, and Rb proteins in the G1 cell cycle check point, *Sox2* expression may be reduced in G1 phase of the cell cycle. Future studies should consider whether *SOX2* expression is cell cycle regulated.

In ESC, a critical enhancer region (referred to as SCR – *Sox2* control region) required for *Sox2* transcription is located ~100 kb downstream of the *Sox2* gene [8]. Previous studies had predicted 10 enhancers surrounding the *Sox2* gene, including two that overlapped *SRR1* and *SRR2*. When tested in promoter/reporter gene constructs, three of the 10 putative enhancers, which are located 18, 107, and 111 kb downstream of the *Sox2* gene, were found to drive the expression of the reporter gene more potently than *SRR1* and *SRR2* in ESC. Importantly, more definitive results were obtained by generating deletions of these enhancers in one allele of the *Sox2* gene using clustered regularly interspaced short palindromic repeats (CRISPR) based gene editing [8]. Deletion of *SRR1*, *SRR2*, or the enhancer located 18 kb downstream of *Sox2* did not affect the expression of the targeted allele. In strong contrast, deletion of the SCR reduced expression in the targeted allele. (For these studies, expression of the targeted and non-targeted alleles was monitored separately by PCR in a heterozygous ESC line containing one allele from mouse strain *Mus musculus* and one allele from *Mus castaneus*.) Consequently, targeting one *Sox2* allele in ESC did not impact the maintenance of pluripotent ESC, due to upregulation of the non-targeted *Sox2* allele. This finding and earlier studies involving Sox2 overexpression in ESC (see below) indicate that Sox2 influences its own expression in ESC by a feedback loop. In the future, it will be important to determine whether the SCR that is active in ESC, is also active in other SOX2 expressing cells, in particular SOX2-positive tumor cells. Thus far, only *SRR2* has been reported to be active in SOX2-positive tumor cells.

Sox2 not only positively influences Sox2 expression in ESC when it is under expressed, it has the opposite effect when Sox2 is overexpressed in ESC. As discussed later, ESC engineered

for inducible overexpression of Sox2 undergo differentiation when Sox2 is elevated by 2-fold or more. Interestingly, overexpression of exogenous Flag-tagged Sox2 in ESC reduces endogenous Sox2 expression at the protein level [17] and at the transcriptional level [18]. Specifically, it was determined that elevation of exogenous Sox2 activated a negative feedback loop mediated at least in part by increased phosphorylation of AKT and one of its downstream targets, FoxO1, which regulates transcription of *Sox2* [18]. When FoxO1 is phosphorylated, it translocates out of the nucleus, thus reducing *Sox2* transcription. Thus in ESC, Sox2 can regulate its own expression at the transcriptional levels by both positive and negative feedback loops when Sox2 expression is too low and when Sox2 expression is too high, respectively. While it is evident that the transcriptional regulation of *SOX2* has been extensively studied, there is far more to learn how this gene is regulated at the transcriptional level.

1.2.2 MicroRNAs and SOX2 Expression

A large body of data has implicated microRNAs (miRs) in the function of normal embryonic and adult cells, as well diseased tissues, in particular cancer. More than 10 years ago, ChIP-Chip studies conducted by Boyer et al determined that SOX2 associates with the regulatory regions of many miR genes in human ESC [19]. This finding was extended by ChIP-seq analysis of Sox2 chromatin binding in mouse ESC [20]. More recently, Fang et al determined by ChIP-seq that SOX2 is bound to over 100 miR genes in a glioblastoma cell line [21]. Further study is expected to show that SOX2 regulates the transcription of a large number of miRs in a wide variety of SOX2-positive tumors. However, the specific miR genes regulated by SOX2 are expected to differ widely between tumor cell types due to differences in their transcriptional circuitries.

In addition to the regulation of miRs by SOX2, there is a growing list of miRs that are capable of regulating *SOX2* at the post-transcriptional level. In the case of cancer, at least 18 miRs have been reported to regulate *SOX2* expression in tumor cell lines (Table 1.1), include

Table 1.1: MicroRNAs regulating SOX2 in cancer

Tumor Type	miR	Effects Observed	Reference
Breast cancer	miR-140	can target SOX2 3'UTR altered expression alters SOX2 expression	Zhang et al., 2012
	miR-378	Enhances SOX2 expression indirctly	Deng et al., 2013
Colorectal cancer	miR-200c	can target SOX2 3'UTR altered expression alters SOX2 expression	Lu et al., 2014
	miR-638	can target SOX2 3'UTR altered expression alters SOX2 expression	Ma et al., 2014
	miR-450b	down regulated in recurrent tumors	Jin et al., 2016
	miR-429	can target SOX2 3'UTR altered expression alters SOX2 expression	Li et al., 2013
Embryonal carcinoma	miR-211	targets both SOX2 and SOX2OT	Shafiee et al., 2016
Embryonic stem cells	miR-145	targets SOX2 3'UTR upon differentiation	Xu et al., 2009
Esophageal cancer	miR-625	can target SOX2 3'UTR altered expression alters SOX2 expression	Wang et al., 2014
Ewing sarcoma	miR-145	altered expression alters SOX2 expression	Riggi et al., 2010
Gastric cancer	miR-371	can target SOX2 3'UTR altered expression alters SOX2 expression	Li et al., 2016
	miR-126	can target SOX2 3'UTR altered expression alters SOX2 expression	Otsubo et al., 2011
Glioblastoma	miR-21	low miR-21/high SOX2 in one subgroup high miR-21/low SOX2 in different subgroup	Sathyan et al., 2015
	miR-145	SOX2 and miR-145 regulate each other	Fang et al., 2011
Glioma stem cells	miR-218	elevated miR-218-5p reduced SOX2 miR-218-5p may not target SOX2 directly	Wu et al., 2016
	miR-9*	ID4 decreases miR-9* and increases SOX2 SOX2 3'UTR activity elvated as ID4 increases	Jeon et al., 2011
Hepatocellular carcinoma	miR-126	can target SOX2 3'UTR	Zhao et al., 2015
	miR-145		Jia et al., 2012
Nasopharyngeal carcinoma	miR-30a	targets SOX2 3'UTR	Qin et al., 2015
Neuroblastoma	miR-340	can target SOX2 3'UTR miR-340 gene is methylated in this tumor	Daa et al., 2013
Non-small cell lung carcinoma	miR-638	can target SOX2 3'UTR	Xia et al., 2014
	miR-145	altered expression alters SOX2 expression	Campayo et al., 2013
Osteosarcoma	miR-126	can target SOX2 3'UTR altered expression alters SOX2 expression	Yang et al., 2013
Pancreatic cancer	miR-145	can target SOX2	Sureban et al., 2013
	miR-1181	directly targets SOX2	Jiang et al., 2015
Prostate cancer	miR-145	altered expression alters SOX2 expression	Ozen et al., 2015
	miR-34b	unclear if it directly targets SOX2	Forno et al., 2015
Urothelial carcinoma	miR-145	altered expression alters SOX2 expression	Fujii et al., 2015

NT/2). Of these, miR-145 has been implicated directly or indirectly in ESC and at least seven cancers, glioblastoma, prostate cancer, non-small cell lung carcinoma, Ewing sarcoma, hepatocellular carcinoma, pancreatic adenocarcinoma, and urothelial carcinoma [22-29]. Interestingly, in glioblastoma, SOX2 and miR145 have been reported to form a negative feedback loop with one another. In this tumor, Sox2 can associate with the gene regulatory regions of *miR145*, where it is believed to repress *miR145* transcription; whereas miR145 reduces the expression of SOX2 by interfering with its translation [21]. In colorectal cancer, miR-200c and SOX2 also appear to regulate one another by a negative feedback loop [30]. With one notable exception [31], SOX2-targeting miRs are associated with downregulation of SOX2.

The association of SOX2 and miRs in specific cancers has been inferred predominately from the correlation between elevated SOX2 expression and low miR expression. In most studies, this association is supported by two additional lines of evidence, down regulation of SOX2 when the miR in question is ectopically elevated in tumor cell lines, and down regulation of a reporter gene construct, typically luciferase, containing a portion of the SOX2 3' untranslated region (UTR) when the miR is ectopically expressed in tumor cell lines (Table 1.1).

For some cancers only a single miR has been implicated thus far in the regulation of SOX2. For example, when miR-30a is upregulated in nasopharyngeal carcinoma cells it appears to be capable of reducing SOX2 protein levels by targeting the 3' UTR of SOX2 mRNA [32]. However, it is likely that SOX2 can be regulated by several miRs in the same cell type. In gastric carcinoma, prostate cancer, and colorectal cancer, more than one miR has been implicated in the regulation of SOX2 (Table 1.1). In prostate cancer, SOX2 expression is associated with low expression of both miR-145 and miR-34b [24, 33]. However, unlike miR-145, which has been shown to target the 3' UTR of the *Sox2* transcript [22], it is unclear whether miR-34b targets SOX2 directly. In the case of colorectal cancer, miR-200c, miR-638, miR-450-5p, and miR-429 have been reported to regulate SOX2, but with different outcomes (Table 1.1). Lu et al reported that miR-200c, which is expressed at lower levels in colorectal specimens and highly metastatic

colorectal cell lines, exhibits an inverse relationship with SOX2 [30]. Similarly, Ma et al has also shown that miR-638, which is expressed at a lower level in colorectal tumors than adjacent non-tumorigenic tissue, is able to target SOX2 [34], and Jin et al reported that miR-450-5p, which is downregulated in recurrent colorectal cancer, is capable of downregulating SOX2 [35]. In contrast, Li et al reported that higher levels of miR-429 and lower levels of SOX2 mRNA in colorectal cancer are correlated with poor survival after surgery [36]. Interestingly, these investigators argued that high miR-429 expression exerts its anti-apoptotic function by downregulating SOX2. However, this is inconsistent with the apparent oncogenic role of SOX2 in a subgroup of in colorectal cancer patients. In this regard, Lundberg et al reported that SOX2-positive colorectal cancer patients do not survive as long as SOX2-negative colorectal cancer patients; and this differential is larger for patients with BRAFV600E mutations who survive for substantially shorter periods than those who are SOX2-positive, but lack the BRAF mutation [37]. Going forward, it will be important to determine whether the levels of miR-429 are lower in colorectal cancer patients with BRAFV600E mutations. One of the mechanisms by which miR-429 promotes colorectal cancer may be to help maintain SOX2 within optimal levels in the BRAFV600E-mutant subgroup of colorectal tumors, which is discussed in greater detail later.

More than one miR has also been reported to target SOX2 in gastric carcinoma. For both miRs, high miR expression is associated with low SOX2 expression. Li et al reported that miR-371-5p, which is elevated in gastric carcinoma compared to adjacent normal tissue, targets SOX2 [38]. In addition, these investigators reported that miR-371-5p downregulated a luciferase reporter gene construct containing a short sequence from the SOX2 3’ UTR; whereas blocking expression of this miR in gastric tumor cell line increased SOX2 expression and cell proliferation *in vitro*. A similar conclusion was reached for miR-126. Otsubo et al reported that transiently elevating miR-126 in gastric cancer cell lines decreased SOX2 and increased cell proliferation *in vitro* [39]. They also demonstrated that miR-126 reduced the expression of a luciferase reporter gene containing regions taken from the SOX2 3’ UTR. Furthermore, these investigators reported

low SOX2 expression and elevated miR-126 in some gastric tumor specimens, but the results reported do not appear to show a clear pattern. Although, elevated miR-126 expression and low SOX2 expression was observed in several gastric cancer tumor specimens, low miR-126 expression was accompanied by low SOX2 expression in several other gastric tumor specimens. Thus, a larger number of tumors specimens will need to be evaluated to resolve the relationship between miR-126 and SOX2. In addition, the relationship between miR-126, SOX2, and patient survival remains to be determined. As expanded on below, high SOX2 in gastric cancer has been reported to be associated with longer patient survival [40-42]. Interestingly, there are reports that miR-126 can act as a tumor suppressor in other types of cancer. For example, Yang et al and Zhao et al reported that miR-126 behaves as a tumor suppressor in osteosarcoma and hepatocellular carcinoma, respectively, by targeting SOX2 [43, 44]. Additionally, Hamada et al reported that loss of mR-126 expression is observed in invasive pancreatic ductal adenocarcinoma [45].

Although miRs are recognized as important regulators of SOX2 expression, two important issues need to be considered. First, unless the cell of origin and its expression of miRs have been determined, it remains to be determined whether the miR in question and its putative loss during tumor progression did in fact contribute to elevated SOX2 expression. Second, the full spectrum of SOX2 targeting miRs is likely to be far greater than those already identified.

1.2.3 Long Noncoding RNAs and SOX2 Expression

In addition to miRs, several long noncoding RNAs (lncRNAs) have been reported to influence the levels of SOX2 in tumor cells. LncRNAs are a class of RNAs that are greater than 200 nucleotides that lack protein-coding sequences. They are transcribed by RNA polymerase II and they are spliced, 5’ capped, and 3’ polyadenylated. The human genome contains several thousand lncRNAs, and there is growing evidence that many play major roles in gene regulation by influencing chromatin structure, gene transcription, and processing of mRNA [46]. More

recently, several lncRNAs have been implicated in the regulation of SOX2 expression and its transcriptional activity. The first direct link between SOX2 and lncRNAs was the discovery that the single exon *SOX2* gene is embedded within an intron of a multi-exon lncRNA gene known as *SOX2 overlapping transcript* (*SOX2OT*, also known as non-protein-coding RNA 43) [47]. Like *SOX2* itself, *SOX2OT* orthologues are expressed widely in other vertebrates, including in mouse, chicken, and zebrafish. *SOX2* and *SOX2OT* are each transcribed in the same direction. *SOX2OT* is reported to possess at least 10 exons with up to four different transcription start sites. Through use of alternative transcription start sites and alternative splicing at least 8 splice variants of *SOX2OT* can be generated [48, 49].

SOX2 and *SOX2OT* have been shown to be co-expressed in ESC, as well as breast, lung, brain, and esophageal tumors [50-54]. In each of these cancers, more than one splice variant is expressed, and the splice variants expressed differ between different cancers. *SOX2* and *SOX2OT* are also likely to be co-expressed in hepatocellular carcinoma. Separate studies have reported that expression of *SOX2* and *SOX2OT* in hepatocellular carcinoma is each associated with poor prognosis [55, 56]. Although the mechanistic relationship between *SOX2* expression and *SOX2OT* remains to be determined, several studies support the conclusion that *SOX2OT* lncRNA contributes to the expression of *SOX2*. Knockdown of *SOX2OT* by small interfering RNA (siRNA) in the lung adenocarcinoma cell line A549 reduced the expression of SOX2 transcripts [53]. Conversely, forced overexpression of SOX2OT in the breast tumor cell line MDA-MB-231 increased the expression of SOX2 transcripts and protein [51]. Intriguingly, *SOX2* and *SOX2OT* expression may both be related by at least one miR. miR-211 has been reported to target the same sequence in transcripts of *SOX2OT* and *SOX2* and lead to their downregulation when miR-211 is overexpressed in the human embryonal carcinoma cell line NT-2 [57].

In addition to *SOX2OT*, several other lncRNAs have been directly implicated in the expression of *SOX2*. The lncRNA *TUNA* (Tcl1 Upstream Neuron-Associated), which can form a complex with three RNA-binding proteins, has been shown by performing chromatin isolation via

RNA purification (ChIRP) to associate with the *Sox2* promoter in mouse ESC [58]. Furthermore, knockdown of *TUNA* by small hairpin RNA (shRNA) reduced the expression of Sox2 and led to the differentiation of mouse ESC. Interestingly, *TUNA* and *Sox2* are also co-expressed in the brain [58]. Thus, it will be interesting to determine whether *TUNA* is expressed in glioblastoma and medulloblastoma and other SOX2-positive tumors, where it may also contribute to SOX2 expression. However, further study will be needed to determine how *TUNA* influences SOX2 expression. The lncRNA *MALAT1* (Metastasis-Associated Lung Adenocarcinoma Transcript 1) also appears to influence the expression of SOX2. *MALAT1* has been shown to be expressed in the glioma tumor cell line SHG139S and in two pancreatic tumor cell lines, AsPC1 and CFPAC-1 [59, 60]. Knockdown of *MALAT1* in each of these tumor cell lines reduced the expression of SOX2. However, it is unclear whether the effect of *MALAT1* on SOX2 in these tumor cells is direct or indirect. Equally interesting is the report that lncRNA *RoR* supports SOX2 expression by functioning as a miRNA sponge. Specifically, *RoR* helps maintain SOX2 expression by serving as an RNA decoy that competes for miRs (e.g. miR145) that target *SOX2* expression [61].

LncRNAs also appear to regulate the transcriptional activity of SOX2. The lncRNA *RMST* (RhabdoMyoSarcoma 2-associated Transcript) has been reported to coregulate Sox2 target genes during neurogenesis [62]. *RMST* interacts physically with SOX2 and promotes the binding of SOX2 to the regulatory regions of neurogenic transcription factors. Impressively, knockdown of *RMST* reduces SOX2 association with approximately half of its chromatin binding sites [62]. Although *RMST* appears to enhance the transcriptional activity of SOX2, at least one lncRNA, *MEG3* (Maternally Expressed Gene 3) that also physically associates with SOX2, can interfere with its action. Knockdown of *MEG3* has been reported to increase the association of SOX2 with the *BMP4* gene and decrease the transcription of this gene [63]. Thus far, the domains of SOX2 that associate with these lncRNA have not been determined, nor has it been determined how they influence the transcriptional activity of SOX2. Going forward, it will be interesting to determine whether *RMST* and *MEG3* are commonly expressed in SOX2-positive tumors, including

glioblastoma and medulloblastoma. Moreover, *MEG3* has been shown to be expressed in pancreatic tumor cell lines, where its knockdown led to a reduction in cell number *in vitro* [64]. Thus, it will be interesting to determine whether knockdown of *MEG3* alters the function of SOX2 in pancreatic tumor cells.

1.2.4 Post-translational Modifications of SOX2

Another important mechanism used to regulate SOX2, including its transcriptional activity, nuclear localization, and stability, is through its post-translational modifications. As discussed in this section, Sox2 is regulated at the post-translational level by a wide range of modifications, including phosphorylation, glycosylation, sumoylation, methylation, ubiquitination, and acetylation. Thus far, nearly all reports of SOX2 post-translational modifications have been conducted with mouse pluripotent stem cells and mostly with ESC. In the future, it will be important to characterize the post-translational modifications of SOX2 in tumor cells. However, at least one study has described a SOX2 post-translational modification (phosphorylation) in human lung squamous cell carcinoma cells [65]. For the purposes of clarity, and to avoid confusion, the reader is reminded that human SOX2 and mouse Sox2 differ in length by 2 amino acids: 317 amino acids and 319 amino acids, respectively due to a two amino acid insertion beginning at residue 23 in mouse Sox2 (Figure 1.1).

The most common and diverse post-translational modification reported for Sox2 is phosphorylation. Sox2 phosphorylation influences its transcriptional activity and its stability. Studies by several research teams have shown that Sox2 can be phosphorylated *in vivo* on at least 6 serine residues (mouse S39, S83, S248, S251, S252, S253) and two threonine resides (mouse Sox2 T118 and human SOX2 T118) (Figure 1.1). Sox2 has also been reported to be phosphorylated when Sox2 is ectopically expressed in 293T cells, which express little if any endogenous SOX2 [66]. It remains to be determined whether these tyrosine residues are

Figure 1.1

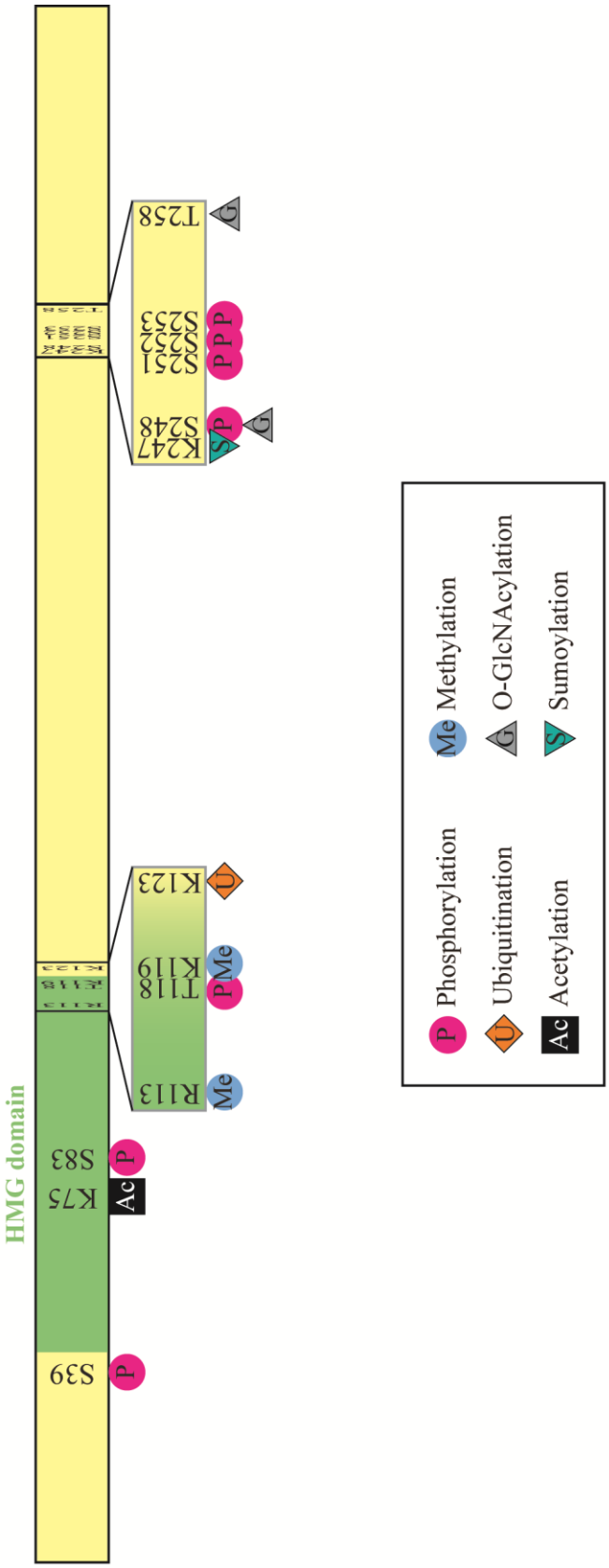

Figure 1.1: Mouse Sox2 structure and post-translational modification sites (319 amino acids)

phosphorylated in cells that endogenously express SOX2. The kinases responsible for serine phosphorylation of SOX2 have only begun to be determined. For example, Cdk2 can phosphorylate both S39 and S253 *in vitro* [66]. Modifying both serine residues by conversion to alanine (S39A, S253A) reduces the ability of mutant Sox2 to reprogram somatic cells into iPS cells. Surprisingly, even though S39 and S253 are phosphorylated in mouse ESC, and most highly phosphorylated during mitosis, a mutant form of Sox2 (S39A/S253A) is able support the self-renewal of mouse ESC when endogenous Sox2 is depleted [66]. It is possible that at different levels of Sox2, phosphorylation of theses serine residues is dispensable.

Serine S248 of mouse Sox2 has been reported to be phosphorylated in mouse ESC. Phosphorylation of this serine is likely to have a significant role in the function of Sox2, because this serine, along with T258 in mouse Sox2 (see below) can also be modified by *O*-GlcNAcylation [67, 68]. Studies conducted thus far suggest that modification of S248 in mouse Sox2 may alter the transcriptional activity of Sox2, but further work will be needed to properly dissect the impact of phosphorylation and *O*-GlcNAcylation of Sox2 S248. Similar to this serine residue, the serine triplet S249-S250-S251 in human SOX2 appears to regulate another post-translational modification, sumoylation. Human SOX2 has been shown to be sumoylated on K245 and K247 in mouse Sox2 [69, 70]. Importantly, sumoylation of K245 is abolished in the SOX2 mutant (S249A-S250A-S251A) [69]. Thus, phosphorylation of one or more serine residues in the triplet appears to serve as a priming step in the subsequent sumoylation of SOX2. Although the role(s) of SOX2 sumoylation remain to be fully characterized, sumoylation of mouse Sox2 has been reported to reduce the ability of Sox2 to increase the transcription of *Fgf4* and *Nanog* [70]. In the future, it will be important to determine whether this reduction in transcriptional activity is restricted to a small number of genes or is true for most Sox2-regulated genes.

The kinases that phosphorylate threonine residues of mouse Sox2 T118 and humanT118 have been identified. These threonine residues are both located within a consensus nuclear localization sequence and the HMG domain of SOX2, which is responsible for DNA binding.

Phosphorylation of human SOX2 onT118 is mediated by the atypical PKCι [65]. Phosphorylation of this threonine is associated with an increase in the transcriptional activity of SOX2, which was shown using SOX2 mutants. The transcriptional activity observed with wild-type SOX2, was not observed with the SOX2 mutant (T118A), but exhibited by the SOX2 phospho-mimetic mutant (SOX2-T118D). Interestingly, human SOX2-T118A does not appear to alter SOX2 stability. In stark contrast, the mouse mutant Sox2-T118A reduces Sox2 stability. Mouse Sox2 can be phosphorylated on T118 by AKT in mouse ESC [71]. Phosphorylation of this serine not only increases the transcriptional activity of Sox2, it also increases its stability. Remarkably, phosphorylation of T118 blocks the monomethylation of Sox2 on the adjacent K119 by the methyltransferase set7 [72]. Methylation of K119 induces the ubiquitination of Sox2 by the E3 ligase WWP2 and the degradation of Sox2 [72]. Thus, the antagonistic phosphorylation-methylation switch mediated by T118-K119 alters the transcription activity and stability of Sox2, respectively. Importantly, we are not aware of any studies reporting that AKT inhibitors reduce the stability of SOX2 in tumor cells. This warrants attention given the use of AKT inhibitors in many cancer clinical trials. As discussed earlier, AKT has been implicated in a negative feedback top that influences the transcription of the Sox2 gene in ESC [18].

Sox2 can also be ubiquitinated on K123, which is located just beyond the C-terminal portion of the DNA binding domain of Sox2 (the HMG domain). The ubiquitin-conjugating enzyme E2S (Ube2s) mediates K11-linked polyubiquitination of Sox2 at this site [73]. When ubiquitinated on K123, Sox2 is targeted for proteasome-mediated degradation. The comparable lysine of human SOX2 is K121. Although SOX2-T118A does not appear to be less stable than wild-type SOX2 in lung squamous cell carcinoma cells, it is possible in some tumor cells that phosphorylation of T118 (human SOX2) may block ubiquitination of SOX2 at K121, as was discussed above for T118 (mouse Sox2), and its influence on the methylation of K119 and the subsequent degradation of Sox2.

In addition to Sox2 methylation and *O*-GlcNAcylation discussed above, SOX2 can also

be methylated and O-GlcNAcylated on other amino acids. Sox2 T258 has been shown to be modified by *O*-GlcNAcylation in mouse ESC. Thus far, the function of T258 *O*-GlcNAcylation has only been studied in the context of double and triple mutants (T258A/S259A and S248A/T258A/S259A). The double mutant reduced the ability of Sox2 to reprogram somatic cells to iPS cells; whereas the triple mutant did not [68]. Additionally, Sox2 can be methylated on R113 by the arginine methyltransferase CARM1, which increases SOX2 self-association and increases the transcriptional activity of Sox2 [74]. However, further study will be needed to determine whether the increase in Sox2 transcriptional activity is linked to its self-association. Furthermore, it is possible that methylation of Sox2 R113 increases its association with other Sox family members [74, 75]. Additionally, R113, which is also located within HMG domain of Sox2, is located within a second Sox2 nuclear localization sequences (NLS2). Interestingly, the Sox2-R113K mutant, which cannot be methylated, did not alter the subcellular location or the stability of Sox2 [74].

Finally, Sox2 has been shown to be acetylated within its DNA binding domain on K75 *in vivo*. Although the acetyltransferase that acetylates Sox2 *in vivo* has not been determined definitively, p300/CBP is a likely candidate, especially since Sox2 can be acetylated by p300/CBP on K75 *in vitro*[76]. Moreover, Sox2 has been shown to recruit p300 to the Fgf4 enhancer in ESC [77]. Blocking acetylation of Sox2 in ESC, as shown with the Sox2-K75A mutant, led to retention of Sox2 in the nucleus and maintenance of its transcriptional activity; whereas, the acetyl-mimic Sox2-K75Q mutant, associates with the nuclear export machinery, specifically Crm1 [76]. Other studies indicate that Sox2 can be deacetylated by Sirt1, a member of the sirtuin family of NAD-dependent protein deacetylases [78, 79]. Acetylation of Sox2 not only affects its function in ESC, a low level of Sox2 acetylation enhances reprogramming of somatic cells to iPS cells [79].

It is clear from the discussion in this section that post-translational modifications of SOX2 dramatically alter its function, and undoubtedly play key roles in helping to adjust the

levels of SOX2 needed to support cellular function. However, many questions remain to be addressed. In addition to the enzymes responsible for creating the variety of SOX2 post-translation modifications, enzymes that remove some of these modifications of SOX2 have not been identified. Besides phosphatases, likely candidates include deubiquitinating enzymes (DUBs). Interestingly, proteomic analysis of the SOX2-interactome indicates that SOX2 associates with several DUBs that exert important roles in tumor cells, including USP9X, USP7, USP15, USP24, and USP34 [80-82]. In the future, defining the roles of each of the SOX2 modifications and the enzymes involved in tumor cells may provide valuable insights into possible strategies for targeting SOX2 in a large number of cancers. An equally important question that warrants careful attention is the extent to which any given SOX2 molecule is simultaneously modified by more than one post-translational modification. By analogy to the histone code, a "SOX2 code" of post-translational modifications is likely to play a key role in orchestrating the formation of the multitude of SOX2-protein complexes (SOX2-interactome) needed to properly control the level, transcriptional activity, subcellular localization, and stability of SOX2.

1.3 SOX2 is Essential during Mammalian Embryogenesis

The essential role of Sox2 during mammalian embryogenesis was first shown by knockout of both Sox2 alleles and it was determined that Sox2 null embryos reach the blastocyst stage in which the inner cluster of cells in the developing embryo has formed an inner cell mass, but these developing embryos do not survive after implantation [2]. However, the stage at which Sox2 knockout embryos arrest may be influenced by Sox2 from maternal sources, which is present in mature oocytes as well as at fertilization [2]. The role of maternal Sox2 during embryogenesis was the subject of two studies that reached different conclusions. Keramari et al. used Sox2 siRNA to knockdown both maternal and zygotic Sox2 [83]. This study led to the conclusion that Sox2 is needed for the embryo to transition into the blastocyst stage. However, a

subsequent study reached a different conclusion. Initially, Wicklow et al used Zp3-Cre to delete maternal Sox2 in the early embryo [84]. Importantly, mating Sox2fl/fl/Zp3Cre females to wild-type males led to healthy offspring at numbers expected for normal litters. Thus, maternal Sox2 is not required for normal development. Furthermore, Wicklow et al determined that combined knockout of both maternal and zygotic Sox2 did not block formation of the blastocyst [84]. Thus, despite the study by Keramari et al, Sox2 does not appear to be needed for the specification of the inner cell mass and surrounding trophectoderm in the early blastocyst [83]. It is also unclear why the use of Sox2 siRNA generated different results. The two most likely explanations, off-target effects of Sox2 siRNAs and the general toxicity of siRNA, were experimentally addressed by Keramari et al [83]. Thus, caution must be exercised when interpreting results where siRNA is used to study mammalian embryogenesis. Overall, studies of Sox2 null embryos established that Sox2 is essential during embryogenesis and argues that the first lineage decision during embryogenesis, specifically the formation of blastocysts, does not require Sox2.

However, additional studies have shown that Sox2 influences the second cell lineage decision, in particular primitive (extraembryonic) endoderm gene expression, in an Fgf4-dependent manner. The second major cell lineage decision made during embryogenesis is the generation of the epiblast and primitive (extraembryonic) endoderm from the inner cell mass. As normal development proceeds to the blastocyst stage, Sox2 has already become restricted to the inner group of cells. At the blastocyst stage, the inner cell mass exhibits a restricted “salt and pepper” distribution where Sox2, is expressed in some cells and not in others [85]. Once the embryo reaches the epiblast stage, Sox2, Nanog, and Fgf4 are restricted to the epiblast. Wicklow et al reported that Sox2 null blastocysts express ~70% less Fgf4 than their wild-type counterparts [84]. This led them to test whether treatment of cultured Sox2 null embryos with Fgf4 would promote the expression of primitive (extraembryonic) endoderm markers. Treatment of wild-type embryos with Fgf4 increases Sox17 expression, but the increase in Sox17 expression exhibited by Sox2 null embryos treated with Fgf4 was substantially higher. In this connection, it is important

to note that although E3.75 Sox2 null embryos exhibit low Sox17 expression, E4.25 Sox2 null embryos express Sox17 and several other PrE-expressed genes (Gata6, Gata4, and Pdgfra). In fact, Sox2 null embryos at E4.25 express Sox17 and Gata6 at levels similar to wild-type embryos. Thus, Sox2 influences primitive (extraembryonic) endoderm differentiation. However, it is unclear whether Sox2 accelerates the onset of primitive (extraembryonic) endoderm gene expression or, as suggested by Wicklow et al, Sox2 is needed for initial expression of these genes, but not for their later expression [84]. Importantly, these investigators also reported that Sox2 is needed to maintain the epiblast starting at E4.25, again confirming the important role of multiple stages of mammalian embryogenesis.

1.4 Sox2 levels are optimized to support both self-renewal and pluripotency of ESC

In addition to the critical functions of Sox2 in the developing embryo, numerous reports have examined the role of Sox2 in the self-renewal and pluripotency of ESC. Studies in this laboratory, which focused on the transcriptional activity of Sox2, led to the prediction that elevating Sox2 in ESC would induce differentiation [86, 87]. Initially, we determined that increasing the levels of Sox2 in F9 ECC inhibits the activity of the Fgf4 promoter [85]. Subsequently, we determined that elevating Sox2 decreased the activities of Oct4, Nanog, UTF1, and Sox2 promoter/reporter gene constructs that were transiently transfected into F9 ECC, as well as in ESC [87]. Interestingly, although elevating Nanog or Oct4 could inhibit their own promoters, only when Sox2 levels were elevated were the activities of all five promoters inhibited. Importantly, small increases in the levels of Sox2 not only decreased the activities of the five promoters, it also reduced the transcripts for Sox2, Oct4, Nanog, Utf1, and Fgf4 [87].

Currently, relatively little is known about the mechanisms by which elevated levels of Sox2 inhibit Sox2, Oct4, Nanog, Utf1, and Fgf4 promoters. However, using promoter/enhancer-reporter gene constructs, elevated Sox2 has been shown to act on the enhancers that are bound by Sox2 and Oct4 in each of these genes [87]. Additionally, we has shown that elevating Sox2 in i-

OSKM-ESC inhibits the transcription of the endogenous Sox2 gene by activating a negative feedback loop that leads to the phosphorylation and the translocation of FoxO1 (an activator of Sox2 transcription) into the cytoplasm [18]. More recent studies also point to Sox2 autoregulation of Sox2. Allele-specific analysis of Sox2 transcripts in ESC indicates that inactivation of one allele leads to increased transcription of the remaining allele [8]. Thus, ESC are able to carefully regulate Sox2 transcription. It is proposed that this also occurs during embryogenesis.

The recognition that small increases in Sox2 reduce the expression of several genes known to be essential for the self-renewal of ESC led us to the hypothesis that small increases in Sox2 would trigger their differentiation. To test this hypothesis, we engineered ESC for inducible overexpression of Sox2 [17]. When Sox2 levels were elevated ~2-fold, the cells underwent pronounced morphological changes indicative of differentiation. Gene expression analysis demonstrated that pluripotency-associated genes (e.g. Nanog) were downregulated; whereas genes associated with neuroectoderm, mesoderm, and trophectoderm, but not endoderm, were upregulated. Interestingly, massive cell death occurs when Sox2 levels were elevated ~5-fold in ESC. Thus, it is imperative that Sox2 levels are limited in ESC, even when differentiation is initiated.

Reducing Sox2 levels in ESC has also been reported to induce differentiation. Chew et al demonstrated that stable transfection of ESC with a vector, which constitutively expresses Sox2 siRNA, reduced Sox2 levels (~50%) and led to a loss of self-renewal [3]. Unlike Oct4 in ESC, ~50% reduction in Sox2 appears to be sufficient to cause the ESC to differentiate. The differentiated cells exhibited a marked change in morphology and expressed trophectocerm markers Cdx2 and Hand1. Together, the knockdown and overexpression studies argue that Sox2 levels are optimized in ESC: both small decreases and small increases disrupt their self-renewal and induce differentiation.

1.5 SOX2-Protein Interactomes Identify Critical Understudied Proteins, like Musashi2, in Multiple Cell Types

During the past 10 years, considerable effort has been devoted to understanding the mechanisms by which essential transcription factors mediate their effects. More recently, significant strides have been made toward mapping protein-protein interaction landscapes of essential transcription factors in a number of cellular systems. For example, extensive progress has been made in determining the proteome of transcription factors, in particular Sox2, Oct4, and Nanog, necessary for maintaining the self-renewal and pluripotency of ESC [82, 88-93]. The integration of interactomes for Sox2, Oct4, and Nanog, argues that these pluripotency associated transcription factors are part of a highly integrated protein-protein interaction landscape, which includes many other transcription factors, chromatin remodeling machinery, DNA repair machinery, and RNA binding proteins [82, 93-95]. Previous studies in our laboratory identified proteins which interact with SOX2 in multiple cell types, including ESC, ESC undergoing differentiation, DAOY, and U87 brain tumor cells. We hypothesized that proteins that interact with SOX2 in multiple cell types are likely to be necessary for the continued growth and function of these cells.

These unbiased proteomic screens have proven to be a powerful approach for identifying under-studied proteins, such as Banf1 and Musashi2 (MSI2), that significantly influence the fate of ESC [93-97]. Msi2 is part of a family of RNA-binding proteins that includes Musashi1 (Msi1). Msi1 and Msi2 each contain two RNA recognition motifs, and both Msi1 and Msi2 can be expressed as more than one isoform due to alternative splicing [98]. Although the roles of Musashi proteins are far from clear, Msi1 has been shown to block the translation of Numb by binding to the 3’ UTR of Numb mRNA [99]. Interestingly, knockdown of Msi2 in two leukemic cell lines led to an increase in Numb at the protein level, but it remains to be determined whether this is a direct effect of Msi2 [100].

Musashi proteins appear to play important roles during development. Msi1 and Msi2

have been shown to contribute to the development of the nervous system, where they appear to work cooperatively to promote the maintenance of neural stem cells [101]. More recently, Msi2 has been shown to influence the behavior of hematopoietic stem cells (HSC) and their progenitors. Overexpression of Msi2 in HSC in a transgenic mouse model increased the population of HSC progenitors and decreased the population of their downstream derivatives [100]. In contrast, knockdown of Msi2 by shRNA in lymphomyeloid progenitors led to an increase in the proportion of more mature differentiated myeloid cells [102]. The importance of Msi2 during hematopoiesis is also evident from the finding that Msi2 null mice exhibit significant defects in HSC. Interestingly, Msi2 null mice are smaller than their wild-type counterparts, they are produced at lower than expected frequencies, and Msi2 null mice are infertile when mated together [103, 104]. The reasons for each of these defects remain to be determined.

In addition to influencing development, Msi2 has also been linked to tumorigenesis. Importantly, recent studies have shown Msi2 is overexpressed in chronic myelogenous leukemia (CML) and acute myeloid leukemia (AML) [100, 103]. In CML, Msi2 is elevated ~10-fold during the more aggressive blast crisis phase than in the chronic phase, and elevated Msi2 expression in CML has been linked to relapse and poorer prognosis [103]. Consistent with these findings, knockdown of Msi2 by shRNA in blast crisis CML cells led to a more differentiated cell population and diminished proliferation of the diseased cells [103]. Additionally, knockdown of Msi2 in several leukemic cell lines reduced their proliferation and led to increased differentiation and apoptosis [100]. While these studies have advanced our understanding of roles of Msi2 in multiple cell types, much remains to known, particularly in ESC and brain tumor cells.

1.6 SOX2 Expression in Cancer: Amplification, Prognosis, and Survival

SOX2 expression has been reported at both the RNA and protein levels in over 20 cancers. Data available from The Cancer Genome Atlas indicates that SOX2 mRNA is elevated in many cancers, relative to normal tissue. For example, *SOX2* is reported to be elevated in >85%

of glioblastoma multiforme samples compared to normal controls [105]. Interestingly, hypomethylation of the *SOX2* promoter was detected in over 250 glioblastoma specimens compared to normal controls [105]. In tumors such as glioblastoma, ovarian, esophageal, lung, oral, prostate, and sinonasal carcinoma, *SOX2* has been shown to be amplified in some subsets of patient tumors [105-116]. One study found *SOX2* to be amplified in 26% of serous ovarian cancers [107], and the *SOX2* locus (3q26.33) was amplified in ~8% of glioblastoma cases [105], suggesting an increase in copy number is only a piece of a much larger puzzle regarding *SOX2* expression in cancer.

For most cancers, SOX2 expression has also been documented at the protein level by immunohistochemistry [42, 105, 106, 108, 111, 113, 114, 117-127]. For example, in a study of breast cancer patients, SOX2 was strongly detected by immunohistochemistry in the nucleus of breast carcinoma cells compared to weak or no SOX2 staining in normal mammary epithelial tissue [117, 128]. Although SOX2 expression has been reported in many cancers [105-126, 128-131], the percent of SOX2-positive cells within SOX2-positive tumors is not consistently reported. However, in the case of ovarian cancer, both the percent of SOX2-positive tumors and the percent of SOX2-positive cells within these tumors have been reported [123]. Interestingly, the percent of SOX2-positive cells differs between different ovarian tumor subtypes [123].

In several cancers, the levels of SOX2 expression at different stages of the cancer has been examined [122]. In pancreatic ductal adenocarcinomas (PDAC), SOX2 is rarely expressed in pre-malignant pancreatic intraepithelial neoplasia (PanIN), but its expression has been reported to increase to ~60% in poorly differentiated and neurally invasive components [125]. Similarly, studies in glioblastoma, esophageal, breast, and prostate cancers have reported that SOX2 levels increase with increasing tumor grade [106, 126, 128, 132, 133], and the percentage of SOX2-positive cells correlates with Gleason score [134]. In the case of ovarian epithelial carcinoma, SOX2 expression was reported to increase from ~55% of normal ovarian epithelia to over 90% of serous and mucinous cystadenocarcinomas [123]. Interestingly, in the case of gastric cancer,

reports regarding the levels of SOX2 expression during tumor progression are conflicting. In one study, *SOX2* mRNA was reported to be significantly elevated compared to adjacent benign tissues [129]. In contrast, other studies reported lower SOX2 expression in gastric cancer and its metastatic lesions compared to matched, normal gastric mucosa [40, 42, 127, 135]. However, SOX2 expression also appears to vary with different mucosal subgroups in gastric cancer [127, 136]. Thus, for several cancers, there is a need to more carefully determine how SOX2 levels change during tumor progression. Undoubtedly, recognizing how SOX2 expression is altered between normal and tumorous tissues is important for understanding molecular changes necessary for tumor development and progression.

In addition to determining how SOX2 levels change during tumor progression, it is essential to determine whether SOX2 levels influence clinical prognosis for cancer patients. Studies reported thus far indicate that high SOX2 levels correlate with poor prognosis for patients with many different cancers, including breast (triple negative), colorectal, esophageal, ovarian, and lung tumors, as well as nasopharyngeal and sinonasal carcinoma (Table 1.2) [124, 133, 137-141]. Furthermore, a higher incidence of recurrence was significantly correlated with SOX2 amplification in sinonasal carcinomas [116], and rectal cancer patients with elevated SOX2 displayed significantly lower disease-free survival following chemoradiotherapy [138]. Additional studies in esophageal, hepatocellular, oral/tongue, and some lung cancers, and have also found a correlation between elevated SOX2 and decreased survival [124, 133, 141-145]. In addition to survival and recurrence, in the majority of cancers examined thus far, high SOX2 expression has been linked to the infiltrative and metastatic capacity of tumor cells [41, 42, 143, 146-148]. For example, in the case of colorectal cancer, SOX2-expressing tumors have been shown to correlate with increased distant and lymphatic metastases [146]. Similarly, in esophageal squamous cell carcinomas, tumors in which more than 50% of the cells express SOX2 were significantly correlated with increased lymphatic and vascular invasion, poor differentiation, and incomplete surgical resection [148]. However, high SOX2 levels may not be uniformly

Table 1.2: SOX2 Expression in Cancer: Amplification, Prognosis, and Survival

Cancer Type	Amplified/Increased Expression	Decreased Expression	Poor Prognosis/ High Tumor Grade	Good Prognosis/ Low Tumor Grade
Breast (triple negative, basal)	Chen et al., 2008 Rodriguez-Pinilla et al., 2007		Chen et al., 2008 Piva et al., 2014	
Colorectal	Long et al., 2009		Saigusa et al., 2009 Lundberg et al., 2014 Talebi et al., 2015	
Embryonal (testicular germ cell) carcinoma	Biermann et al., 2007			
Esophageal	Gen et al., 2010 Bass et al., 2009 Long et al., 2009		Wang et al., 2009	
Gastric	Tian et al., 2014	Chen et al., 2016 Wang et al., 2015 Otsubo et al., 2008 Tsukamoto et al., 2005 Li X et al., 2004		Zhang et al., 2010 Chen et al., 2016 Wang et al., 2015
Glioblastoma	Alonso et al., 2011 Schmitz et al., 2007 Phi et al., 2008 Annovazzi et al., 2011		Annovazzi et al., 2011 Ma et al., 2008	
Head and neck squamous cell carcinoma	Bourguignon et al., 2012		Lee et al., 2014	Bayo et al., 2015
Hepatocellular carcinoma			Sun et al., 2013	
Lung adenocarcinoma	Sholl, Long et al., 2010		Sholl, Barletta et al., 2010	
Lung cancer, non-small cell			Chou et al., 2013	
Lung cancer, small cell	Güre et al., 2000 Rudin et al., 2012			
Lung cancer, squamous cell	Bass et al., 2009 Hussenet et al., 2010 Yuan et al., 2010 Sholl, Long et al., 2010 Wilbertz et al., 2011			Lu Y et al., 2010 Wilbertz et al., 2011
Lung cancer, neuroendocrine	Sholl, Long et al., 2010			
Melanoma	Laga et al., 2011		Chen et al., 2013	
Nasopharyngeal carcinoma			Wang et al., 2012	
Oral squamous cell carcinoma	Freier et al., 2009		Du et al., 2011	
Ovarian	Belotte et al., 2015 Ye et al., 2011 Zhang et al., 2012		Wang et al., 2014 Zhang et al., 2012	Belotte et al., 2015
Pancreas	Sanada et al., 2006			
Prostate	Sattler et al., 2000 Jia et al., 2011		Kregel et al., 2013 Jia et al., 2011	
Sinonasal carcinoma	Schrock et al., 2013		Schrock et al., 2013	

indicative of poor patient prognosis. For at least four cancers, including gastric cancer and squamous cell lung cancer, low SOX2 expression has been reported to correlate with poor prognosis (Table 1.2) [41, 42, 113, 149]. Moreover, for gastric cancer elevated SOX2 levels are linked to reduced lymph node and distant metastases. The reasons for the contrasting results for SOX2 levels in different cancers remain to be determined.

Disappointingly, for some cancers, in particular head and neck squamous cell carcinomas and in ovarian cancer, there are conflicting reports regarding the levels of SOX2 expression and patient survival [107, 142, 145, 150]. In the case of head and neck squamous cell carcinomas, initial studies by Lee et al showed that SOX2 expression is correlated with poor prognosis and a nearly 5-fold higher risk of recurrence [142], but subsequent studies by Bayo et al determined that $SOX2^{high}$ tumors had a median progression-free survival of 51 months compared to $SOX2^{low}$ tumors (16 months) and that $SOX2^{high}$ tumors had a >110 month improved overall survival compared to $SOX2^{low}$ tumors [150]. Questions also exist in the case of ovarian cancer. Belotte et al reported that tumors with SOX2 amplification had statistically significant improved survival [107]; however, an earlier study from Wang et al reported that high SOX2 levels in both primary and metastatic tumor components statistically correlated with significantly worse survival [145]. It is evident from the discussion in this section that there is a clear need for further investigation into the clinical implications of SOX2 expression, particularly in how SOX2 levels influence tumor progression and patient survival.

1.7 SOX2 and Tumor-Initiating Cells/Cancer Stem Cells

SOX2 is not only expressed in many types of cancer, it has also been implicated in the tumor-initiating populations (proposed cancer stem cell population) of many of these tumors (Table 1.3). Many studies have used putative cancer stem cell markers, such as ALDH1, CD44, ABCG2, and side population via Hoechst efflux assay, to isolate and enrich for cells capable of forming tumors [tumor-initiating cells (TIC), sometimes considered cancer stem cells]

Table 1.3: SOX2 in tumor initiating cells, drug resistance, and tumor cell growth

Cancer Type	Cancer Stem Cells/ Tumorigenicity	Drug Resistance	Alter Growth
Bladder	Hepburn et al., 2012 *	Hepburn et al., 2012	
Breast (triple negative, basal)	Piva et al., 2014	Piva et al., 2014	Leis et al., 2012
	Simões et al., 2011		Chen et al., 2008
Cervical	Liu et al., 2014 ***		
Colorectal	Lundberg et al., 2016		
Esophageal			Gen et al., 2013
			Bass et al., 2009
Gastric	Tian et al., 2012	Tian et al., 2012	Hütz et al., 2013
		Tian et al., 2014	Tian et al., 2014
			Wang et al., 2015
Glioblastoma	Jeon et al., 2011	Hagerstrand et al., 2011	Fang et al., 2011
		Jeon et al., 2011	Cox et al., 2012
			Alonso et al., 2011
			Hagerstrand et al., 2011
			Gangemi et al., 2009
Head and neck squamous cell carcinoma	Lee et al., 2014 ***	Lee et al., 2014	
	Bourguignon et al., 2012		
Lung adenocarcinoma	Nakatsugawa et al., 2011 ***		
Lung cancer, non-small cell	Singh et al., 2012	Chou et al., 2013	Chou et al., 2013
	Xiang et al., 2011	Singh et al., 2012	
Lung cancer, small cell			Rudin et al., 2012
Lung cancer, squamous cell	Hussenet et al., 2010?		Bass et al., 2009
			Hussenet et al., 2010
Medulloblastoma	Vanner et al., 2014 ***		Cox et al., 2012
Melanoma	Santini et al., 2014***		Laga et al., 2010
Osteosarcoma	Basu-Roy et al., 2015		Basu-Roy et al., 2012
Ovarian	Ma et al., 2010 *	Ma et al., 2010	Wang et al., 2014
	Yasuda et al., 2013 *	Yasuda et al., 2013	Yasuda et al., 2013
	Bareiss et al., 2013 ***	Bareiss et al., 2013	
Pancreas	Herreros-Villanueva et al., 2013	Wuebben et al., 2016	Wuebben et al., 2016
Prostate	Rybak et al., 2013	Li et al., 2014	Cox et al., 2012
		Jia et al., 2011	Jia et al., 2011
Skin squamous-cell carcinoma	Boumahdi et al., 2014***		

[112, 130, 151-155]. For example, in the case of an ovarian cancer cell line, the side population exhibited elevated levels of SOX2 mRNA and a higher percentage of TIC when assayed using a limiting cell dilution tumor assay, the gold standard for assessing the frequency of TIC within a tumor [154]. However, for most cancers, the link between SOX2 and their TIC has not been firmly established. For several tumor types, knockdown of SOX2 and/or ectopic expression of SOX2 have been used to implicate SOX2 in the biology of the TIC [137, 156-160]. For example, Lee et al and Santini et al determined that stable knockdown of SOX2 in limiting cell dilution tumor assays dramatically reduced tumor initiation/formation in both head and neck squamous cell carcinomas, and melanomas, respectively [142, 161]. Conversely, others [162, 163] generated lung and ovarian tumor cells that stably overexpress SOX2 and reported an elevated number of TIC when these cells were tested in limiting cell dilution tumor assays. However, as discussed below, there are concerns over the use of tumor cells engineered to stably overexpress SOX2.

Arguably the most conclusive studies have linked SOX2 to TIC by isolating the SOX2-positive cell population rather than experimentally altering the levels of SOX2 within cells. This is important, because SOX2 is expressed heterogeneously throughout the cells of many tumors [137, 150, 164]. Moreover, for some tumors, only a percentage of the cells express SOX2. This is particularly evident for SOX2-positive tumor cell lines [137, 164, 165]. Thus far, the SOX2-positive cells isolated from heterogeneous populations were engineered to either express GFP that was knocked into the endogenous *SOX2* gene (GFP-SOX2 fusion protein) [164, 165] or GFP driven by a transgene under the control of the *SOX2* promoter and enhancer [166]. In these three studies, SOX2-positive cells exhibited a higher frequency of TIC compared to the SOX2-negative cells of the same tumor cells population in a limiting cell dilution tumor assay. Furthermore, studies by Vanner et al not only showed SOX2-positive cells to be important for the tumor initiation, but also used thymidine-analog retention (label-retaining cells) to show that SOX2-positive cells are members of a quiescent, slowly-cycling population within the tumor [164].

Importantly, a recent study in bladder cancer has shown that the quiescent label-retaining cancer stem cell population does not respond to cytotoxic therapy and is capable of repopulating the tumor following drug removal [221]. Such studies not only call attention to a role for SOX2 in the TIC population, but also that this slowly-cycling, SOX2 positive population may be responsible for repopulating the tumor after drug treatment is suspended.

1.8 SOX2 and Drug Resistance

Several recent studies have shown that exogenous elevation of SOX2 can promote resistance to chemotherapeutics currently being used clinically [126, 129, 137, 141, 142, 152, 158, 163, 167-169]. In a report from Bareiss et al, ovarian cancer cell lines that did not express SOX2 and that were sensitive to carboplatin, cisplatin, and paclitaxel became resistant following stable, ectopic expression of SOX2 [163]. Furthermore, in a SOX2-expressing ovarian cancer cell line, SOX2 knockdown using shRNAs provided susceptibility to these drugs, which was reversed upon re-expression of SOX2 ectopically [163]. Similar results were seen in breast cancer cell lines, as stable overexpression of SOX2 in MCF-7 cells promoted resistance to tamoxifen, while stable downregulation of SOX2 using shRNAs enhanced the sensitivity of MCF-7 cells to tamoxifen [137]. While SOX2 may be acting to protect tumor cells through antiapoptotic signaling or quiescent-like phenotypes [126, 152, 158, 164], SOX2 may also be promoting drug resistance through various ATP-binding cassette transporters, including ABCG2, ABCC3, and ABCC6. In particular, ABCG2 has been shown in various tumors to be upregulated in the side population TIC [151, 154] and is, in some tumors, considered to be an additional cancer stem cell marker. Furthermore, studies in lung cancer, as well as head and neck squamous cell carcinoma, have shown that stable downregulation of SOX2 via shRNAs decreases ABCG2, which implicates this transporter in SOX2-related drug resistance [142, 159]. Recognizing and focusing on the role of SOX2 in drug resistance could greatly improve the treatment options for patients with a multitude of cancers, especially those with highly refractory tumors, as the ability to

eradicate the TIC population is likely to be the only way to prevent recurrence.

1.9 SOX2 Levels and Tumor Growth

Many studies have used stable overexpression and/or knockdown of SOX2 in tumor cell lines to better understand the roles of this transcription factor in cancer. Knockdown of SOX2 using either siRNA or shRNA have been used in multiple studies [12, 21, 105, 109, 110, 122, 126, 128, 129, 141, 145, 154, 167, 170, 171]. Importantly, even partial reductions in SOX2 levels have been reported to significantly decrease cell viability, clonal growth, sphere formation, and tumorigenicity in multiple cancer types. Clearly, knockdown studies have established that SOX2 plays important roles in these cancers. However, SOX2 overexpression studies have generated conflicting results. For example, stable overexpression of SOX2 in the gastric tumor cell line N87 was reported to increase growth both *in vitro* and *in vivo* [129]. In contrast, stable overexpression of SOX2 in the gastric cell line MKN28 was reported to decrease growth both *in vitro* and *in vivo*. Currently, the reasons for the conflicting results are unclear. In both studies, SOX2 was substantially overexpressed in gastric tumor cell lines that endogenously express relatively little SOX2. Part of the explanation may be due to differences in the cell lines used. However, as discussed below other factors related to experimental design may also be a contributing factor.

Conflicting reports from SOX2 overexpression studies have also been reported for breast, prostate and pancreatic cancers. Stable overexpression in MCF7 (breast), DU145 (prostate), and Patu8988t (pancreatic ductal adenocarcinoma) cells have been reported to increase growth *in vitro* [126, 128, 155]. However, overexpression of SOX2 from a doxycycline-inducible transgene demonstrated that overexpression of SOX2, where one can monitor the early consequences of elevating SOX2, does not increase cell proliferation. Inducibly elevating SOX2 (~5 to 7-fold) in glioblastoma (U87, U118), medulloblastoma (DAOY), breast carcinoma (MCF7), and prostate carcinoma (DU145) cell lines led to growth inhibition in each case [172]. Thus, the immediate effect of elevating SOX2 in these tumor cell lines is growth inhibition. Clearly, it is important to

reexamine the effects of SOX2 in other cancers using inducible overexpression of SOX2, in particular where stable overexpression has been reported to increase tumor cell growth.

1.10 Background on Brain Tumors and PDAC

While SOX2 has been shown to be important in over two dozen tumors, the studies described in this dissertation have focused on brain tumors and PDAC. Brain tumors, specifically glioblastomas (GBM) and medulloblastomas (MB) are highly debilitating diseases that are very difficult to treat. Despite improved therapeutic regimes, patients diagnosed with GBM, the most common primary adult brain tumor, have a median survival of 10-14 months [173]. Treatment of patients with MB, the most common pediatric brain cancer, poses an additional problem. Current therapies for MB cause dramatic impairment of cognitive function and predispose patients to future treatment-associated neoplasms [174]. Hence, there is a pressing need to identify novel proteins and signaling pathways that can serve as new targets for improved treatment of GBM and MB. As briefly outlined above, elevated levels of the transcription factor SOX2, which plays critical roles in the development of the nervous system, have been shown to correlate with poor clinical outcome for brain tumor patients [175]. The critical role of SOX2 in brain tumors is supported by the finding that knockdown of SOX2 by RNA interference reduces the *in vitro* and *in vivo* growth of GBM cells [170]. Moreover, SOX2 is expressed in MB cells [175] and, recently, it has been determined that the knockdown of SOX2 in DAOY MB cells reduces their proliferation (Cox and Rizzino, unpublished results).

Pancreatic ductal adenocarcinoma (PDAC) also expresses SOX2 and it is one of the most lethal malignancies. For several decades, the 5-year survival of patients with PDAC has remained at or below 7% with a median survival of less than one year for patients with locally advanced or metastatic disease [176]. In the United States, PDAC is the fourth most common cause of cancer deaths (~40,000/year), and it is predicted to become the second leading cause of cancer deaths in the United States by 2030 [177]. The high mortality of PDAC patients is due in large measure to

late diagnosis of the disease when tumor resection is not feasible and resistance of PDAC to chemotherapy designed to target aberrantly regulated signaling networks. Consequently, there is a desperate need to identify new therapeutic targets that influence drug-resistance. Thus far, a wide range of genes and signaling pathways have been shown to be aberrantly activated in PDAC. The most common mutation is in the coding region of the KRAS gene, which generates constitutively activated KRAS in >90% of all PDAC [178]. These tumors are highly dependent on upregulated AKT and RAF/MEK/ERK signaling, which are downstream of KRAS [179-182]. This led to a large number of PDAC clinical trials testing AKT inhibitors (AKTi), e.g. MK-2206, and at least five MEK inhibitors (MEKi), e.g. trametinib [183]. Disappointingly, these drugs have not produced significant responses in PDAC clinical trials, which has led to the general belief that PDAC is largely resistant to AKTi and MEKi.

As outlined earlier, SOX2 expression increases significantly during tumor progression rising from ~20% in pre-malignant PanIN3 lesions to nearly 60% of poorly differentiated PDAC [125]. Subsequent studies reported that SOX2 is expressed in many different human PDAC cell lines, with high expression in some PDAC cell lines, but little or no expression in others [155]. Importantly, this study demonstrated that SOX2 expression is closely associated with putative cancer stem cell markers previously reported to be expressed by PDAC tumor-initiating cells [155]. This study also demonstrated that knocking down SOX2 in PDAC cell lines reduced their growth *in vitro*; whereas, stable expression of SOX2 in a PDAC cell line, which does not endogenously express detectable levels of SOX2, increased their anchorage-independent growth [155]. Although this study provided support *in vitro* for a critical role of SOX2 in the stemness of PDAC, the effects of SOX2 on the tumorigenicity of PDAC tumor cells were not examined. Thus, our understanding of SOX2 in PDAC is incomplete, and many important questions remain unanswered.

1.11 Dissertation Objectives

The studies presented in this dissertation extend our knowledge of SOX2 and its function as a master regulator in multiple cell types, and further elaborate on the finding that proteins like Msi2, which associate with SOX2 in multiple cell types, are also required for continued cell growth and function.

In this dissertation, two major bodies of work are presented. The first major objective examined the role of the SOX2-associated protein Msi2 in both ESC and brain tumor cells. As described in Chapter 3, the results of these studies demonstrate that ESC require Msi2 to maintain self-renewal and pluripotency. This work regarding Msi2 in ESC was carried forward into studies of MSI2 in brain tumors, as MSI2 was also identified as a SOX2-associated protein in DAOY MB cells and GBM cells. Work outlined in Chapter 3 of this dissertation demonstrates that MSI2 is required to support the growth and survival of DAOY cells and two GBM tumor cell lines, U87 and U118.

The second major objective of this dissertation focused on SOX2 and its roles in PDAC. As described earlier, one study reported that elevation of SOX2 promoted PDAC cell growth; however previous studies in our laboratory found that inducible elevation of SOX2 in multiple other cancer cell lines in fact reduces tumor cell growth. To resolve this issue, multiple PDAC cell lines were engineered for either inducible overexpression of SOX2 or inducible knockdown of SOX2. Initially, these inducible PDAC cell lines were used to examine the growth *in vitro* and tumorigenicity following changes in SOX2 levels. Through these studies, we determined that SOX2 functions as a biphasic molecular rheostat in PDAC, as described in Chapter 4. Furthermore, we determined that the sensitivity of PDAC cells to small molecule inhibitors currently being tested in clinical trials can be altered by changes in SOX2 levels. Specifically, Chapter 5 details how inducible elevation of SOX2 protects PDAC cells from the growth inhibitory effects of MEK and AKT inhibitors; conversely, growth inhibition due to these inhibitors is enhanced by the inducible knockdown of SOX2.

Altogether, the work presented in this dissertation strengthens our knowledge of SOX2 and SOX2-associated proteins in multiple cell types, and provides useful platforms for the continued study these highly deadly malignancies.

CHAPTER 2: Materials and Methods

2.1 Cell Culture Conditions

D3 mouse ESC (obtained from T. Doetschman, 1985) and their genetically modified derivatives (see below) were cultured on tissue culture plastic pretreated with 0.1% gelatin (G2500, Sigma-Aldrich, St. Louis, MO), in Dulbecco's Modified Eagle's Medium (DMEM, 12100046, Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS, SH30910.03, Hyclone, Logan, UT), 5 ng/mL leukemia inhibitory factor (LIF, ESG1107, Millipore, Billerica, MA) and 100 μM β-mercaptoethanol (M7522, Sigma-Aldrich), as described previously [17]. DAOY (HTB-186, ATCC, Manassas, VA), U87 (HTB-14, ATCC), U118 (HTB-15, ATCC), HEK293T (CRL-11268, ATCC), T3M4 (obtained from T. Hollingsworth, UNMC), BxPC3 (obtained from T. Hollingsworth, UNMC), HPAF-II (obtained from T. Hollingsworth, UNMC), L3.6 (obtained from D. Billadeau, Mayo Clinic, Rochester, MN) cells and their genetically modified derivatives (see below) were cultured in DMEM supplemented with 10% FBS, as described previously [81, 184]. The identity of each of the PDAC cell lines was verified by genetic analysis, which was performed by the Molecular Diagnostics Laboratory at UNMC. To differentiate ESC, the cells were treated with 5 μM retinoic acid (RA, 207341000, Acros Organics, Morris Plains NJ) for 4 days. Doxycycline (Dox; Sigma-Aldrich) was suspended in phosphate buffered saline at the indicated concentrations. In all engineered lines, Flag-tagged SOX2 or SOX2 shRNA was induced by supplementing the culture medium with Dox for the times and at the concentrations indicated. Kinase inhibitors were obtained from companies listed in Table 2.1. The EC_{50} for each drug used for each cell line was determined by measurements of cell growth over a 4 day period (Table 2.1). Photomicrographs taken of cultured cells were obtained with a Canon Rebel XTi camera at 10X and/or 25X, where indicated.

Table 2.1: Inhibitors used and the EC50 for each drug

Inhibitor Name	Target	Company, Location	T3M4	HPAF-II	BxPC3	L3.6
GSK-1120212, trametinib	MEK	Selleck, Houston, TX	40 nM	20 nM	4 nM	4 nM
AZD-6244, selumetinib	MEK	Selleck, Houston, TX	10 μM	ND	ND	ND
RDEA-119, refametinib	MEK	Active BioChem, Hong Kong	1 μM	ND	ND	ND
GDC-0623	MEK	Active BioChem, Hong Kong	20 nM	ND	ND	ND
GDC-0973, cobimetinib	MEK	Active BioChem, Hong Kong	200 nM	ND	ND	ND
MK-2206	AKT	Selleck, Houston, TX	2 μM	5 μM	200 nM	80 nM

*ND, not determined

2.2 Lentiviral Vector Engineering

2.2.1 pLVX-PGK-TetOn-Advanced

The CMV promoter responsible for driving the expression of the neomycin resistance gene in pLVX-Tet-On-Advanced (Clontech, Mountain View, CA, #632162) was replaced with a PGK promoter. For this purpose, the PGK promoter from pLVX-Tight-Puro (Clontech, #632162) was amplified by PCR, with primers that introduce ClaI and BamH1 restriction sites, upstream and downstream of the promoter, respectively. The sequence of the upstream primer for amplifying the PGK promoter was: CAGTTT**ATCGAT**TACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGTCTGG (ClaI site in bold font), and the sequence for the downstream primer was: CATGGT**GGATCC**CGAAAGGCCCGGAGATGAGGAAGAGGAGAACAGCGCGG (BamHI site in bold font). The PGK PCR product was digested with ClaI and BamH1 restriction enzymes, and the fragment was ligated into pLVX-Tet-On-Advanced, previously treated with ClaI and BamHI to remove the CMV promoter. The resulting modified plasmid is thenceforth referred to as pLVX-PGK-TetOn-Advanced.

2.2.2 pLVX-Tight-Puro-Flag-Msi2 isoform 1 and pLVX-Tight-Puro-Flag-Msi2 isoform 2

To produce viruses for inducible expression of Flag-tagged Msi2 isoform 1 and Flag-tagged Msi2 isoform 2, the coding sequence for Msi2 isoform1 and the coding sequence for Msi2-isoform2 were first cloned from RNA isolated from D3 ESC and cDNA synthesized, as described below. The Msi2 coding sequences were amplified by PCR. For this purpose, primers were designed to add a BamHI restriction enzyme site, Kozak sequence, and a Flag peptide to the N-terminus of each Msi2 coding sequence, as well as 3 stop codons and an EcoRI restriction enzyme site to the C-terminus. The upstream PCR primer used was: ATCGC**GGATCC**GCCACCATGGACTACAAGGACGACGATGACAAGATGGAGGCAAAT

GGGAGCCCA (BamHI restriction enzyme site is shown in bold font, followed by the Kozak sequence, then the Flag peptide sequence, which is underlined). The downstream primer used was: TACCG**GAATTC***TTATTATCA*GTGGTATCCATTTGTAAAGGCCGTTGC (EcoRI restriction enzyme site in bold font and stop codons in italicized font). The Flag-Msi2 products were digested with BamH1 and EcoRI restriction enzymes, and the fragments were ligated into pBluescript II KS+ (Stratagene), previously digested with BamH1 and EcoRI in the multiple cloning site. Due to the design of the primers, both Msi2 isoforms 1 and 2 were amplified via PCR, and ligated into pBluescript II KS+. Sequencing of this library was performed by UNMC High-Throughput DNA Sequencing Core to identify Flag-Msi2 isoform 1 or Flag-Msi2 isoform 2 clones. Once identified, Flag-Msi2 isoform 1 or Flag-Msi2 isoform 2 fragments were isolated from the pBluescript II KS+ plasmids by digestion with BamH1 and EcoRI, and ligated into pLVX-Tight-Puro previously digested with the same enzymes. These plasmids were then called pLVX-Tight-Puro-Flag-Msi2 isoform 1 and pLVX-Tight-Puro-Flag-Msi2 isoform 2.

2.3 Transient Transfection

Transient transfection of HEK293T (293T) cells was performed using the calcium phosphate precipitation method, as described previously [185]. Specifically, plasmid DNA for transfection and 150 µl of 2.5M $CaCl_2$ are added to 1.35 mL H_2O. This mixture is then added to 1.5 mL of HEPES buffered saline, pH 7.1 (HBS, 1.64% (w/v) NaCl, 1.19% (w/v) HEPES (H4034, Sigma-Aldrich), dropwise with bubbling to mix. The 3 mL DNA precipitate solution is then added dropwise over the surface of a 150 mm dish of cells plated 24 hours earlier.

2.4 Lentiviral Production and Transduction

To produce lentivirus, HEK293T cells were transiently transfected (as detailed above) with 45 µg transfer vector (pLVX-PGK-TetOn-Advanced, pLVX-Tight-Puro-Luc (632162, Clontech), pLVX-Tight-Puro-Flag-Msi2 isoform 1, or pLVX-Tight-Puro-Flag-Msi2 isoform 2,

RMM4534-NM_054043, RMM4534-NM_011443, pLVX-tetO-(fs)SOX2, RHS4696-201902991, or RHS4696-201899634, as specified below), 30 µg packaging vector psPAX2 (12260, Addgene, Cambridge, MA), and 15 µg envelope vector pMD2.G (12259, Addgene) using the calcium phosphate precipitation method described above. Twenty-four hours later, the cells were refed with 15 mL DMEM, 10% FBS, 10 mM HEPES, and 10 mM sodium butyrate (303410, Sigma-Aldrich). After 24 hours, the media was collected, filtered (0.45 µm) and stored at 4°C overnight and transfected cells were refed with 15 mL of same media as day before. The following day, the second day media was again collected and filtered. The filtered media was combined, and viral particles were collected by ultra-speed centrifuge with an SW-28 rotor at 26,000 rpm at 4°C for 2 hours. Lentiviruses were resuspended in 550 µL pre-chilled DMEM with 10% FBS and 10 mM HEPES at 4°C overnight, aliquoted into 50 µL, and stored at −80°C. To infect cells, one ~50 µl aliquot of lentivirus was diluted in ~7 ml of the appropriate cell medium for a given cell type supplemented with 10 mM HEPES and 6 µg/ml polybrene (H9268, Sigma-Aldrich).

2.5 Engineering ESC that Inducibly Express Msi2 isoform 1 or isoform 2

Lentiviral particles were produced from pLVX-Tight-Puro-Flag-Msi2 isoform 1, pLVX-Tight-Puro-Flag-Msi2 isoform 2, or pLVX-Tight-Puro-Luc (632162, Clontech) in 293T cells as described above. To produce ESC that express either Flag-tagged Msi2 isoform 1 or Flag-tagged Msi2 isoform 2, D3 ESC were first infected with the lentiviral vector pLVX-PGK-TetOn-Advanced to express the reverse tet transactivator. Virally transduced cells, referred to as D3-rtTA ESC, were isolated after treatment with 300 µg/mL G418 sulfate (A1720, Sigma-Aldrich) for 6 days. To produce i-Msi2.1-D3 ESC and i-Msi2.2-D3 ESC, D3-rtTA ESC were infected with the virus pLVX-Tight-Puro-Flag-Msi2 isoform 1 or pLVX-Tight-Puro-Flag-Msi2 isoform 2. Twenty-four hours after infection, D3-rtTA ESC were cultured in the presence of 5 µg/ml puromycin (P8833, Sigma-Aldrich) for 48 hours to select for infected cells. Flag-tagged Msi2 isoform 1 and Flag-tagged Msi2 isoform 2 were individually expressed from inducible transgenes

stably integrated into i-Msi2.1-D3 ESC and i-Msi2.2-D3 ESC, respectively. The recombinant proteins were induced by the addition of 1 μg/ml Dox.

2.6 Knockdown of Msi2 in D3 mouse ESC

Msi2 was knocked down in D3 ESC that had been seeded at a density of 105 cells per well in a 6-well plate. A previously validated non-specific shRNA (Scrambled) was used as a negative control in knockdown experiments [186]. One day later, cells were infected with lentiviruses that express either the scrambled shRNA or shRNAs targeting Msi2 (Msi2 shRNA #1, #4, or #5). Lentiviral vectors for expression of shRNA sequences that target mouse Msi2 were obtained from Open Biosystems (RMM4534-NM_054043, Huntsville, AL). Msi2 shRNA lentiviral constructs #1, #4, and #5 used in this study correspond to TRCN0000071973, TRCN0000071976, and TRCN0000071977, respectively. Sequences of these shRNAs are provided in Table 2.2. Production of these shRNA lentiviral particles in HEK293T cells is described above.

2.7 Knockdown of MSI2 in Glioblastoma and Medulloblastoma Tumor Cells

To knockdown MSI2 in DAOY, U87, and U118 tumor cells, cells were infected with lentivirus made using vectors containing shRNAs that target MSI2 (RMM4534-NM_011443, Open Biosystems), as well as the scrambled control shRNA. Sequences for these shRNAs are provided in Table 2.2. Notably, shRNA #4 and shRNA #5 target both mouse and human forms of MSI2 with the same targeting sequence. Production of these shRNA lentiviral particles in HEK293T cells is described above.

2.8 Engineering PDAC cells for SOX2 overexpression and knockdown

PDAC cell lines were engineered for Dox-inducible SOX2 expression as described previously [172]. T3M4 cells were first transduced with the reverse tet transactivator lentiviral

Table 2.2: shRNA constructs used for Msi2 knockdown

Msi2 shRNA	Open Biosystems Accession Number	Mature sense sequence	Mature Anti-Sense
Msi2 shRNA #1	TRCN0000071973	CCCAGCTTAATATCTAGTTAA	
Msi2 shRNA #4	TRCN0000071976	GCTACAGTGCTCAACCGAATT	AATTCGGTTGAGCACTGTAGC
Msi2 shRNA #5	TRCN0000071977	CCACCATGAGTTAGATTCCAA	TTGGAATCTAACTCATGGTGG

vector (pLVX-PGK-TetOn-Advanced) and a G418-resistant clone selected for in medium containing 300 µg/mL G418 sulfate for 9-12 days. Secondly, these cells were infected with lentivirus containing the pLVX-tetO-(fs)SOX2 lentiviral vector (Figure 2.1) and were selected for in medium containing 5 µg/ml puromycin for 48 hours, resulting in i-SOX2-T3M4 cells. The cell lines i-SOX2-BxPC3 and i-SOX2-HPAF-II were engineered by infection with both viruses simultaneously prior to selection.

For inducible knockdown of SOX2, T3M4 and L3.6 cell lines were engineered for Dox-inducible expression of an shRNA using a TRIPZ lentiviral vector obtained from Open Biosystems (now GE Dharmacon, Lafayette, CO). This vector, RHS4696-201902991, has a mature antisense sequence of ACATGCTGATCATGTCCCG, which targets the ORF of both human and mouse SOX2. In T3M4 cells a second, independent lentiviral vector was used. This second vector, RHS4696-201899634, has a mature antisense sequence of TTCTTGTCGGCATCGCGGT. The TRIPZ vector results in puromycin resistance and constitutive expression of a reverse tet transactivator as well as Dox-inducible expression of the shRNA and red fluorescent protein (RFP). I-KdSOX2-T3M4 and i-KdSOX2-L3.6 cell lines were isolated after puromycin selection, as described above. The i-KdSOX2-T3M4 cell population was further enriched by flow cytometry for cells with higher RFP expression following an 18 hour induction with 1 µg/mL Dox.

2.9 Colony Forming Assays

ESC were plated at clonal densities and maintained in ES-cell media for up to 6 days, at which point the number of colonies that exhibited only the morphology of ESC, a mix of ESC and differentiated cells, or only cells with a differentiated morphology were counted in 10 random 40X fields by an observer unaware of sample designation. For PDAC cloning efficiency assays, cells were plated at clonal densities (80 cells per cm^2) and maintained in serum containing media (as indicated above). After 8-12 days, the number of colonies (8 or more cells per colony)

Figure 2.1

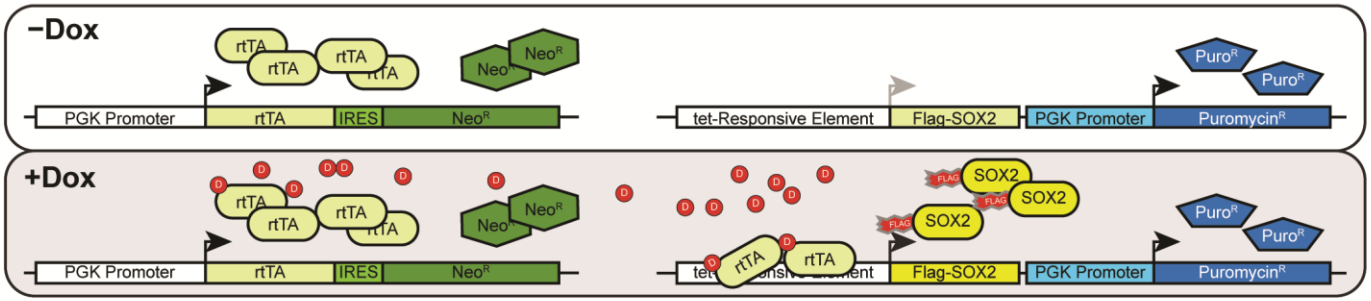

Figure 2.1: Inducible SOX2 overexpression system
Illustration of the two lentiviral vectors used to engineer PDAC cells for Dox-inducible, Flag-tagged SOX2 expression: one vector constitutively expresses reverse-tet transactivator (rtTA) and the second expresses Flag-SOX2, but only when its promoter is bound by rtTA complexed with Dox.

was determined in 15-20 random 40X fields by an observer unaware of sample designation. For replating efficiency assays, cells were grown at subconfluent densities for 6 days with or without treatment in normal media (as indicated above), at which point the cells were trypsinized and replated at clonal densities in normal media. After an additional 7 or 11 days, the number of colonies that exhibited 8 or more cells per colony was determined in 8-15 random 40X fields by an observer unaware of sample designation.

2.10 Soft Agar Growth Assays

Soft agar growth assays were performed in serum-free, stem cell medium, as described previously [81]. The number of spheres that exhibited 8 or more cells per sphere was determined, and spheres larger than 50 μm in diameter were scored as large.

2.11 Alkaline Phosphatase Staining

ESC were stained for alkaline phosphatase using an AP-staining kit, according to the manufacturer's protocol (00-0009, Stemgent, San Diego, CA).

2.12 MTT Assays

MTT assays of triplicate samples were used to assess relative cell growth, as described previously [187, 188]. MTT assays were used to assess proliferation using mitochondrial dehydrogenase activity as a measure of cell number, was described previously [187, 188]. Cells were cultured in 12-well plates and, when appropriate depending on experimental design, cells were refed 750 μL culture medium + 250 μL MTT reagent (5 mg/mL MTT (M2128, Sigma-Aldrich) in PBS), and placed in a cell culture incubator for 2 hours. 750 μL of MTT solvent (20% (w/v) SDS in 1:1, water:DMF (D4551, Sigma-Aldrich), pH adjusted to 4.7 with 2.5% (v/v): 80% (v/v) acetic acid (A38C-212, Fisher) and 2.5% (v/v) 1N HCl [A144-500]) was added to each well, and the 12-well plate was returned to the cell culture incubator overnight. The next day, the

absorbance of each well was read at 570 nm using a spectrophotometer. Each sample was treated and measured in triplicate. Controls were averaged and set to 1. Error bars represent standard deviation between the three replicates and p values were determined by student's t-test (*$p<0.05$, **$p<0.01$, and ***$p<0.005$).

2.13 Protein Isolation and Micro BCA Quantification

Nuclear and cytoplasmic protein extracts were prepared using NE-PER kits (Thermo-Scientific, Rockford, IL) according to the manufacturer's protocol to isolate proteins for western blot analysis. Whole cell protein extracts were prepared using RIPA extraction buffer [150 mM NaCl (S9888, Sigma-Aldrich), 50 mM Tris-HCl (pH 7.4, 10812846001, Sigma-Aldrich), 1% IGEPAL (I8896, Sigma-Aldrich), 0.25% sodium deoxycholate (D6750, Sigma-Aldrich), and 1 mM EDTA (E6758, Sigma-Aldrich)] supplemented with the following protease and phosphatase inhibitors: Na_3VO_4 (5 mM, S6508, Sigma-Aldrich), PMSF (1 mM, P7626, Sigma-Aldrich), NaF (10 mM, S7920, Sigma-Aldrich), EDTA (10 mM, E6758, Sigma-Aldrich), leupeptin (1 µg/mL, L2884, Sigma-Aldrich), pepstatin A (2 µM, P5318, Sigma-Aldrich), chymostatin (1 µg/mL, C7268, Sigma-Aldrich), aprotinin (2.5 KIU/mL, A1153, Sigma-Aldrich), soybean trypsin inhibitor (20 µg/mL, T9003, Sigma-Aldrich), and NaPPi (30 mM, P8135, Sigma-Aldrich). Protein extract concentrations were quantified using the Micro BCA™ Protein Assay Kit (23235, Pierce), according to the manufacturer's guidelines.

2.14 Western Blot Analysis

Western blot analysis was performed as described previously [82, 97]. Primary mouse antibodies used were: α-Msi2 (ab76148, Abcam, Cambridge, MA, 1:2,000), α-Sox2 (#2683-1, Epitomics, Burlingame, CA, 1:5,000), and α-Oct 4 (sc8628, Santa Cruz, Santa Cruz, CA, 1:500). Primary human antibodies used were: α-MSI2 (ab83236, Abcam, Cambridge, MA), α-NUMB (ab4147, Abcam), α-SOX2 (ab92494, Abcam, Cambridge, MA, 1:1,000), and α-phospho-p44/42

MAPK antibody (#9106, Cell Signaling Technology, Danvers, MA, 1:1,000). HDAC1 (ab-7028, Abcam, 1:5,000) and α-GAPDH (G8795, Sigma-Aldrich) were used as loading controls. MSI2, SOX2, and HDAC1 primary antibodies were detected with an anti-rabbit-IgG-AP secondary antibody (A3687, Sigma-Aldrich, 1:10,000). Oct4 and Numb primary antibodies were detected with an anti-goat-IgG-AP secondary antibody (A4187, Sigma-Aldrich, 1:10,000). Phospho-ERK1/2 and GAPDH primary antibodies were detected with an anti-mouse-IgG-AP secondary antibody (A4312, Sigma-Aldrich, 1:10,000).

2.15 RNA Isolation and Expression Analysis

D3 ESC were infected with lentiviruses that express either scrambled shRNA or Msi2 shRNA #1 for 24 hours followed by selection with puromycin for 24 hours. Cells were subcultured 48 hours after puromycin selection at a low density (4,500 cells per cm^2). Cells were maintained for 4 days in normal ES cell media followed by RNA isolation and cDNA synthesis as described previously [17]. Expression of ES-related genes and lineage-specific genes in D3 ESC treated with Msi2 shRNA #1 and scrambled shRNA were analyzed by SYBR Green (SuperArrayBioscience Corporation, Frederick, MD) quantitative Real-Time polymerase chain reaction (RT-qPCR) [17, 82]. Similarly, i-Msi2.1-D3 and i-Msi2.2-D3 were treated with or without 1 μg/ml Dox for 48 hours prior to plating at 4,500 cells/cm^2. Cells were then treated with and without Dox in the presence of 5 μM RA for 48 hours, followed by the removal of LIF for 48 hours to further differentiate the cells prior to the RNA extraction and cDNA synthesis. Primers for Msi2 isoforms, Msi1, Numb, Sox7, and Tfec are provided in Table 2.3. Primers for ES cell- and lineage-specific transcripts have been described previously [17, 82, 87]. Gene expression of MSI2 in DAOY, U87, or U118 cells treated with targeting or scrambled shRNA were analyzed by SYBR Green RT-qPCR , as described above. Primers used for the PCR step in the analysis of MSI2 RNA were h-MSI2-F (AAGTATTAGGTCAGCCCCAC) and h-MSI2-R

Table 2.3: Primers for RT-qPCR

Primer Set	Primer name	RT-qPCR Primer Sequence (5'⇒3')	Amplicon size
Msi2 F1/R1	Msi2-F1	CAACGACTCCCAGCACGAC	237 bp
	Msi2-R1	GTCAATCGTCTTGGAATCTAACTC	
Msi2 F2/R2	Msi2-iso1-F	GTGCTCAACCGAATTTTGGC	106 bp
	Msi2-iso-R	GTAGAGATCGGCGACAGGTCC	
Msi2 F3/R3	Msi2-iso2-F1	TCGAGGATCAGGCTCCAACCC	175 bp
	Msi2-iso2-R1	CAAAGGTCCAGCTATGCCGTG	
Msi1	m-Msi1-F1	AGTTGGCAGACCACGCAGGAAG	131 bp
	m-Msi1-R1	TGGTCCATGAAAGTGACGAAGC	
Numb F1/R1	Numb-F1	ACTACGGCAAAGCTTCAGGAG	185 bp
	Numb-R1	ATCTCTTTACGGCATCTTCAC	
Numb F2/R2	Numb-F2	TCGGCGATGGATCTGTCATTG	185 bp
	Numb-R2	TGACACGGAATGATCCTTCTC	

(TTCTCAAAAGTGACAAAGCC). Primers for GAPDH control expression have been described previously [97].

2.16 Microarray Analysis

RNA isolated from D3 ESC infected with lentiviruses that express either scrambled shRNA or Msi2 shRNA #1, as described above, was used for genome-wide RNA expression analysis. Sense-strand cDNA was generated from 300 ng total RNA using the Ambion WT Expression kit for Affymetrix Whole Transcript Expression Arrays (Affymetrix, Santa Clara, CA). This cDNA was fragmented and labeled using the GeneChip® WT Terminal Labeling and Hybridization (Affymetrix) followed by hybridization for 16 hours at 45°C to an Affymetrix GeneChip Mouse Gene 1.0 ST (Affymetrix). Gene chips were washed and stained with the Affymetrix Fluidics Station 450 (Affymetrix) prior to being scanned by the Affymetrix GeneChip Scanner 3000 7G (Affymetrix). Data was analyzed with Affymetrix Expression Console software (Affymetrix) using Robust Multichip Analysis (RMA) for normalization. Data collection and analysis were performed by the University of Nebraska Medical Center DNA Microarray Core Facility. Microarray data was sorted for genes that increase or decrease 2-fold or more when Msi2 shRNA #1 was compared to the control scrambled shRNA. Of these genes, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to classify them into broad-based cellular and molecular functions [189, 190]. All microarray data is available on Gene Expression Omnibus (Accession No. GSE33882, GEO, http://www.ncbi.nlm.nih.gov/geo/).

2.17 Cell Cycle Analysis

I-SOX2-T3M4 cells were seeded at subconfluent densities in the presence or absence of Dox (300 ng/ml) and MEKi were added the following day. After 3 days treatment with each MEKi in the presence or absence of Dox, cells were prepared for cell cycle analysis by the Telford Method, as described previously [191]. Floating cells were included in the cell cycle

analysis. Flow analyses were performed by the UNMC Cell Analysis core facility.

2.18 Determination of Tumorigenicity

Female NCr-nu/nu mice (7 weeks of age) were obtained from Charles River (Wilmington, MA). All animal procedures were approved by the UNMC Institutional Animal Care and Use Committee. Where indicated, $2.5x10^5$ i-SOX2-T3M4 cells or $2.5x10^5$ i-KdSOX2-T3M4 cells were trypsinized, washed, resuspended in 50 µl of sterile PBS and injected subcutaneously into the flank. Tumor growth was monitored daily. After palpable tumors had formed, tumor-bearing mice were randomized to size-matched control and experimental (Dox-treated) groups. Dox-treatment for elevation or knockdown of SOX2 was accomplished by addition of Dox (2 mg/ml) to drinking water that contained 5% sucrose. Untreated mice were provided with 5% sucrose drinking water as a control. Tumor volumes were calculated based on measurements with a digital caliper at the times indicated. At the completion of the tumor growth study, mice were euthanized and tumors excised for weight measurements and immunohistochemical analysis.

2.19 Immunohistochemical Analysis

Formalin-fixed tumor sections were paraffin-embedded and stained for hematoxylin and eosin (H&E), SMA, and Ki-67 by the University of Nebraska Medical Center Tissue Sciences Facility. H&E, SMA, and Ki-67 stained photomicrographs were captured using either an iScan Coreo Au Scanner with iScan Coreo 3.4.0 software (Ventana Medical Systems, Inc., Tucson, AZ), or a Nikon Digital Sight DS-Fi1 camera with NIS Elements 4.0. software (Nikon, Inc., Melville, NY). Quantification of the stromal component of tumors was assessed by overlaying a grid on top of photomicrographs of SMA stained tumor tissues using Adobe Photoshop 2015.0.1. An area of 1 mm2 was divided into 864 squares, which were examined for positive staining, indicating stroma. Two independent tumors from each treatment condition (e.g. i-SOX2-T3M4

cells or i-KdSOX2-L3.6 cells, without Dox and with Dox) were assessed, and the percentages of SMA positive squares were averaged and standard deviation calculated for each condition. Proliferation in tumors was assessed by staining for Ki-67, and counting the number of positively stained cells out of at least 500 cells, only in the carcinoma or stromal components of each tumor growth condition. As with the stromal quantification, two independent tumors from each treatment condition were scored, and Ki-67 incidence in the stroma or carcinoma was averaged and standard deviation calculated. The student's T-test (2-tailed) was used to determine statistical significance ($p < 0.05$) using Microsoft Excel for Mac (15.20).

2.20 Immunofluorescence Assays

Immunofluorescence assays used to assess SOX2 expression in PDAC cell lines. Cells were cultured in 8-well chamber slides (354118, Corning Falcon Fisher) with 300 µL culture medium per well and placed in a cell culture incubator for 3 days. When appropriate, cells were washed with 1X PBS prior to fixation with 200 µl 100% methanol (chilled to -20°C) at room temperature for 5 minutes. Cells were then permeabilized with 0.3% TritonX-100 and blocked in animal serum (5% in 0.3% TritonX-100) corresponding to the animal the secondary antibody was raised in, with washes in 1X PBS between each step. Permeabilized cells were incubated with primary antibody overnight at 4°C (ab92494, AbCam, 1:100 in 1% BSA and 0.3% TritonX-100), washed with 1X PBS, and incubated in secondary antibody for 2 hours at room temperature (anti-rabbit-IgG-FITC, F0382, Sigma, 1:500 in 1% BSA and 0.3% TritonX-100). Cells were also incubated with Hoeschst dye (33258, Fisher) at 2 µg/ml in 1X PBS for 3 min.

CHAPTER 3: Musashi2 is required for the self-renewal and pluripotency of embryonic stem cells

The studies described in this chapter, which were conducted by Erin Wuebben, are described in two studies [80, 96].

3.1 Introduction

Sox2 is required during mammalian embryogenesis and it plays critical roles in the self-renewal and pluripotency of ESC. SOX2 expression also influences the growth of brain tumor cells. Previous studies in our laboratory used unbiased proteomic screens to identify nuclear proteins that associate with Sox2 in ESC undergoing early stages of differentiation, as well as in MB and GBM brain tumor cells [80, 82]. Remarkably, Sox2 associates with >60 proteins during the early stages of differentiation and >280 in DAOY MB cells that participate in a diverse range of cellular processes; one of these Sox2-associated proteins identified in both networks is the RNA binding protein Musashi 2 (Msi2), which also associates with SOX2 in GBM. As discussed in the Introduction of this dissertation, Msi2 has been shown to play important roles in development. Specifically, Msi2 has been shown to contribute to the development of the nervous system, along with Msi1, and they have been shown to work together to promote the maintenance of neural stem cells [101]. Msi2 has also been shown to be elevated in several leukemias such as CML and AML and its elevated expression has been linked with poorer prognosis in these cancers [100, 103, 192]. Given the contribution of Msi2 to tumorigenicity, its roles during development, and its association with Sox2 in ESC and brain tumor cells we predicted that MSI2 plays essential roles in cells where SOX2 and MSI2 associate with one another. Specifically we predicted that proteins that associate with SOX2 in multiple SOX2-dependednt cell types must also be essential in those cells.

3.2 ESC express and require Msi2

To study the role of Msi2 in mouse ESC, we initially examined whether ESC express more than one isoform of Msi2. Studies in other systems [98, 193] identified two isoforms of Msi2: isoform 1 (full length) consisting of 346 amino acids and isoform 2 consisting of 328 amino acids (Figure 3.1A). Using primers specific to Msi2 isoform 1 and isoform 2, we determined that D3 ESC express both isoforms of Msi2 at the RNA level (Figure 3.1B). In

Figure 3.1

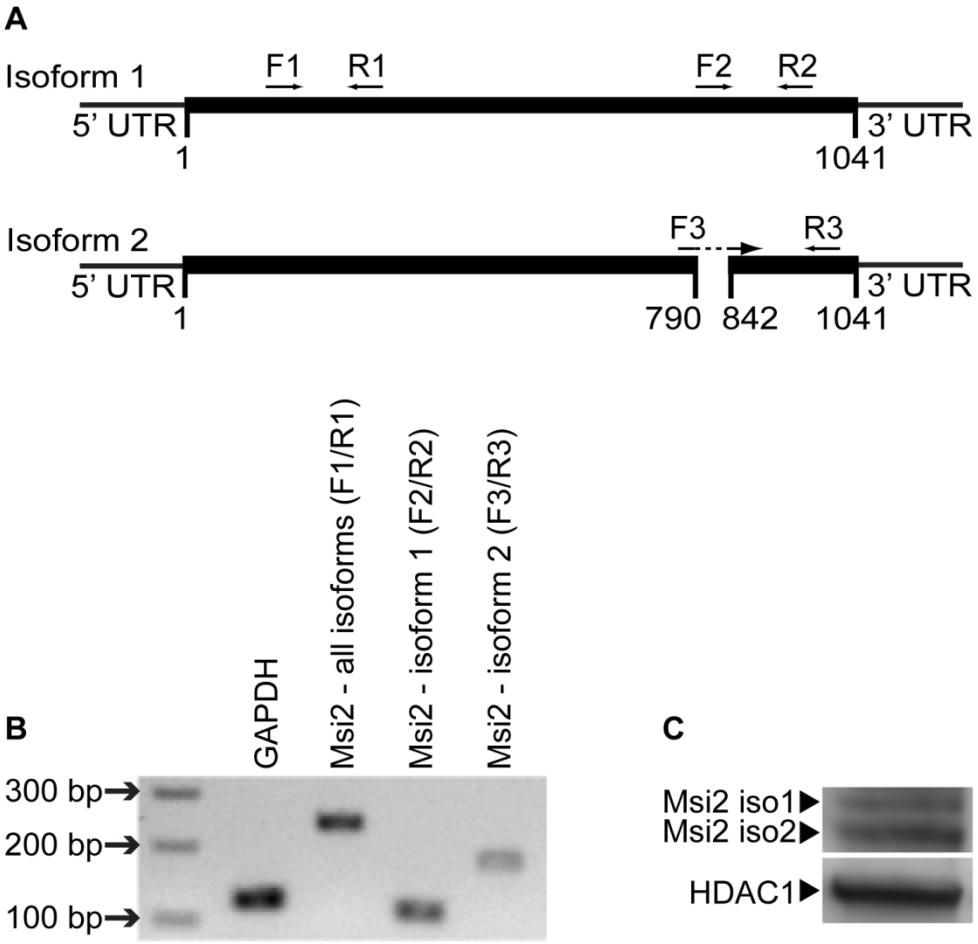

Figure 3.1: Expression of Msi2 mRNA and protein in ESC.
(A) Primers for RT-qPCR were designed to be specific for both isoforms of Msi2, isoform 1 or isoform 2. Primer set F1/R1 amplifies sequences present in both isoform 1 and 2. Primer sets F2/R2 and F3/R3 are specific to isoform 1 and isoform 2, respectively. **(B)** DNA fragments generated via RT-qPCR using RNA isolated from D3 ESC and the primer sets indicated were separated over a 2% agarose gel. **(C)** Western blot analysis of Msi2 isoforms expressed in nuclear extracts isolated from D3 ESC. Isoform 2 runs slightly faster than isoform 1 (35.7 kDa and 36.9 kDa, respectively). HDAC1 protein was used as a loading control.

addition, we determined by western blot analysis that ESC express both isoforms of Msi2 at the protein level (Figure 3.1C).

To investigate the role of Msi2 in ESC, we examined how ESC would be affected by the knockdown of Msi2. For this purpose, we used lentiviral vectors that express shRNA directed at both isoforms (shRNA #1 and shRNA #5) and only isoform 1 (shRNA #4) (Figure 3.2A). Specifically targeting isoform 2 will be more difficult, as isoform 2 shares the entirety of its sequence with isoform 1, differing only through an omission of a small region. Thus, to target isoform 2, an shRNA sequence will need to bridge the region that is spliced out from isoform 1. As a control, we also infected ESC with a lentivirus that expresses a scrambled shRNA sequence, which we previously determined does not influence the behavior of D3 ESC [97]. Initially, we examined the knockdown of Msi2 by comparing the protein levels of Msi2 in ESC infected with the lentiviral vector that expresses the scrambled shRNA control with the levels of Msi2 in cells infected with lentiviral vectors that express shRNA #1, #4 or #5. We determined that infection of ESC with the lentiviral vector that expresses shRNA #1 caused a significant reduction in both isoforms of Msi2, and infection of ESC with the lentiviral vector that expresses #4 caused a significant reduction in Msi2 isoform 1, but not isoform 2 (Figure 3.2B). In contrast, the lentiviral vector that expresses shRNA #5 appears to induce only a modest reduction in isoform 1 (~30%) and small reduction in isoform 2 (~10%).

Examination of the infected cells by light microscopy indicated that ESC infected with the control lentiviral vector, which expresses the scrambled shRNA sequence, did not induce morphological changes in the cells. In contrast, lentiviral vectors #1 and #4 caused extensive morphological differentiation (Figure 3.2C). As expected from the effects on Msi2 protein expression (Figure 3.2B), lentiviral vector #5 caused significantly less differentiation. To further characterize the observed change in morphology, we stained cells infected with the various shRNA constructs with alkaline phosphatase (AP), a cell-surface marker associated with pluripotency. Reduced AP-staining intensity in ESC infected with Msi2 shRNA constructs #1 and

Figure 3.2

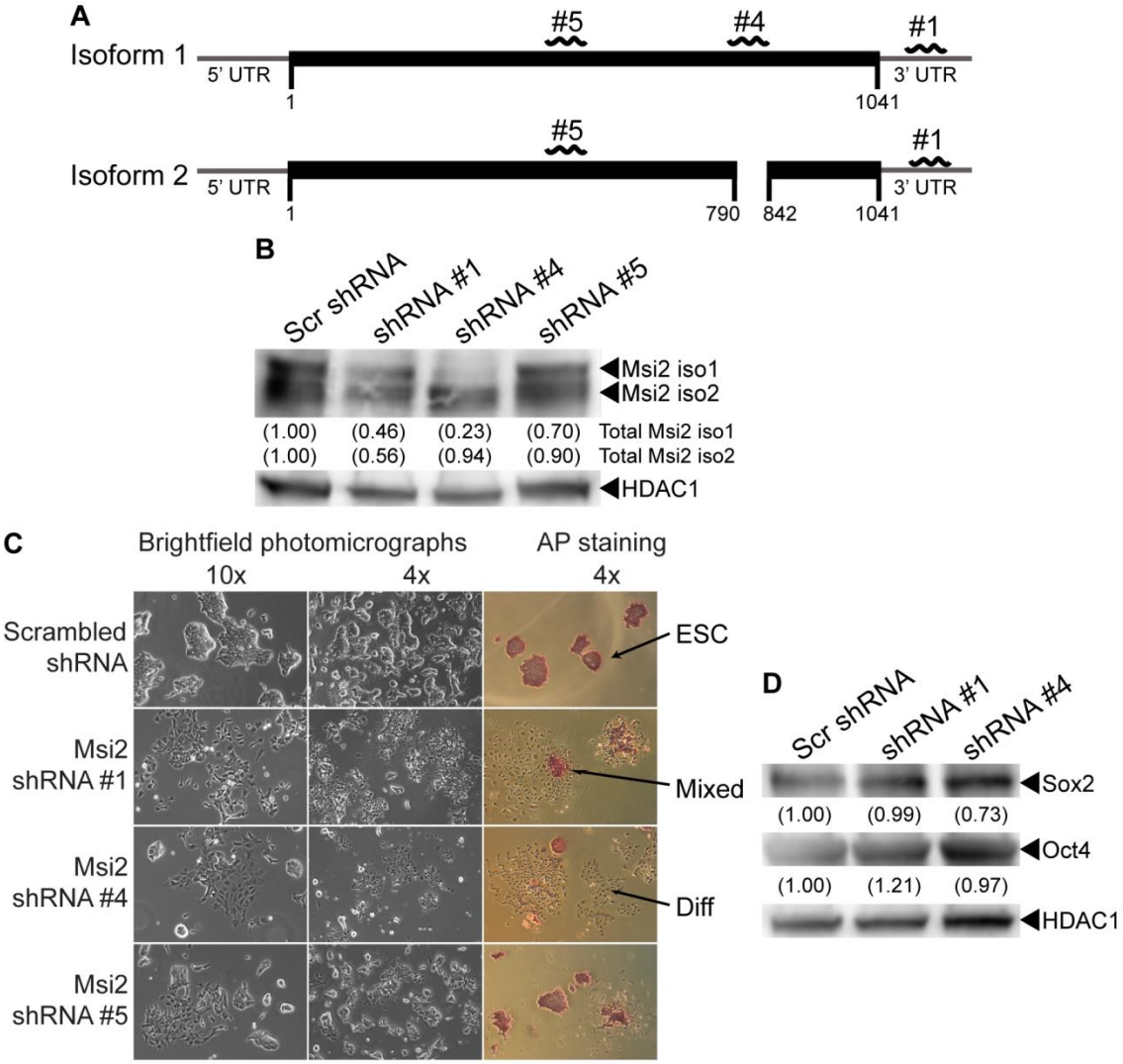

Figure 3.2: Knockdown of Msi2 results in the differentiation of ESC.
(A) Regions of Msi2 mRNA targeted by shRNA #1, shRNA #4, and shRNA #5. **(B)** The D3 ESC were infected with lentiviruses that express scrambled (Scr) shRNA, shRNA #1, shRNA #4, or shRNA #5 sequences. Two days after infection, the cells were subjected to puromycin selection for 24 hours. After selection, the cells were subcultured and grown for an additional 24 hours before nuclear extracts were harvested for western blot analysis. HDAC1 was used as the loading control for quantification. **(C)** Bright field photomicrographs of cells subcultured at 5,000 cells per cm2 were taken 7 days post-infection with each shRNA (left columns). Cells were stained with alkaline phosphatase (right column) 10 days post-infection after being subcultured at 200 cells per cm2. Arrows point to colonies that exhibit a morphology characteristic of ESC (ESC), a morphology consisting of ESC and differentiated cells (Mixed), or a morphology characteristic of differentiated cells (Diff). **(D)** The D3 ESC were infected with lentiviruses that express scrambled (Scr) shRNA, shRNA #1, shRNA #4, or shRNA #5 sequences. Two days after infection, the cells were subjected to puromycin selection for 24 hours. After selection, the cells were subcultured and grown for an additional 24 hours before nuclear extracts were harvested for western blot analysis of pluripotency markers. HDAC1 was used as the loading control for quantification.

#4 corroborated our observation that knockdown of Msi2 impairs the ability of ESC to self-renew (Figure 3.2C). To determine whether the differentiation observed was due to a loss in essential pluripotency factors, we conducted western blot analysis to examine the levels of Sox2 and Oct4 following the knockdown of Msi2. Interestingly, the levels of Sox2 and Oct4 were not significantly altered after the knockdown of Msi2 (Figure 3.2D).

To quantitate the extent of differentiation induced by the knockdown of Msi2, virally infected cells were plated at clonal density. Six days later, colonies were scored by an observer unaware of sample designation as ES cell colonies, differentiated colonies or mixed colonies consisting of both ESC and differentiated cells. Typical of unmodified ESC, a high percentage of D3 ESC expressing the scrambled shRNA formed ES cell colonies (~80%) and relatively few mixed and differentiated colonies (Figure 3.3). In contrast, D3 ESC infected with lentiviral vectors expressing either shRNA #1 or shRNA #4 formed far fewer ES cell colonies (<15% in the case of shRNA #4) and a large percentage of mixed and differentiated cell colonies. In addition, shRNA #5 only modestly reduced the number of ES cell colonies (by ~40%) and increased the number of mixed and differentiated colonies. Importantly, our studies show that the extent of differentiation and the loss of self-renewal capacity correlate with the level to which Msi2 levels were decreased. Equally importantly, our studies indicate that the knockdown of isoform 1 induces the differentiation of ESC. Studies discussed below suggest that the self-renewal of ESC may also require expression of Msi2 isoform 2.

3.3 Msi2 knockdown alters gene expression

To further characterize the differentiation of ESC following the knockdown of Msi2, we initially examined the changes in gene expression by microarray analysis. Changes in global RNA expression were determined by comparing the RNA expression profiles of ESC that express either the scrambled shRNA or shRNA #1 by microarray. Of 29,000 transcripts examined, we determined that cells infected with Msi2 shRNA #1 exhibited increased expression of 40 genes

Figure 3.3

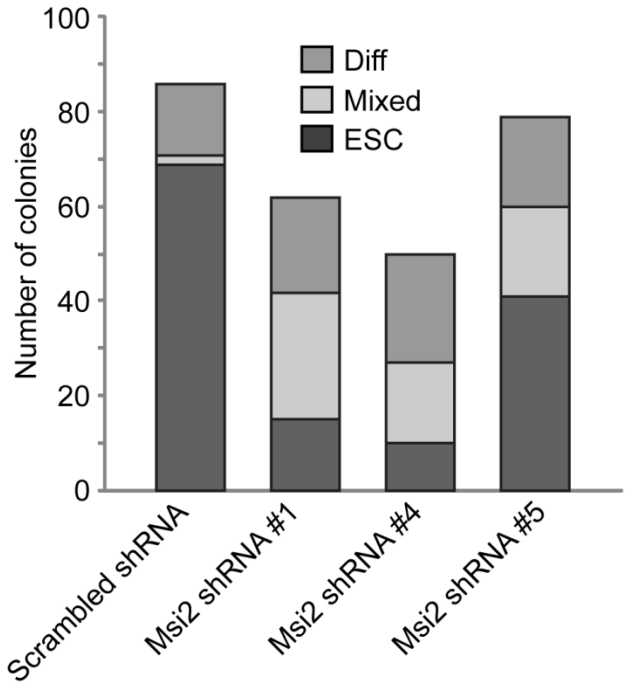

Figure 3.3: Knockdown of Msi2 decreases the cloning efficiency of ESC.
D3 ESC infected with lentiviral constructs that express Scr, Msi2#1, Msi2#4, or Msi2#5 shRNA sequences were subcultured at 200 cells per cm2. Six days later an observer unaware of sample designation scored colonies as ESC, Mixed or differentiated (Diff).

≥2.5-fold (Table 3.1) and decreased expression of 29 genes ≥ 2.5-fold (Table 3.2) compared to cells infected with scrambled shRNA. Broadening our parameters to genes whose expression increased or decreased by ≥ 2-fold (Figure 3.4A, Tables 3.3 and 3.4) expanded these subsets with an additional 49 and 46 genes, respectively. Gene ontology analysis (Figure 3.4B) indicated that a large percentage of the genes that exhibited >2-fold increased gene expression play roles in cell signaling (e.g. Tspan2, Irs2, Sfrp2, Ctgf) and cellular structure (e.g. Acta2, Actc1, Cald1, Myl9); whereas, genes that exhibited >2-fold decrease in expression participate in development (e.g. Amot, Pdgfr, Lama1) and metabolism (e.g. Nostrin, Nrg1, Inhbb). Given the morphological changes that accompany differentiation, in particular the increase in cytoplasmic to nuclear ratio, increases in genes associated with cellular structure is not surprising. Similarly, the large change in the expression of genes associated with metabolic processes when Msi2 is knocked down parallels the large changes in metabolic gene expression when somatic cells are reprogrammed to induced pluripotent stem cells [194]. A complete list of the differentially expressed genes and their associated ontologies are provided in a heatmap [96].

To validate our microarray analysis, 23 genes were examined more closely using RT-qPCR, which is a more quantitative approach to examine transcript expression. For this analysis, we examined a number of genes that increased or decreased according to our microarray data, and a number of genes critical for maintaining pluripotency in ESC. In concordance with our microarray data, analysis by RT-qPCR indicates that a number of genes associated with mesoderm development (Tpm1, Tagln, Brachyury, MyoD1), ectoderm development (Pax6, Nestin) and trophectoderm development (Cdx2, Esx1) were elevated when Msi2 was knocked down (Figure 3.5). Additionally, a number of markers associated with endoderm development (Gata6, Sox17, Gata4, Sox7) were reduced as determined by both our microarray and RT-qPCR (Figure 3.5). We also examined the expression of Numb and Msi1 mRNA by RT-qPCR, both of which exhibited a small increase when Msi2 was knocked down. The small increase in Msi1 mRNA may reflect a compensatory mechanism that coordinates the expression of Msi1 and Msi2.

Table 3.1: Genes more highly expressed (>2.5-fold) in Msi2-shRNA #1 treated ESC

Gene Description	Gene Symbol	Gene Accession	Msi/Scr
actin, alpha 2, smooth muscle, aorta	Acta2	NM_007392	17.87408
fibronectin type III domain containing 3C1	Fndc3c1	NM_001007580	10.42333
cytochrome P450, family 2, subfamily b,	Cyp2b23	NM_001081148	9.956793
guanylate binding protein 2	Gbp2	NM_010260	9.717386
myosin, light polypeptide 9, regulatory	Myl9	NM_172118	9.12257
transgelin	Tagln	NM_011526	8.762207
protogenin homolog (Gallus gallus)	Prtg	NM_175485	5.768678
calponin 1	Cnn1	NM_009922	5.197678
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	Sema3e	NM_011348	5.10546
fermitin family homolog 1 (Drosophila)	Fermt1	NM_198029	4.589138
G protein-coupled receptor 177	Gpr177	NM_026582	4.570582
lymphoid enhancer binding factor 1	Lef1	NM_010703	4.296537
lysophosphatidic acid receptor 4	Lpar4	NM_175271	4.244466
tropomyosin 1, alpha	Tpm1	NM_024427	4.203669
collectin sub-family member 12	Colec12	NM_130449	4.113838
von Willebrand factor A domain containing 5A	Vwa5a	NM_172767	4.005255
cadherin 2	Cdh2	NM_007664	3.834051
inhibitor of DNA binding 2	Id2	NM_010496	3.652261
peripheral myelin protein 22	Pmp22	NM_008885	3.641219
latrophilin 2	Lphn2	NM_001081298	3.493878
sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	Sema6a	NM_018744	3.465361
latrophilin 2	Lphn2	NM_001081298	3.268155
forkhead box I3	Foxi3	NM_001101464	3.266992
brachyury	T	NM_009309	3.258786
calponin 2	Cnn2	NM_007725	3.245409
Meis homeobox 2	Meis2	NM_001136072	3.224858
latrophilin 2	Lphn2	NM_001081298	3.131864
actin, alpha, cardiac muscle 1	Actc1	NM_009608	3.119528
latrophilin 2	Lphn2	NM_001081298	3.00825
latrophilin 2	Lphn2	NM_001081298	2.942925
thrombospondin 1	Thbs1	NM_011580	2.808685
Ras-related GTP binding D	Rragd	NM_027491	2.760515
matrix metallopeptidase 25	Mmp25	NM_001033339	2.712283
secreted frizzled-related protein 2	Sfrp2	NM_009144	2.702162
tetraspanin 7	Tspan7	NM_019634	2.684918
paired box gene 6	Pax6	NM_013627	2.625564
double homeobox B-like	Duxbl	NM_183389	2.590408
double homeobox B-like	Duxbl	NM_183389	2.590408
transmembrane protein 47	Tmem47	NM_175771	2.540623
carbonic anhydrase 4	Car4	NM_007607	2.501597

Table 3.2: Genes more highly expressed (>2.5-fold) in control scr-shRNA treated ESC

Gene Description	Gene Symbol	Gene Accession	Msi/Scr
cubilin (intrinsic factor-cobalamin receptor)	Cubn	NM_001081084	0.173962
nuclear RNA export factor 7	Nxf7	NM_130888	0.185415
nitric oxide synthase trafficker	Nostrin	NM_181547	0.207473
apolipoprotein B mRNA editing enzyme, catalytic polypeptide 2	Apobec2	NM_009694	0.270858
serglycin	Srgn	NM_011157	0.274735
carcinoembryonic antigen-related cell adhesion molecule 1	Ceacam1	NM_001039185	0.28154
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	Kdelr3	NM_134090	0.287021
transcription factor EC	Tcfec	NM_031198	0.291922
klotho beta	Klb	NM_031180	0.292923
solute carrier family 44, member 3	Slc44a3	NM_145394	0.296724
laminin, alpha 1	Lama1	NM_008480	0.309917
forkhead box Q1	Foxq1	NM_008239	0.311016
GLI pathogenesis-related 1 (glioma)	Glipr1	NM_028608	0.320636
HNF1 homeobox B	Hnf1b	NM_009330	0.326332
dickkopf homolog 1 (Xenopus laevis)	Dkk1	NM_010051	0.330495
disabled homolog 2 (Drosophila)	Dab2	NM_023118	0.332967
SRY-box containing gene 7	Sox7	NM_011446	0.345285
fatty acid binding protein 3, muscle and heart	Fabp3	NM_010174	0.346694
inhibin beta-B	Inhbb	NM_008381	0.351159
fatty acid binding protein 3, muscle and heart	Fabp3	NM_010174	0.351295
N-acetylneuraminate pyruvate lyase	Npl	NM_028749	0.35156
stearoyl-Coenzyme A desaturase 1	Scd1	NM_009127	0.36289
ankyrin repeat domain 33B	Ankrd33b	NM_027496	0.36428
legumain	Lgmn	NM_011175	0.372774
epithelial membrane protein 1	Emp1	NM_010128	0.373816
GATA binding protein 4	Gata4	NM_008092	0.385494
pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 1	Pcbd1	NM_025273	0.390757
serine peptidase inhibitor, Kazal type 3	Spink3	NM_009258	0.39558
DNA-damage regulated autophagy modulator 1	Dram1	NM_027878	0.396483

Table 3.3: Genes more highly expressed (>1.9-fold, <2.5-fold) in Msi2-shRNA #1 treated ESC

Gene Description	Gene Symbol	Gene Accession	Msi/Scr
tumor necrosis factor receptor superfamily, member 19	Tnfrsf19	NM_013869	2.4794965
NADH dehydrogenase subunit 6	ND6	ENSMUST00000082419	2.4780579
neurofilament, light polypeptide	Nefl	NM_010910	2.4721711
double homeobox B-like	Duxbl	NM_183389	2.4504792
connective tissue growth factor	Ctgf	NM_010217	2.4156442
chromodomain helicase DNA binding protein 7	Chd7	NM_001081417	2.4118173
plexin domain containing 2	Plxdc2	NM_026162	2.3861052
CDC42 effector protein (Rho GTPase binding) 5	Cdc42ep5	NM_021454	2.3790399
solute carrier family 40 (iron-regulated transporter), member 1	Slc40a1	NM_016917	2.3782097
interleukin 17 receptor D	Il17rd	NM_134437	2.3733912
adenosine kinase	Adk	NM_134079	2.3319867
insulin receptor substrate 2	Irs2	NM_001081212	2.328649
peroxidasin homolog (Drosophila)	Pxdn	NM_181395	2.3241663
ephrin B2	Efnb2	NM_010111	2.2976924
paternally expressed 3	Peg3	NM_008817	2.2495816
sarcoglycan, epsilon	Sgce	NM_001130190	2.2413525
chromodomain helicase DNA binding protein 7	Chd7	NM_001081417	2.2303059
fibroblast growth factor 5	Fgf5	NM_010203	2.2019409
PDZ and LIM domain 7	Pdlim7	NM_001114088	2.1878111
neuropilin (NRP) and tolloid (TLL)-like 2	Neto2	NM_001081324	2.1832726
sulfatase 1	Sulf1	NM_172294	2.1632546
FAT tumor suppressor homolog 1 (Drosophila)	Fat1	NM_001081286	2.1574785
zyxin	Zyx	NM_011777	2.1554205
epithelial splicing regulatory protein 1	Esrp1	NM_194055	2.1506269
Kruppel-like factor 7 (ubiquitous)	Klf7	NM_033563	2.120471
stratifin	Sfn	NM_018754	2.1089497
LIM domain containing preferred translocation partner in lipoma	Lpp	NM_178665	2.1061581
proviral integration site 2	Pim2	NM_138606	2.1007711
caldesmon 1	Cald1	NM_145575	2.0994667
annexin A3	Anxa3	NM_013470	2.0893397
MAP7 domain containing 3	Mtap7d3	NM_177293	2.0848384
chromodomain helicase DNA binding protein 7	Chd7	NM_001081417	2.0741013
proteasome (prosome, macropain) 28 subunit, beta	Psme2	NM_011190	2.0708415
proteasome (prosome, macropain) 28 subunit, beta	Psme2	NM_011190	2.0704021
PERP, TP53 apoptosis effector	Perp	NM_022032	2.0646108
tetraspanin 2	Tspan2	NM_027533	2.058896
unc-5 homolog B (C. elegans)	Unc5b	NM_029770	2.0573781
immunoglobulin superfamily, DCC subclass, member 4	Igdcc4	NM_020043	2.0352323
Wilms tumor 1 homolog	Wt1	NM_144783	2.0347951
GLI-Kruppel family member GLI3	Gli3	NM_008130	2.0280686
necdin	Ndn	NM_010882	2.0245506
sterol O-acyltransferase 1	Soat1	NM_009230	2.0183945
chromodomain helicase DNA binding protein 7	Chd7	NM_001081417	2.0178176
chromodomain helicase DNA binding protein 7	Chd7	NM_001081417	2.0156997
potassium voltage-gated channel, subfamily G, member 3	Kcng3	NM_153512	2.013439
filamin C, gamma	Flnc	NM_001081185	2.0116606
mesoderm specific transcript	Mest	NM_008590	2.0042277
solute carrier family 43, member 1	Slc43a1	NM_001081349	2.0024233
discoidin domain receptor family, member 1	Ddr1	NM_007584	2.0010318

Table 3.4: Genes more highly expressed (>1.9-fold, <2.5-fold) in control scr-shRNA treated ESC

Gene Description	Gene Symbol	Gene Accession	Msi/Scr
nephrosis 1 homolog, nephrin (human)	Nphs1	NM_019459	0.4005573
platelet derived growth factor receptor, alpha polypeptide	Pdgfra	NM_011058	0.4022971
runt related transcription factor 1	Runx1	NM_001111023	0.4053648
bone morphogenetic protein 6	Bmp6	NM_007556	0.4063212
SRY-box containing gene 17	Sox17	NM_011441	0.4065884
solute carrier organic anion transporter family, member 2a1	Slco2a1	NM_033314	0.4091104
yippee-like 2 (Drosophila)	Ypel2	NM_001005341	0.4110711
trichorhinophalangeal syndrome I (human)	Trps1	NM_032000	0.4187246
vesicle-associated membrane protein 8	Vamp8	NM_016794	0.4188315
lymphocyte antigen 6 complex, locus A	Ly6a	NM_010738	0.4193457
jumonji C domain-containing histone demethylase 1 homolog D (S. cerevisiae)	Jhdm1d	NM_001033430	0.4228574
RIKEN cDNA 1600029D21 gene	1600029D21Rik	NM_029639	0.4256209
neuregulin 1	Nrg1	NM_178591	0.4261635
angiomotin	Amot	NM_153319	0.4272724
solute carrier family 13 (sodium-dependent citrate transporter), member 5	Slc13a5	NM_001004148	0.429784
immunoglobin superfamily, member 21	Igsf21	NM_198610	0.4325762
glutathione peroxidase 2	Gpx2	NM_030677	0.4354825
family with sequence similarity 129, member A	Fam129a	NM_022018	0.4367573
muscle and microspikes RAS	Mras	NM_008624	0.4368234
growth arrest specific 6	Gas6	NM_019521	0.4370603
fructose bisphosphatase 2	Fbp2	NM_007994	0.4427842
START domain containing 8	Stard8	NM_199018	0.4429606
muscle and microspikes RAS	Mras	NM_008624	0.443599
RIKEN cDNA 2610305D13 gene	2610305D13Rik	NM_145078	0.4564995
leukemia inhibitory factor receptor	Lifr	NM_013584	0.4567801
GATA binding protein 6	Gata6	NM_010258	0.4620735
myoferlin	Myof	NM_001099634	0.4621322
DnaJ (Hsp40) homolog, subfamily C, member 6	Dnajc6	NM_198412	0.4622408
cathepsin L	Ctsl	NM_009984	0.4625777
nidogen 1	Nid1	NM_010917	0.4639019
ephrin A3	Efna3	NM_010108	0.4672158
collagen, type IV, alpha 1	Col4a1	NM_009931	0.4675327
phospholipid transfer protein	Pltp	NM_011125	0.4685716
polymerase (DNA directed), alpha 1	Pola1	NM_008892	0.4735306
histone cluster 1, H2bc	Hist1h2bc	NM_023422	0.4762064
procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	P4ha1	NM_011030	0.4791994
amino-terminal enhancer of split	Aes	NM_010347	0.4793438
junction adhesion molecule 2	Jam2	NM_023844	0.4798303
glycerophosphodiester phosphodiesterase domain containing 5	Gdpd5	NM_201352	0.483551
apolipoprotein C-I	Apoc1	NM_007469	0.4835545
predicted gene 9904	Gm9904	DQ973493	0.4861883
secreted phosphoprotein 1	Spp1	NM_009263	0.4888129
dipeptidylpeptidase 4	Dpp4	NM_010074	0.4916286
transcobalamin 2	Tcn2	NM_015749	0.4925643
cordon-bleu	Cobl	NM_172496	0.4961215
T-box 3	Tbx3	NM_011535	0.4993247

Figure 3.4

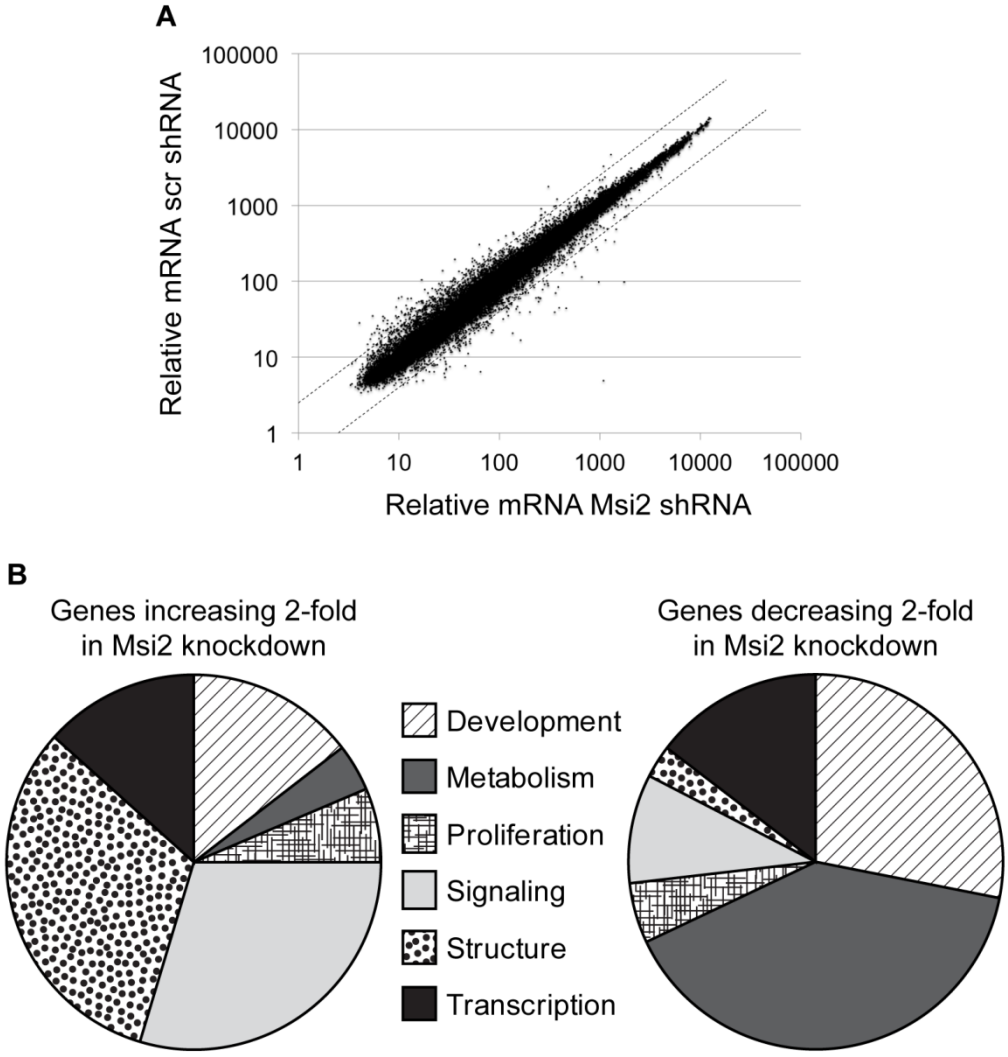

Figure 3.4: Knockdown of Msi2 results in global changes in gene expression.
(A) Seven days post-infection, RNA was isolated from the D3 ESC infected with lentiviruses that express either the scrambled (Scr) shRNA sequence or the Msi2 shRNA#1 sequence. RNA was used for microarray analysis as described in the Materials and Methods. The dotted lines represent a 2-fold increase (bottom) or 2-fold decrease (top) in RNA expression relative to RNA isolated from the D3 ESC infected with the scrambled shRNA control sequence. **(B)** Gene ontology analysis of genes whose expression changes when Msi2 is knocked down. Gene ontology analysis was conducted for the genes whose expression increased (left side) or decreased (right side) two-fold or more in the Msi2 knockdown cell population. For this analysis, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Figure 3.5

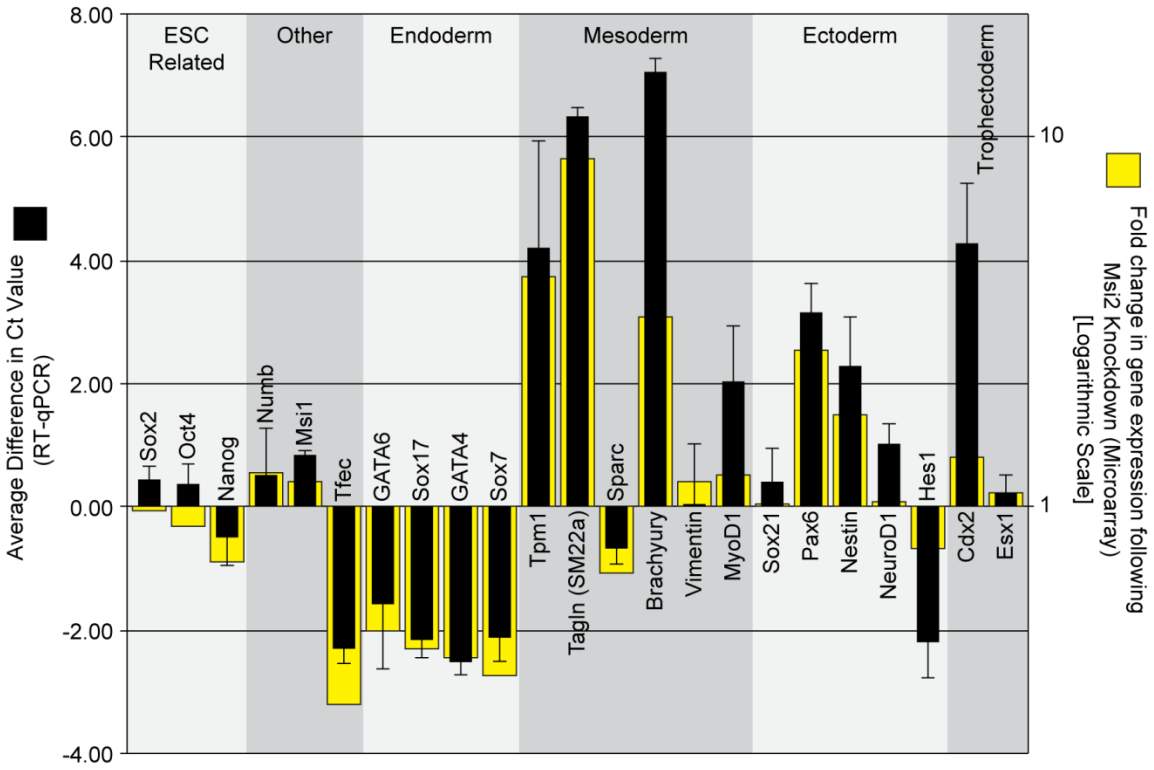

Figure 3.5: Knockdown of Msi2 in ESC leads to the expression of lineage-specific markers. Seven days post-infection, RNA was isolated from the D3 ESC infected with lentiviruses that express either the scrambled (Scr) shRNA sequence or the Msi2 shRNA #1 sequence. Microarray analysis was used to assess global changes in gene expression (Fig. 3.4A). RNA expression for a select set of genes (yellow bars) is indicated as fold change, where a value >1 represents an increase in expression in cells treated with shRNA #1. All microarray data is available on Gene Expression Omnibus (Accession No. GSE33882, GEO, http://www.ncbi.nlm.nih.gov/geo/). Expression of this gene subset was verified by RT-qPCR (black bars). Threshold cycle (Ct) values were calculated by normalizing all Ct values to GAPDH then subtracting the Ct value for cells infected with Msi2 shRNA #1 from the Ct value for cells infected with the scrambled shRNA lentivirus. A positive Ct value indicates an increase in the level of the transcript in the Msi2 knockdown cells. Multiple rounds of RT-qPCR were used to calculate an average change in Ct value, and error bars represent standard deviation.

3.4 Msi2 enhances the cloning efficiency of ESC

Because of our observation that the knockdown of Msi2 leads to the loss of self-renewal in ESC, we examined whether the elevation of Msi2 would enhance the self-renewal of ESC. Recent studies have shown that elevating Msi2 helps in the maintenance of hematopoietic and tumor stem cells [100, 102, 103]. However, it was not clear whether Msi2, in particular isoform 1 or isoform 2 could enhance ESC self-renewal. To examine this possibility, we engineered ESC for Dox-inducible overexpression of Flag-tagged Msi2, as described in the Methods section. Briefly, D3 ESC were infected with a lentivirus that constitutively expresses the reverse tet transactivator (rtTA), which binds Dox to mediate inducible transgene expression. These cells were then infected with a second lentivirus that expresses either Flag-tagged Msi2 isoform 1 (i-Msi2.1-D3 ESC) or isoform 2 (i-Msi2.2-D3 ESC) when the cells are exposed to Dox.

Using this system, we determined that addition of Dox leads to a small increase (~1.8 fold) in Flag-Msi2-isoform 1 (Figure 3.6A, left) and increases the cloning efficiency of ESC, specifically the number of ES cell colonies that form (Figure 3.6B, left). Conversely, ~2-fold overexpression of Flag-Msi2-isoform 2 (Figure 3.6A, right) in i-Msi2.2-D3 ESC had no significant effect on cloning efficiency, in particular the number of ES cell colonies that formed (Figure 3.6B, right). As a control, D3 ESC engineered for the inducible expression of luciferase did not demonstrate significant changes in the distribution of colony morphologies upon the addition of Dox (data not shown).

3.5 Msi2 isoform 1 or Msi2 isoform 2 on their own do not rescue the knockdown of both isoforms of Msi2

Our initial experiment in which shRNA #4 was used to target only isoform 1 suggested that Msi2 isoform 2 may not be required to support the self-renewal of ESC (Figure 3.2). This finding, coupled with the observation that Msi2 isoform 1 enhances the self-renewal of ESC, led us to examine whether isoform 1 alone is sufficient to support the self-renewal of ESC.

Figure 3.6

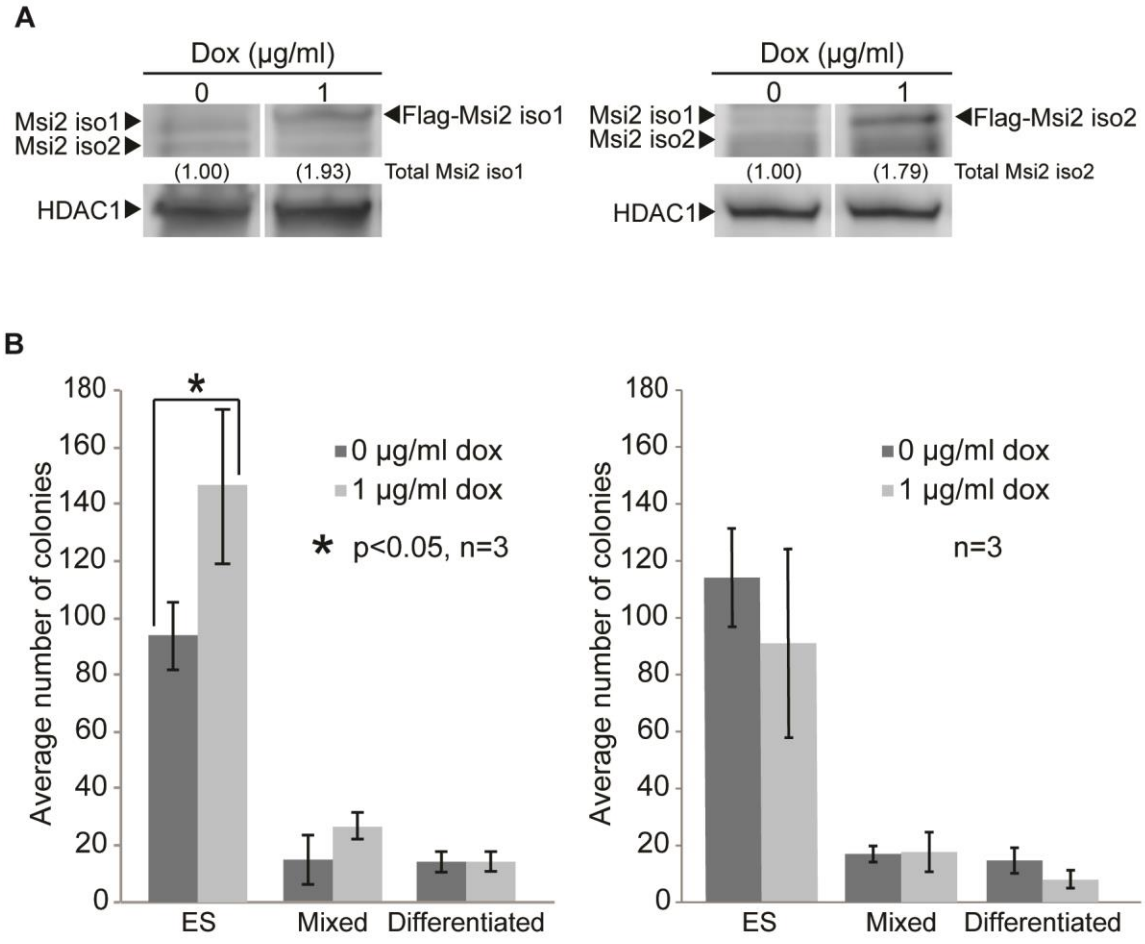

Figure 3.6: Overexpression of Msi2 in ESC increases the cloning efficiency of ESC.
(A) Western blot analysis of Msi2 present in D3 ESC stably infected with a lentivirus for the inducible expression of Flag-tagged Msi2 isoform 1 (left) or Flag-tagged Msi2 isoform 2 (right) in the presence of 1 µg/ml Dox. **(B)** Effects of inducing Flag-tagged Msi2 isoform 1 (left) or Flag-tagged Msi2 isoform 2 (right) on the cloning efficiency of D3 ESC. The cells were plated at clonal density (200 cells per cm2) and exposed to 1 µg/ml Dox for 5 days. The error bars are standard error of the mean. This experiment was repeated twice and similar results were obtained.

To determine whether Msi2 isoform 1 is sufficient to support ESC self-renewal, i-Msi2.1-D3 ESC and i-Msi2.2-D3 ESC were cultured in the absence or presence of Dox (2 μg/mL) for 24 hours. Next, the cells cultured in the presence or absence of Dox were infected with Msi2 shRNA #1 lentivirus, which targets both isoforms of Msi2 on their 3' UTR. Cells pretreated with Dox were cultured in the presence of Dox for the entire experiment. Western blot analysis of proteins isolated from i-Msi2.1-D3 ESC verified that total Msi2 levels were reduced (~60%) in cells cultured in the absence of Dox; whereas, total Msi2 levels were near normal (~90%) when the infected cells were maintained in the presence of Dox, due in part to exogenous expression from the transgene (Figure 3.7A). Additionally, in i-Msi2.2-D3 ESC infected with shRNA #1, Msi2 levels were knocked down (~60%, relative to endogenous Msi2) in the minus Dox control, but elevated ~2-fold when cultured in the presence of Dox (Figure 3.7B). The reason for the intensely staining band observed at the level of Msi2 isoform 2 in i-Msi2.2-D3 ESC cultured in the presence of Dox is not completely clear. We suspect that this is due to alternative translation start from our exogenous Flag-Msi2 isoform 2 transcript, which retains the endogenous Msi2 start codon.

To quantify any changes in self-renewal efficiency, i-Msi2.1-D3 ESC and i-Msi2.2-D3 ESC expressing their respective isoforms of Msi2 and infected with Msi2 shRNA #1 were subcultured 72 hours after infection with shRNA lentivirus, and plated at clonal density. Our findings indicate that elevation of Msi2 isoform 2 did not block the differentiation of ESC following the knockdown of both isoforms of Msi2 (Figure 3.7C). Interestingly, isoform 1 was also unable to block the differentiation of ESC following the knockdown of Msi2 (Figure 3.7C). Thus, our data suggests that the expression of isoforms 1 and 2 are both necessary to support the self-renewal of ESC.

3.6 Elevation of Msi2 during differentiation does not bias differentiation

Because of the role of Msi2 in hematopoietic stem cell maintenance and neural

Figure 3.7

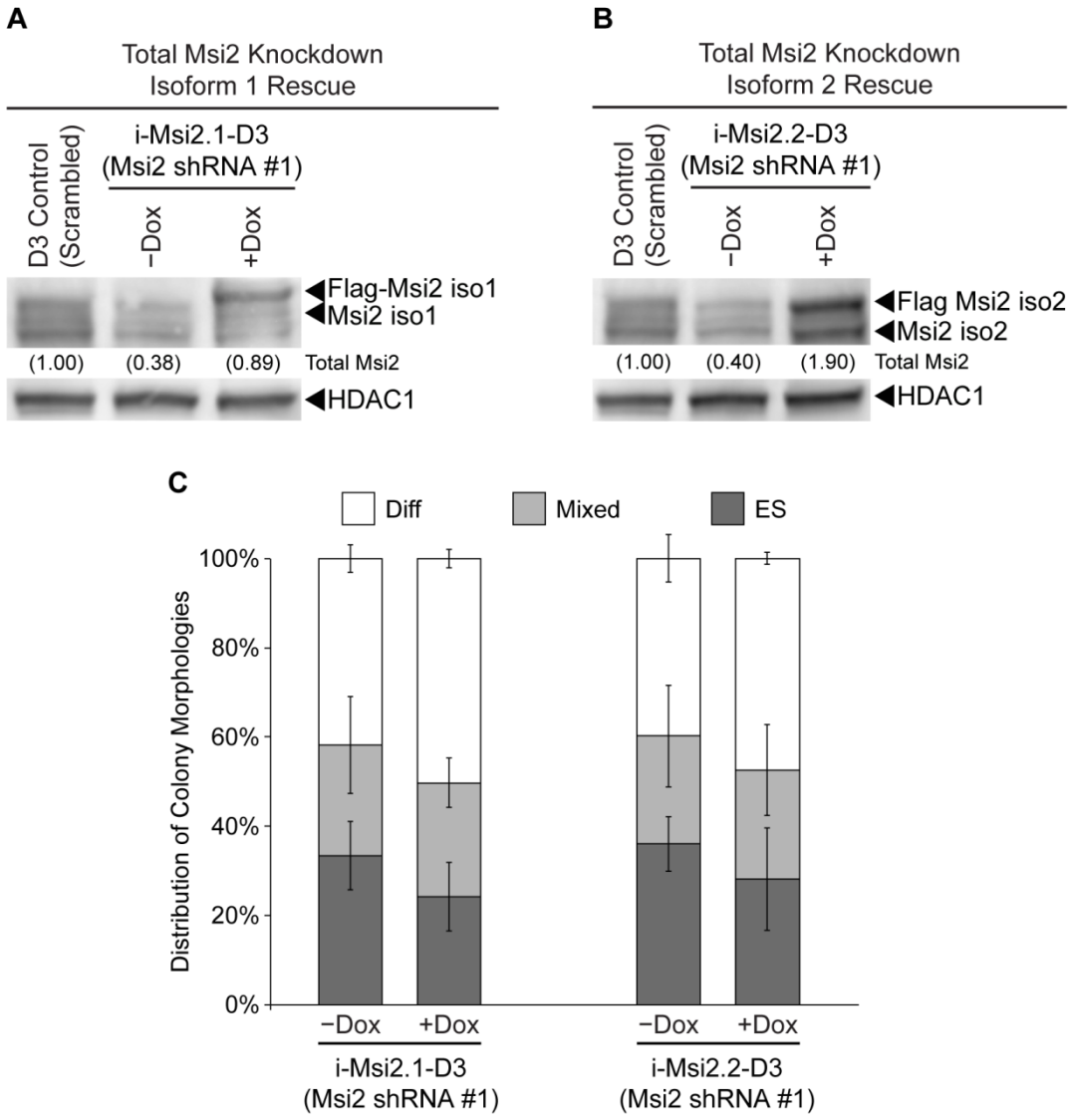

Figure 3.7: Rescue of Msi2 knockdown using an epitope-tagged overexpression construct. Cells engineered to overexpress Flag-tagged Msi2 isoform 1 or Flag-tagged Msi2 isoform 2, i-Msi2.1-D3 and i-Msi2.2-D3 respectively, were plated at 10,500 cells/cm2 in the presence or absence of 2 μg/ml Dox. Cells were infected with lentiviral constructs that express Msi2 shRNA #1 for 48 hours. One day later, the cells were subcultured at 600 cells/cm2 in the continued presence or absence of 2 μg/ml Dox and the remaining cells harvested for protein extracts. Western blot analysis was used to monitor the levels of Msi2 in i-Msi2.1-D3 **(A)** or i-Msi2.2-D3 **(B)** stably infected with shRNA #1 lentivirus in the presence or absence of 2 μg/ml Dox. D3 ESC infected with the lentiviral vector that expresses the scrambled shRNA was used as a control. **(C)** Effects of inducing Flag-tagged Msi2 isoform 1 (left) or Flag-tagged Msi2 isoform 2 (right) on the cloning efficiency of D3 ESC following Msi2 knockdown. Six days following subculture, two observers unaware of sample designation scored colonies as ESC, Mixed or differentiated (Diff). The error bars are standard deviation between the average percentages as scored by the two observers. This experiment was repeated twice and similar results were obtained.

development, we examined whether induction of Msi2 could skew the differentiation of ESC toward specific developmental lineages. For this purpose, i-Msi2.1-D3 and i-Msi2.2-D3 ESC, expressing their respective forms of Msi2, were differentiated using retinoic acid (RA). More specifically, i-Msi2.1-D3 and i-Msi2.2-D3 ESC were cultured continuously in the absence or presence of Dox. Two days after the addition of Dox to cells, RA was added to the culture medium, and cells were allowed to grow an additional 4 days. After treatment of cells with RA for 4 days (with and without Dox), RNA was isolated and examined by RT-qPCR analysis as described in the Methods. As expected, the pattern of differentiation induced by RA in the absence of Dox was highly similar for i-Msi2.1-D3 and i-Msi2.2-D3 ESC. More importantly, treatment of these ESC with Dox, which induces ectopic expression of Msi2 isoform 1 and Msi2 isoform 2, respectively (Figures 3.6 and 3.7), did not alter the pattern of RA-induced differentiation (Figure 3.8).

3.7 Musashi2 is necessary for the proliferation of DAOY MB cells

Previous reports demonstrated that MSI2 is essential for the progression of CML [100, 103, 192], and the knockdown of another family member, Musashi1 (MSI1), disrupts the viability of DAOY MB cells and GBM cells [195, 196]. To determine whether MSI2 is necessary for the proliferation of DAOY MB cells, shRNA constructs were used to knock down endogenous MSI2. Specifically, lentiviruses that constitutively express shRNA against MSI2 were used to infect DAOY MB cells. Two independent shRNA constructs (described in Chapter 2 of this dissertation) were used to knockdown MSI2, and a previously characterized non-specific shRNA (Scrambled) was used as a control [97, 184]. Following selection of the infected cells with puromycin, western blot analysis demonstrated that MSI2 isoforms 1 and 2 were substantially knocked down (Figure 3.9A). This reduction in MSI2 was verified at the RNA levels by RT-qPCR (Figure 3.10A). In addition, when compared to the growth of DAOY cells infected with the Scrambled shRNA lentiviral vectors, we observed a large reduction in growth when the cells

Figure 3.8

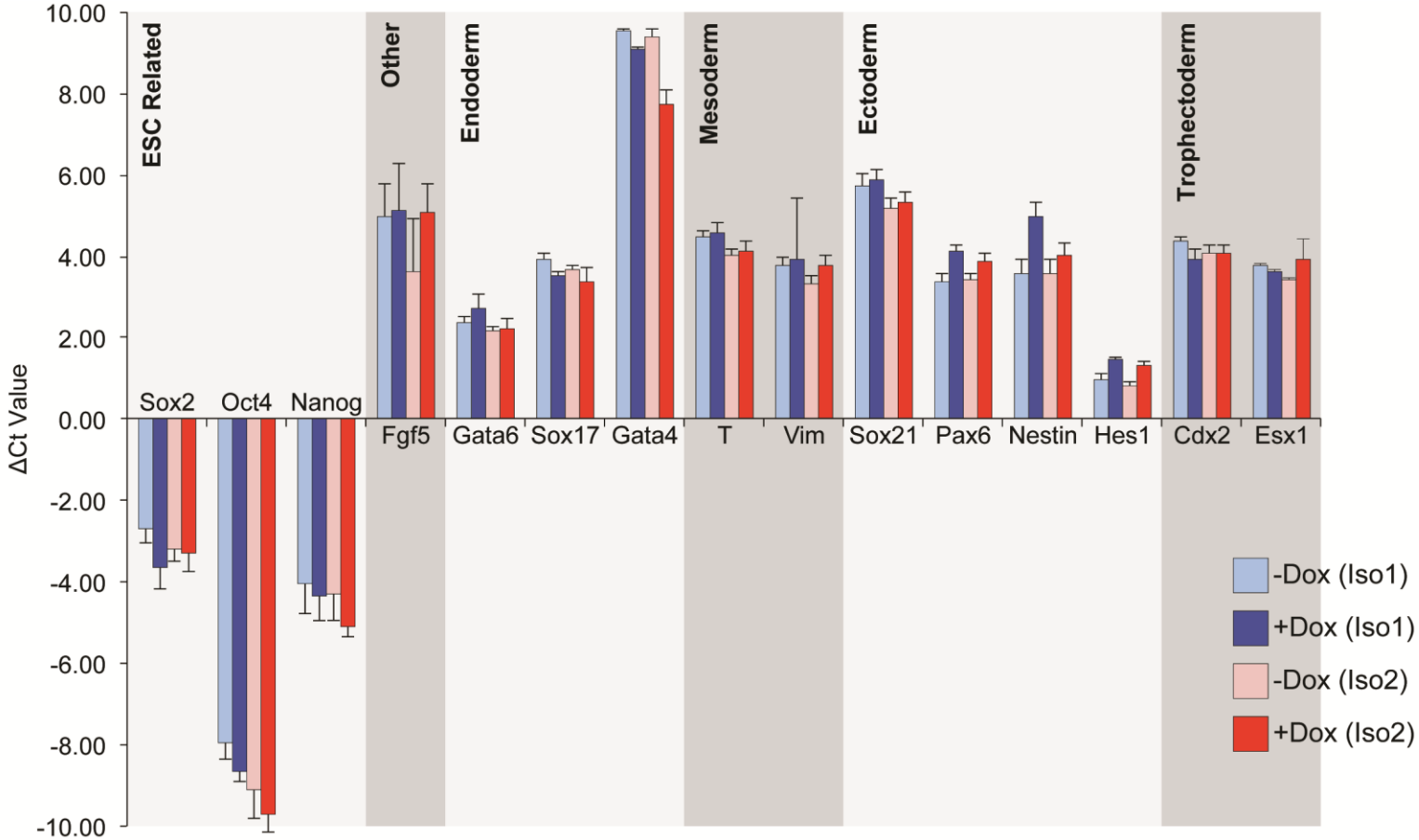

Figure 3.8: Effects of Msi2 overexpression on the differentiation of ESC.
i-Msi2.1-D3 and i-Msi2.2-D3 were treated with or without 1 μg/ml Dox for 48 hours prior to plating at 4,500 cells/cm2. Cells were treated with and without Dox in the presence of 5 μM RA for 48 hours, followed by the removal of LIF for 48 hours to further the differentiation of the cells. RNA was isolated from these cells and the expression of a subset of differentially expressed genes was verified by RT-qPCR, as described in the Materials and Methods. Threshold cycle (Ct) values were normalized to GAPDH and represent the difference in transcript levels between the indicated treatment condition and undifferentiatied ESC. A positive Ct value indicates an increase in the level of the transcript in the differentiated cells, as indicated. Multiple rounds of RT-qPCR were used to calculate an average change in Ct value, and error bars represent standard deviation.

Figure 3.9

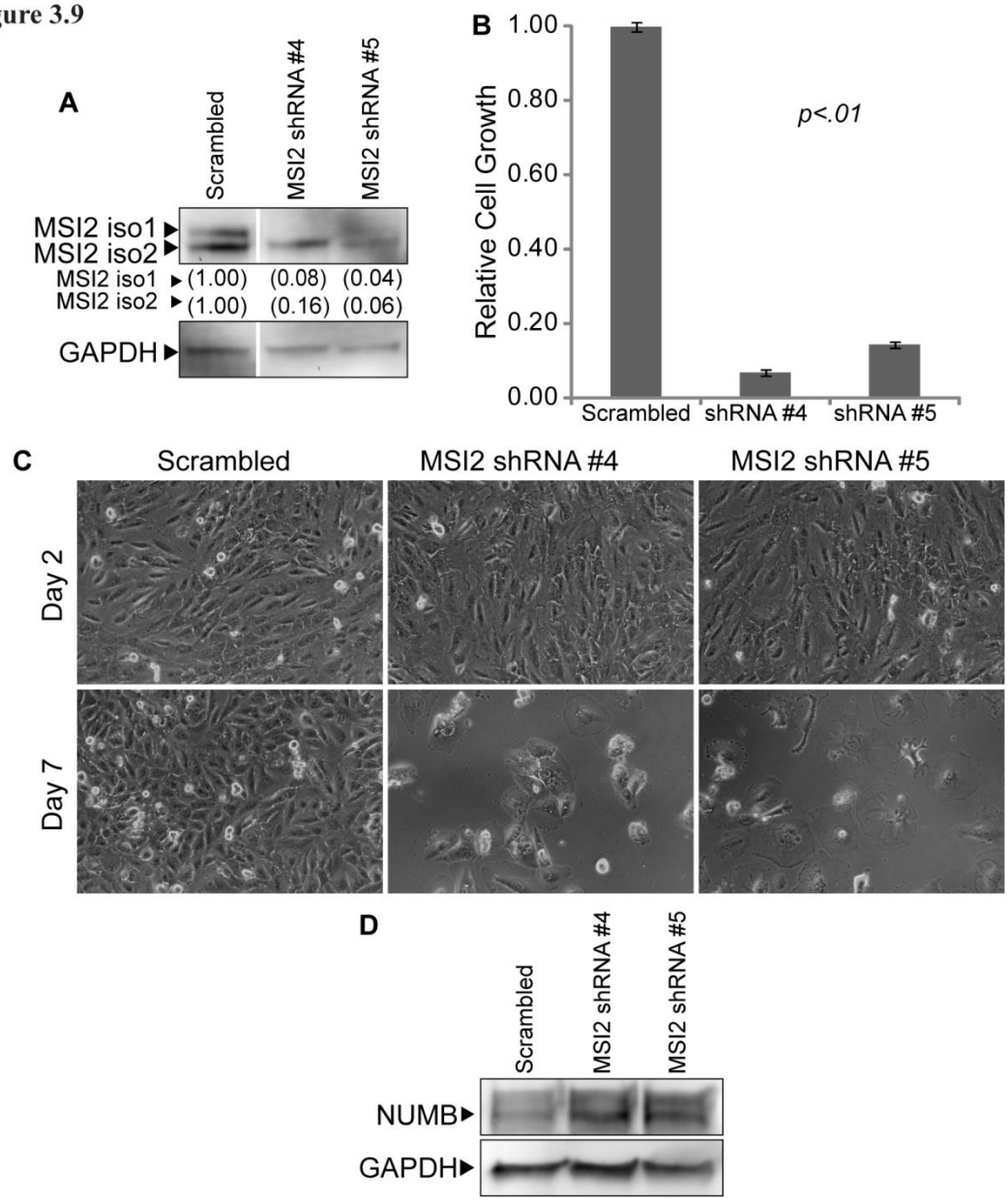

Figure 3.9: Knockdown of MSI2 in DAOY MB cells.
(A) Western blot analysis of MSI2 levels in DAOY whole cell extracts 96 hours after infection with Scrambled or MSI2 shRNA lentiviruses. Two isoforms were detected: isoform 1 (MSI2 iso1) and isoform 2 (MSI2 iso2). GAPDH was probed as a loading control. MSI2 levels are quantified, with levels found in the Scrambled control set to 1.00. **(B)** Cell growth was examined in triplicate by MTT assay 6 days after being plated at 10^4 cells per well of a 12-well plate. The data shown are averages relative to the Scramble control. Error bars represent standard deviation. P values were determined by student t-test and found to be <.01 for both MSI2 shRNA #4 and #5. **(C)** Photomicrographs of DAOY MB cells following infection with either non-specific (Scrambled) or MSI2 targeting (#4, #5) shRNA lentiviruses. Cells were infected on Day 0, selected using medium supplemented with puromycin on Day 1 and refed fresh medium on Day 2. Cells were photographed on days 2 and 7 after infection. **(D)** Western blot analysis of NUMB in DAOY MB extracts used in Figure 4.1A.

Figure 3.10

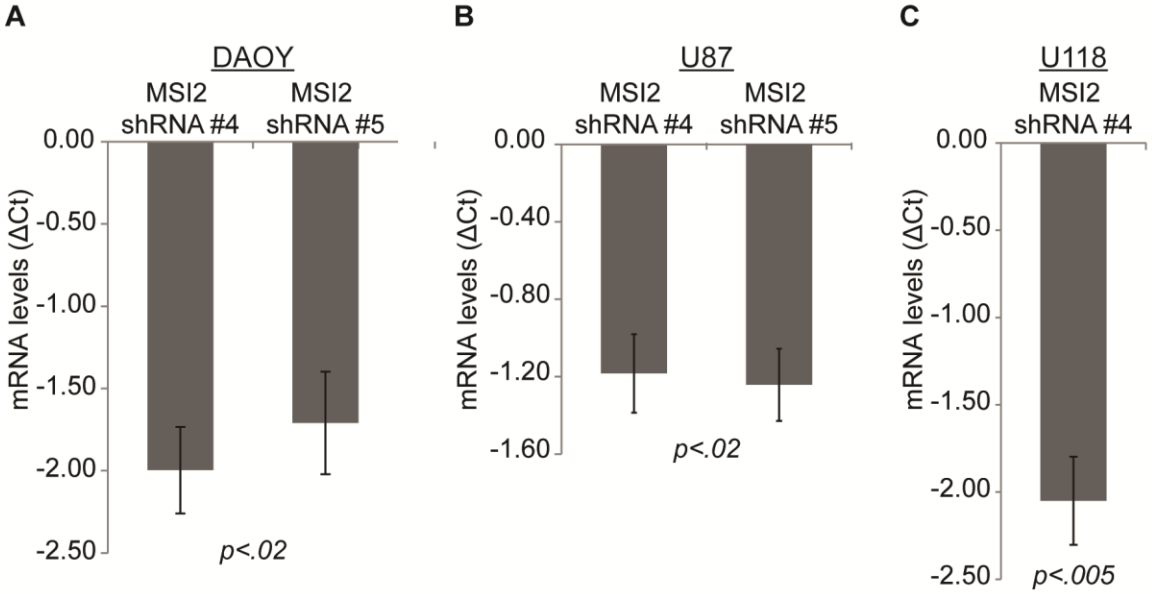

Figure 3.10: Validation of MSI2 knockdown in DAOY, U87, and U118 cells.
Cells were infected with lentiviruses that express either the Scrambled shRNA sequence or the MSI2 shRNA #4 or shRNA #5 sequence. RNA was isolated from DAOY cells **(A)**, U87 cells **(B)**, and U118 cells **(C)**. Expression levels of total MSI2 RNA was determined by RT-qPCR. Threshold cycle (Ct) values were calculated by normalizing all Ct values to GAPDH then subtracting the Ct value for cells infected with MSI2 shRNA #4 or shRNA #5 from the Ct value for cells infected with the Scrambled shRNA lentivirus. A negative Ct value indicates a decrease in the level of the transcript in the MSI2 knockdown cells. Multiple rounds of RT-qPCR were used to calculate an average change in Ct value, error bars represent standard error of the mean, and p values were determined by student t-test.

were infected with MSI2 shRNA lentiviral vectors (Figure 3.9B). Moreover, photomicrographs taken 7 days after infection demonstrated that cells in which MSI2 had been knocked down were flatter and larger, reminiscent of post-mitotic cells, when compared to the Scrambled control (Figure 3.9C).

Currently, relatively little is known about the roles of MSI2, but in mouse model of leukemia it is believed to down-regulate the protein Numb [103], which has been shown to regulate both Notch and Wnt signaling [197, 198]. Therefore, we examined whether knockdown of MSI2 in DAOY cells influences the expression of NUMB. We determined that knockdown of MSI2 with shRNA lentiviral vectors #4 and #5 caused an increase in the protein levels of NUMB (Figure 3.9D). Thus, knockdown of MSI2 causes both a large reduction in the growth of DAOY MB tumor cells and increases the expression of NUMB.

3.8 Musashi2 is necessary for the proliferation of GBM cells

We also examined the consequences of knocking down MSI2 in GBM tumor cells, because MSI2 was also identified as a SOX2-associated protein in U87 GBM cells (Wilder and Rizzino, unpublished results). For this purpose, we initially infected U87 GBM tumor cells with the same MSI2 shRNA lentiviral vectors described earlier. Again, a scrambled shRNA was used as a control. Three days after infection with the lentiviral vectors, western blot analysis determined that MSI2 isoform 1 and isoform 2 were both substantially reduced (Figure 3.11A) and the reduction in MSI2 was verified at the RNA levels by RT-qPCR (Figure 3.10B). As in the case of DAOY cells, U87 GBM cells infected with MSI2 shRNA constructs exhibited a marked decrease in cell proliferation (Figure 3.11B) and a significant increase in cell size (Figure 3.11C). To extend these findings, U118 GBM cells were infected with MSI2 shRNA lentiviruses. Similar to DAOY and U87 cells, knockdown of MSI2 in U118 cells resulted in a decrease in MSI2 protein and RNA, a large reduction in cell growth, and a significant increase in cell size (Figure 3.10C and Figure 3.12). Taken together, our data indicate that MSI2 is required to sustain the

Figure 3.11

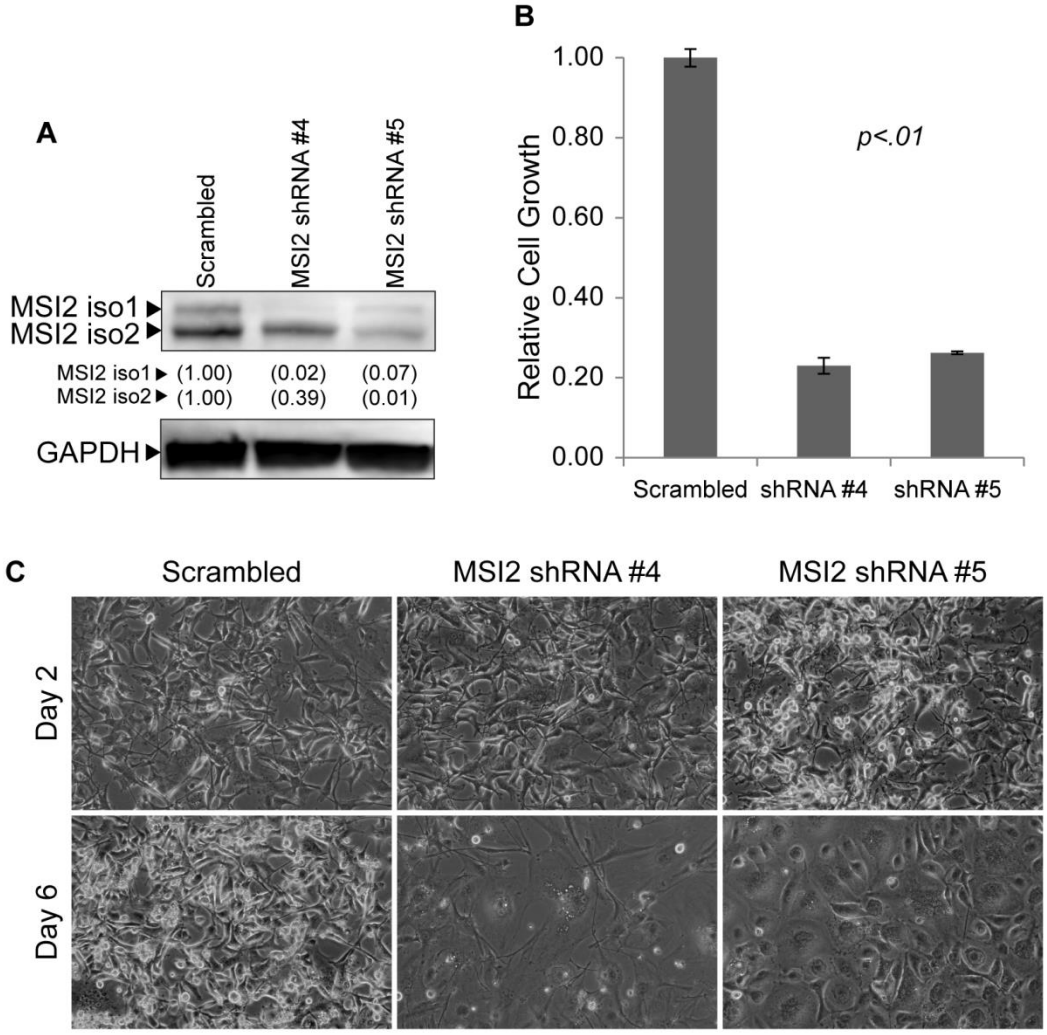

Figure 3.11: Knockdown of MSI2 in U87 GB cells.
(A) Western blot analysis of MSI2 levels in U87 nuclear extracts 96 hours after infection with Scrambled or MSI2 shRNA lentiviruses. MSI2 levels are quantified, with levels found in the Scrambled control set to 1.00. **(B)** Cell growth was examined in triplicate by MTT assay 5 days after being plated at $1.5x10^4$ cells per well of a 12-well plate. The data shown are averages relative to the Scramble control. Error bars represent standard deviation. P values were determined by student t-test and found to be <.01 for both MSI2 shRNA #4 and #5.
(C) Photomicrographs of U87 GB cells following infection with either non-specific (Scrambled) or MSI2 targeting (#4, #5) shRNA lentiviruses. Cells were infected on Day 0, selected using medium supplemented with puromycin on Day 1 and refed fresh medium on Day 3. Cells were photographed on days 2 and 6 after infection.

Figure 3.12

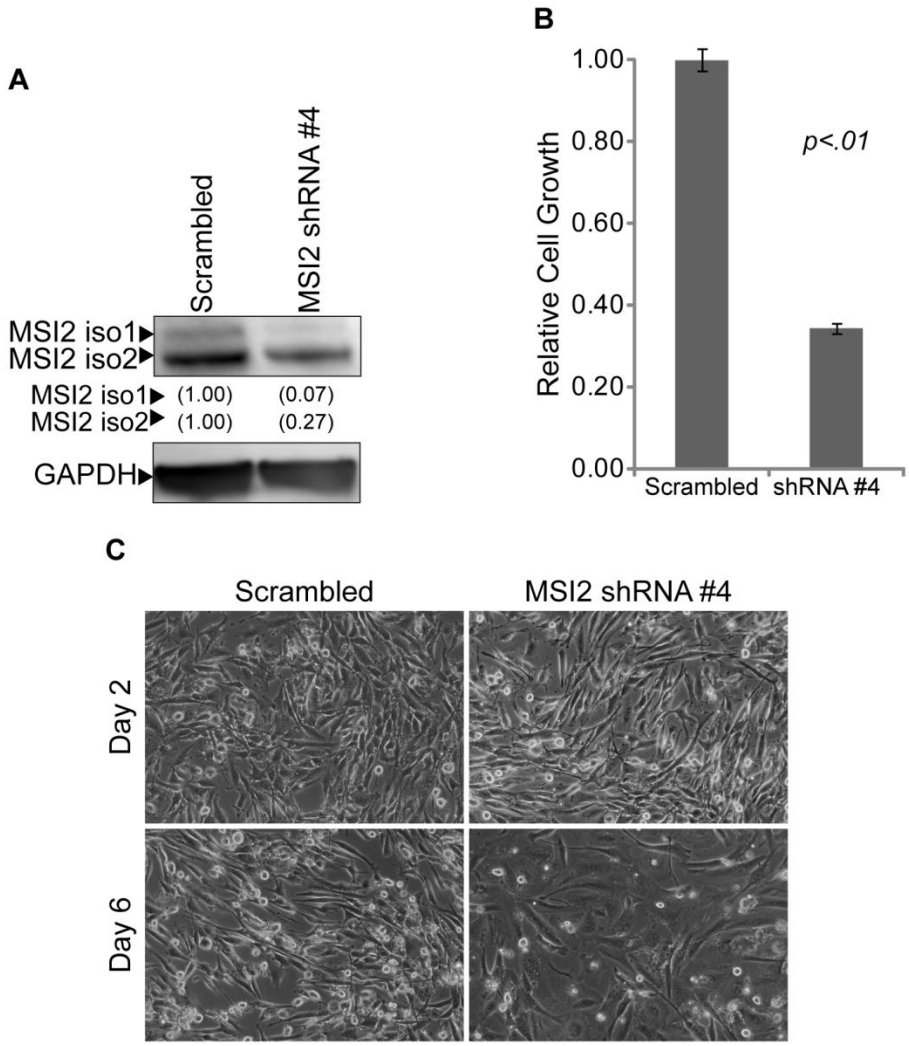

Figure 3.12: Knockdown of MSI2 in U118 glioblastoma cells.
(A) Western blot analysis of MSI2 levels 96 hours after infection with Scrambled or MSI2 shRNA lentiviruses. Two isoforms were detected: isoform 1 (MSI2 iso1) and isoform 2 (MSI2 iso2). GAPDH was probed as a loading control. MSI2 levels are quantified, with levels found in the Scrambled control set to 1.00. **(B)** Cell growth was examined in triplicate by MTT assay 5 days after being plated at $2.5x10^4$ cells per well of a 12-well plate. The data shown are averages relative to the Scramble control. Error bars represent standard deviation and p values were determined by student's t-test. P values were <.01 for MSI2 shRNA #4. **(C)** Photomicrographs of U118 GB cells were taken days 2 and 6 following infection with either non-specific (Scrambled) or MSI2 targeting shRNA lentiviruses.

survival of DAOY MB cells, and the proliferative capacity of U87 and U118 GBM cells.

3.9 Discussion

In this study, we demonstrate that ESC express two isoforms of Msi2, and we determined that the knockdown of Msi2 disrupts the self-renewal of ESC and induces their differentiation. Moreover, our studies demonstrate that the extent of differentiation and the loss of self-renewal capacity correlates with the extent to which Msi2 levels were decreased. Remarkably, the knockdown of Msi2 causes ESC to differentiate despite continued expression of both Sox2 and Oct4.

Although knockdown of Msi2 isoform 1 is sufficient to induce the differentiation of ESC (Fig. 3.2 shRNA #4), a more rigorous demonstration that ESC strictly require Msi2 isoform 2 will require considerably more work. The most direct method for addressing this question would be to selectively knockdown Msi2 isoform 2. As mentioned earlier, addressing this question will require an shRNA that only targets isoform 2, which is currently not available. Thus far, this question has not been addressed in any study, including the recent reports that demonstrated prominent roles of Msi2 in the function HSC, CML, and AML [100, 102, 103]. In these seminal studies, the shRNA sequences used targeted both isoforms of Msi2.

We also determined that overexpression of Msi2 isoform 1, but not isoform 2, enhances the cloning efficiency of ESC, which is a measure of their self-renewal capacity. However, we determined that ectopic expression of either Msi2 isoform 1 or isoform 2 does not block the differentiation of ESC when both isoforms of Msi2 are knocked down. Moreover, ectopic expression of Msi2 isoform 1 or isoform 2 does not appear to alter the pattern of differentiation induced by the treatment of ESC with RA. Thus, our findings suggest that both isoforms of Msi2 are required to maintain the self-renewal of ESC.

Finally, the studies presented in this Chapter support that MSI2 is required for the proliferation of medulloblastoma cells as well as glioblastoma cells. Although several SOX2-

associated proteins have been implicated in brain cancer, the roles of the vast majority of SOX2-associated proteins have not been examined in brain cancer. In this Chapter, we examined the SOX2-associated protein MSI2, which has been implicated in supporting the growth of other cancers. Knockdown of MSI2 in MB and GB cells impairs their ability to proliferate. Currently, it is unclear why the knockdown of MSI2 reduces the growth of brain tumor cells. Recent studies suggest that the translation of NUMB mRNA, which is a known target of MSI1 [99], inversely correlates with MSI2 expression in leukemia [192]. Interestingly, we observed an increase in the level of NUMB when MSI2 was knocked down in DAOY cells. Moreover, others have reported that overexpression of NUMB in DAOY cells reduces their colony-forming ability [199]. Thus, it is tempting to speculate that knockdown of MSI2 reduces the viability of DAOY cells because of the increase in NUMB. However, further study will be needed to verify whether this is in fact the case, because MSI2 is likely to affect the expression of other important genes. In this regard, studies conducted in HEK293T cells identified >60 mRNA that associate with MSI1 [200]. Hence, MSI2 is also likely to regulate the translation of a significant number of mRNA. The reason for the reduction in the proliferation of U87 and U118 GB cells when MSI2 is knocked down is also unclear. Recently, it has been reported that elevating NUMB in U87 cells does not affect their proliferation [201]. Thus, further study will be needed to define the roles of MSI2 in brain tumor cells. Importantly, the studies described in this Chapter substantiate our belief that identifying proteins that interact with SOX2 in multiple cellular contexts is a useful approach for identifying critical understudied proteins for diseases such as cancer.

CHAPTER 4: SOX2 functions as a molecular rheostat in pancreatic ductal adenocarcinoma

The studies described in this chapter, which were conducted by Erin Wuebben, and are published in Oncotarget [202].

4.1 Introduction

PDAC is presently one of the most lethal malignancies in the United States, as outlined in Chapter 1 of this dissertation, and SOX2 expression has been shown to increase as PDAC progresses. More recent studies reported that SOX2 is expressed in many different human PDAC cell lines, with high expression in some PDAC cell lines, but little or no expression in others [155]. Importantly, this study demonstrated that SOX2 expression is closely associated with putative cancer stem cell markers previously reported to be expressed by PDAC tumor-initiating cells [155]. This study also demonstrated that knocking down SOX2 in PDAC cell lines reduced their growth *in vitro*; whereas, stable expression of SOX2 in a PDAC cell line, which does not endogenously express detectable levels of SOX2, increased their anchorage-independent growth [155]. Although this study provided support *in vitro* for a critical role of SOX2 in the stemness of PDAC, the effects of SOX2 on the tumorigenicity of PDAC tumor cells were not examined.

Here, we examined the growth responses of multiple PDAC cells lines engineered for either inducible overexpression of SOX2 or inducible knockdown of SOX2. In addition to examining how altering SOX2 expression influences PDAC cell growth *in vitro*, we examined how tumorigenicity is affected when SOX2 levels are increased and decreased.

4.2 Engineering PDAC Cell Lines for SOX2 Overexpression

To determine how elevating the levels of SOX2 influences the behavior of PDAC cells, we initially engineered T3M4 PDAC cells for inducible overexpression of epitope-tagged SOX2. Epitope-tagged SOX2 enabled us to distinguish exogenously expressed SOX2 from endogenous SOX2. SOX2 was tagged at its N-terminus with a sequence that codes for a Flag-Strep tag. Previous studies have shown that placement of this tag at the N-terminus does not interfere with its function [80, 87, 93, 172, 203]. T3M4 cells were selected because they express SOX2 at intermediate levels, ~15-fold lower than L3.6 cells (data not shown), which have been shown previously to express SOX2 at levels significantly higher than most other PDAC cell lines [155].

Additionally, L3.6 cells express mutant KRAS (G12D) [204]; whereas, T3M4 cells heterozygously express a different KRAS mutant (Q61H/WT) [205]. Using T3M4 cells, we could determine how both inducible overexpression of SOX2, as well as inducible knockdown of SOX2 (see below), influences the behavior of PDAC cells. T3M4 cells were engineered for inducible overexpression with the aid of two lentiviral vectors, which are similar to those used previously to engineer brain tumor cells for inducible expression of exogenous SOX2 [172]. One lentiviral vector codes for the expression of the reverse tet-transactivator driven by a PGK promoter, and the second lentiviral vector codes for the expression of epitope-tagged SOX2, which is driven by a Dox-inducible promoter (Figure 2.1). After viral transduction of T3M4 cells, cells stably transduced with both lentiviral vectors were isolated as described in Chapter 2. These cells are referred to as i-SOX2-T3M4 cells.

4.3 Overexpression of SOX2 Reduces PDAC Cell Growth *in vitro* and *in vivo*

To determine how inducible elevation of SOX2 influences the *in vitro* growth of i-SOX2-T3M4 cells, we initially examined a Dox-dose response curve. As the concentration of Dox was increased, there was a dose dependent increase in the expression of Flag-SOX2. At 300 ng/ml of Dox there was a ~7.5-fold increase in total SOX2 (endogenous plus exogenous SOX2) (Figure 4.1A). Treatment of i-SOX2-T3M4 cells with Dox over a 4 day period led to decreased cell growth at all Dox concentrations tested, reaching nearly 40% reduction in cell proliferation at 300 ng/ml of Dox (Figure 4.1B). A significant reduction in cell growth was evident after 72 hr (not statistically different at 48 hr, Figure 4.1C). As a control, we tested the effects of Dox on parental T3M4 cells. At concentrations as high as 1 μg/ml, there were no effects on the growth of parental T3M4 cells (Figure 4.1B). To extend these studies, we assessed the effects of elevating SOX2 on the clonal growth of i-SOX2-T3M4 cells in both monolayer culture and under anchorage-independent growth conditions. When plated at clonal densities in monolayer culture, inducible overexpression of SOX2 after 8 days significantly reduced the number of colonies, as well as the

Figure 4.1

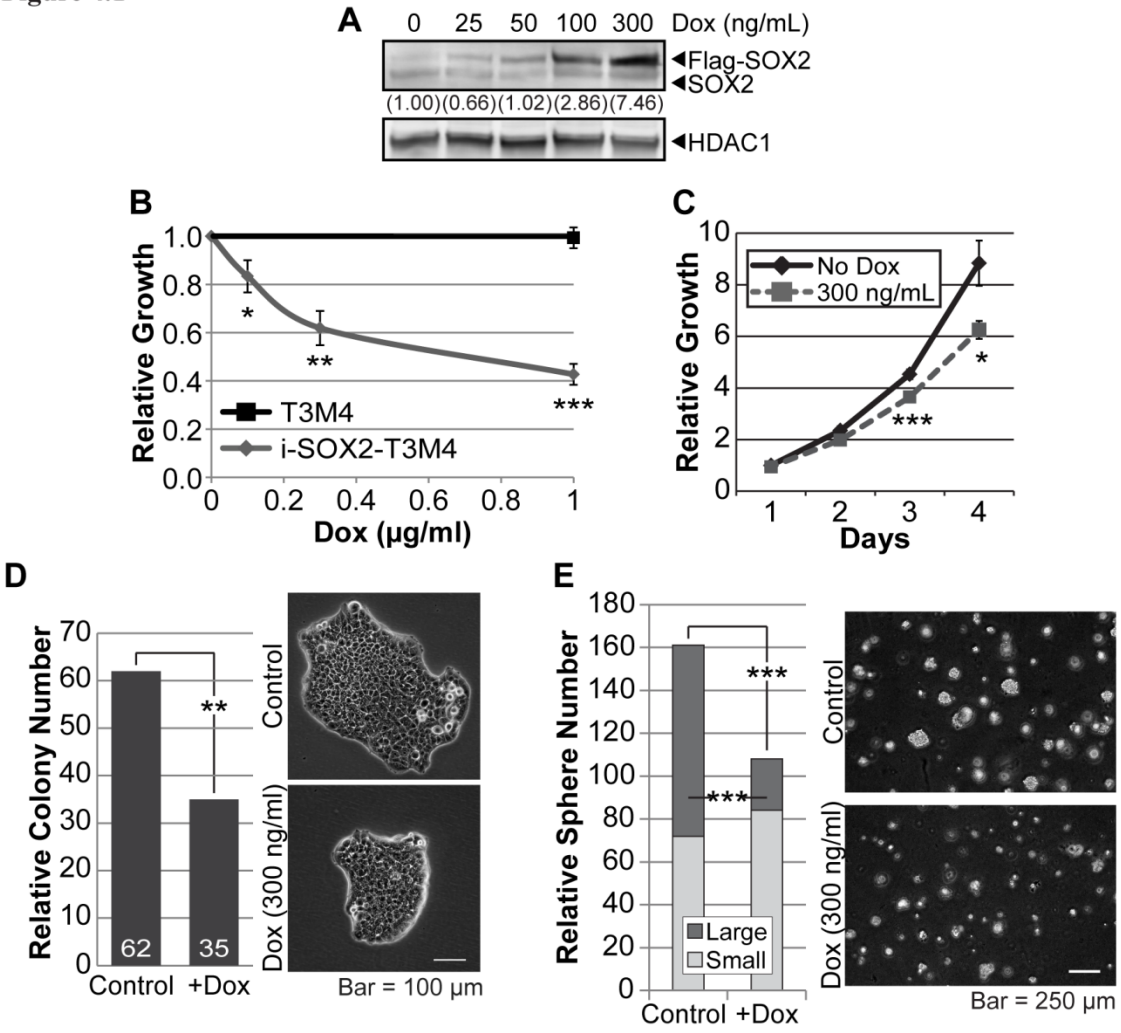

Figure 4.1: Overexpression of SOX2 in PDAC cells reduces proliferation.
(A) Western blot analysis of SOX2 expression in whole cell extracts from i-SOX2-T3M4 cells. The overexpression of Flag-SOX2 after 24 hr of Dox treatment was compared to endogenous SOX2 in the untreated sample. HDAC1 protein was used as a loading control. **(B)** Cell proliferation of i-SOX2-T3M4 was determined by MTT assay following 4 days growth at the indicated Dox concentrations. Growth in the absence of Dox was set to 1. **(C)** Proliferation of i-SOX2-T3M4 cells over a 4 day period was determined by MTT assay following growth in the presence or absence of Dox (300 ng/ml). **(D)** Cloning efficiency of i-SOX2-T3M4 cells was determined by the number of colonies formed after 8 days of growth in the presence or absence of Dox (300 ng/ml) as described in Chapter 2. Representative photomicrographs (25X) were taken on day 8 and the cells in both panels were photographed at the same magnification. **(E)** Soft agar growth in serum-free, stem cell medium and representative photomicrographs (10X) of i-SOX2-T3M4 cells in the presence or absence of Dox (300 ng/ml) after 9 days. The colonies in both panels were photographed at the same magnification. An observer unaware of sample designation scored colonies containing >8 cells in 10 random fields. Spheres larger than 50 µm were scored as "large". Error bars represent standard deviation; statistical significance was determined by student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.005$). The studies shown in A, C, D, and E were repeated and similar results were obtained.

size of the colonies (Figure 4.1D). Importantly, even after repeated passage in the presence of Dox (> 10 passages), we failed to observe the emergence of cells that exhibited accelerated growth due to elevation of SOX2. After each passage, there was a reduction in the growth of cells treated with Dox when compared to cells cultured in the absence of Dox (data not shown). Not surprisingly, inducible elevation of SOX2 also failed to increase the growth of i-SOX2-T3M4 cells under anchorage-independent growth conditions. After treatment with Dox for 9 days in serum-free, stem cell medium, the number and size of the colonies formed in soft-agar was reduced significantly (Figure 4.1E). Under these conditions, there was a reduction in the total number of colonies, where the largest reduction was in the number of large colonies.

To determine whether the effects of SOX2 overexpression were PDAC cell line dependent, we engineered two additional PDAC cell lines, BxPC3 and HPAF-II, for inducible overexpression of SOX2. BxPC3 cells endogenously express SOX2 at levels ~5-fold higher than T3M4 cells; whereas, HPAF-II cells express endogenous SOX2 at levels lower than T3M4 cells (data not shown). HPAF-II cells express activated, mutant KRAS (G12D) [206]; whereas, BxPC3 cells express wild-type KRAS [207, 208]. Thus, BxPC3 cells could help determine whether the effects of inducible overexpression of SOX2 were related to the KRAS status of PDAC cells. BxPC3 cells and HPAF-II cells were each transduced with the same lentiviral vector set (Figure 2.1) used to engineer T3M4 cells. As shown for i-SOX2-T3M4, we observed tunable induction of exogenous SOX2 when i-SOX2-HPAF-II cells and i-SOX2-BxPC3 were exposed to increasing concentrations of Dox (Figure 4.2A, D). In addition, at all Dox concentrations tested, elevation of SOX2 in i-SOX2-HPAF-II and i-SOX2-BxPC3 cells reduced both their short-term monolayer growth and their growth at clonal density (Figure 4.2B, C, E, and F). Elevating SOX2 in i-SOX2-HPAF-II, led to ~40% reduction in growth (Figure 4.2E). In the case of i-SOX2-BxPC3 cells, reduction in growth was smaller, but statistically significant Figure 4.2B). Importantly, under no conditions examined did we observe an increase in proliferation when SOX2 levels were elevated in three different PDAC cell lines. Altogether these studies demonstrate that inducible

Figure 4.2

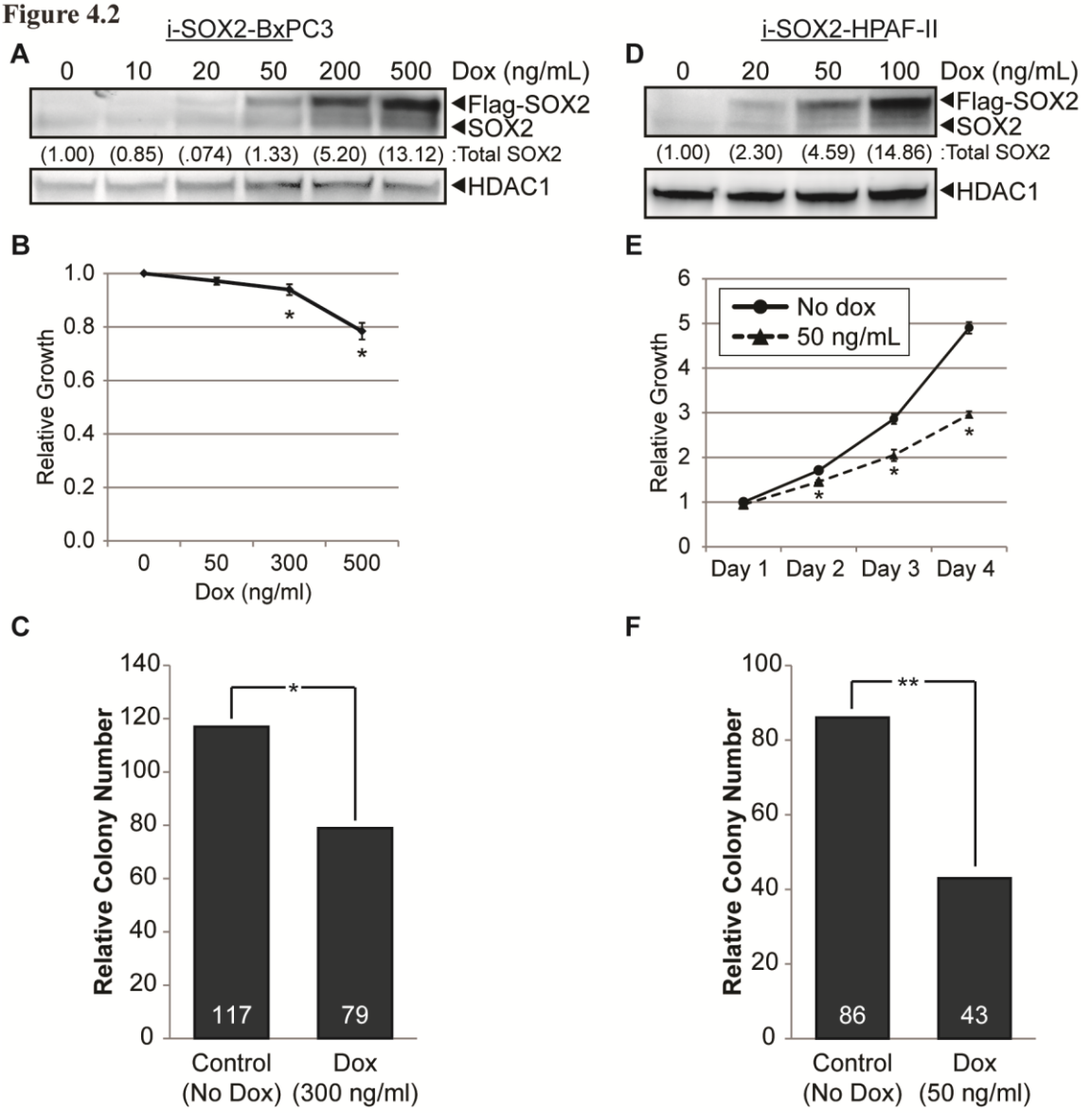

Figure 4.2: Overexpression of SOX2 in PDAC cells reduces cellular proliferation.
(A) Western blot analysis of SOX2 expression in whole cell extracts from i-SOX2-BxPC3 cells following 3 days of Dox-induction of the transgene. The overexpression of Flag-SOX2 was compared to endogenous SOX2. HDAC1 protein was used as a loading control. **(B)** Proliferation of i-SOX2-BxPC3 cells was determined by MTT assay following 4 days growth at the indicated Dox concentrations. Growth in the absence of Dox was set to 1. **(C)** Clonal growth of i-SOX2-BxPC3 cells was determined after 8 days in the presence or absence of Dox (300 ng/ml). **(D)** Western blot analysis of SOX2 expression in whole cell extracts from i-SOX2-HPAF-II cells following 2 days of Dox-treatment. The overexpression of Flag-SOX2 was compared to endogenous SOX2. HDAC1 protein was used as a loading control. **(E)** Growth of i-SOX2-HPAFII cells over 4 days was determined by MTT assay following growth in the presence or absence of Dox (50 ng/ml). **(F)** Clonal growth of i-SOX2-HPAF-II cells was determined after 8 days in the presence or absence of Dox (50 ng/ml). Error bars represent standard deviation; statistical significance was determined by student's t-test (*$p<0.05$, **$p<0.01$). The studies shown in A, C, D, and F were repeated and similar results were obtained.

overexpression of SOX2 in PDAC cells reduces their growth *in vitro*.

4.4 SOX2 Overexpression Decreases Subcutaneous Tumor Growth

A key property of cancer cells is tumorigenicity. To assess the impact of overexpression of SOX2 on the tumorigenicity of PDAC cells, 2.5x105 i-SOX2-T3M4 cells were engrafted subcutaneously into NCr-nu/nu mice, as described in Chapter 2. Nine days after palpable tumors had formed, sized-matched tumors were randomly assigned to the control or the Dox-treated group. After 9 additional days, tumors in the control group (11 mice) had grown to an average > 450 mm^3; whereas tumors in the Dox-treated group (11 mice) exhibited much less growth, reaching on average ~90 mm^3 (~80% smaller, $p<0.001$) (Figure 4.3A). In addition, tumor weight was reduced ~70% in the Dox-treated group (Figure 4.3B). There was also ~2-fold increase in fraction of the tumor consisting of desmoplastic stroma relative to that observed in the control tumor group, which were not treated with Dox, as determined by smooth muscle actin (SMA) staining ($p<0.05$, Figure 4.3D). Interestingly, the proliferation marker Ki-67 was ~75% lower in the tumor cell compartment of the Dox-treated tumors compared to untreated tumors ($p<0.01$); whereas, Ki-67 staining was ~2-fold higher in the stromal compartments of Dox-treated tumors compared to untreated tumors ($p<0.05$, Figure 4.3E). Altogether, our findings argue that inducible overexpression of SOX2 in PDAC cells does not increase cell growth, but, in fact, reduces their growth in culture as well as their tumorigenicity.

4.5 Knockdown of SOX2 Decreases PDAC Cell Growth *in vitro* and *in vivo*

We also assessed the impact of knocking down SOX2 on the growth and tumorigenicity of T3M4 cells. For this purpose, T3M4 cells were transduced with a single lentiviral vector that codes for an inducible promoter driving expression of a SOX2 shRNA, as well as coding for constitutive expression of the reverse tet-transactivator that is capable of binding to the Dox-inducible promoter when Dox is added to the culture medium. Productively transduced T3M4

Figure 4.3

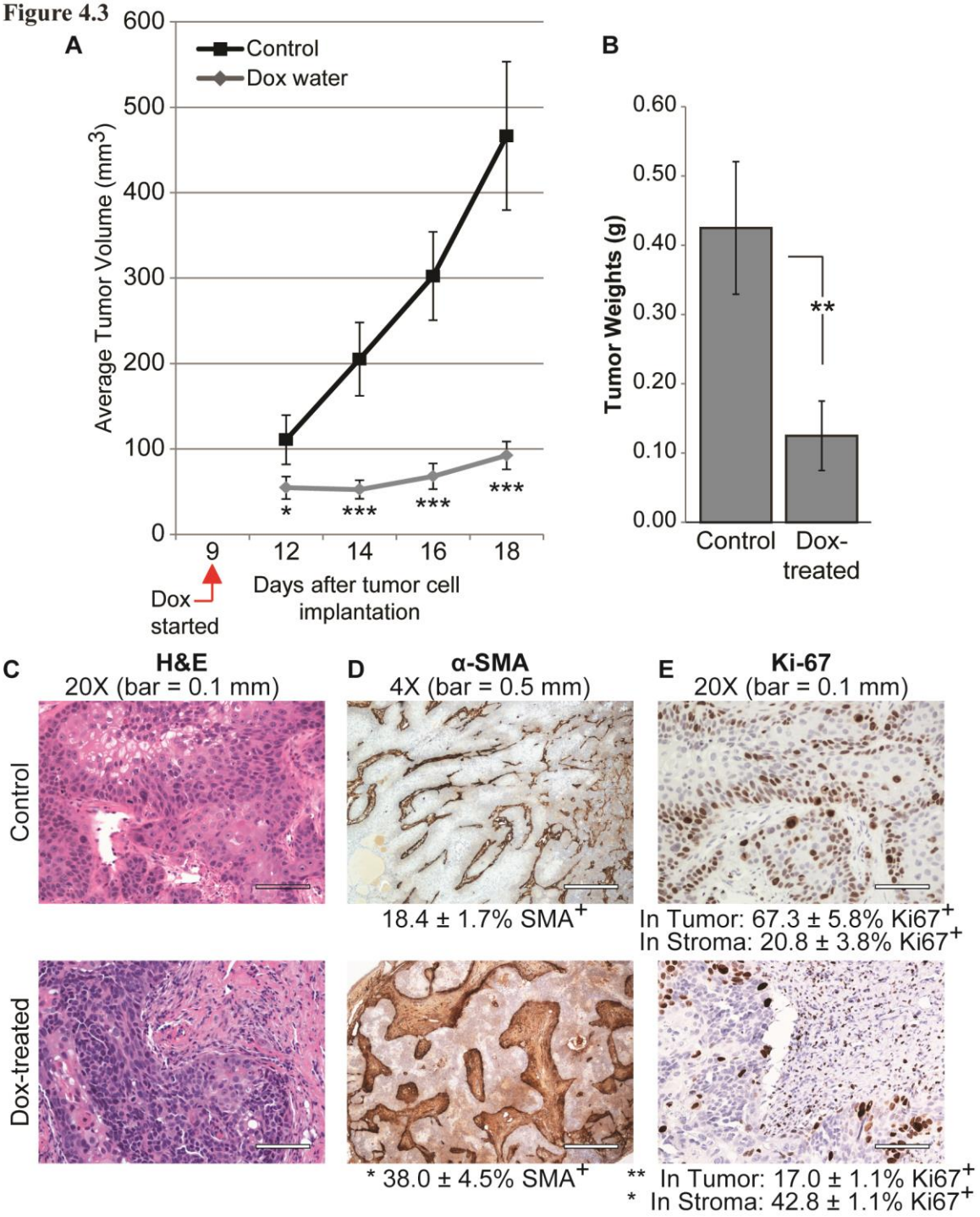

Figure 4.3: Overexpression of SOX2 in i-SOX2-T3M4 cells reduces subcutaneous tumor growth. (A) Subcutaneous tumor volumes were determined for each group of 11 mice as described in Chapter 2. Average tumor volumes are presented for control and Dox-treated groups. Error bars represent standard error of the mean. **(B)** Tumor weights were measured following excision after a total 19 days of tumor growth. Error bars represent standard deviation. Subcutaneous tumor sections were stained and quantified for **(C)** H&E, **(D)** SMA, and **(E)** Ki-67 as described in Chapter 2. Statistical significance was determined by student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

cells, referred to as i-KdSOX2-T3M4 cells, were isolated as described in Chapter 2. Treatment of these cells with increasing concentrations of Dox led to dose dependent reductions in the expression of endogenous SOX2 protein (Figure 4.4A) and dose dependent reductions in cell growth (Figure 4.4B). After 3 days of growth, there was a statistically significant reduction of growth, reaching >50% inhibition after 4 days (Figure 4.4C) when SOX2 was reduced ~60% (Figure 4.4A). As discussed below, treatment with Dox at this concentration also reduced the number of colonies as well as the size of colonies when plated at clonal density in monolayer culture. Additionally, a second, independent shRNA lentiviral vector was used in T3M4 cells to validate that observed effects were due to the knockdown of SOX2. As described above, increasing the concentration of Dox resulted in dose-dependent reductions in SOX2 protein expression and in cell growth after 4 days when using this second shRNA vector; however, this shRNA was less effective at knocking down SOX2 (~40% reduction) and less effective at reducing growth (<30%, Figure 4.5). Thus, in the studies described below, the cells engineered with the first SOX2 shRNA were used.

Additionally, we examined whether knocking down SOX2 in another PDAC cell line would also alter their growth. For this purpose, L3.6 cells, which express high levels of SOX2, were transduced with the same Dox-inducible SOX2 shRNA lentiviral vector used to generate i-KdSOX2-T3M4 cells. Treatment of i-KdSOX2-L3.6 cells with increasing concentrations of Dox led to a dose dependent decrease in the expression of SOX2 protein and a decrease in the growth of the cells in monolayer culture (Figure 4.4D-F).

Next, we assessed the impact of knocking down SOX2 on the tumorigenicity of i-KdSOX2-L3.6 cells. i-KdSOX2-L3.6 cells were engrafted subcutaneously into NCr-nu/nu mice. Once palpable tumors had formed by engrafted i-KdSOX2-L3.6 cells, mice with sized-matched tumors were randomly assigned to the control or the Dox-treated group. After an additional 8 days, the tumors in the control group increased from an average of 20 mm^3 to an average of 230 mm^3; whereas tumors in the Dox group increased from an average of 20 mm^3 to an average of 70

Figure 4.4

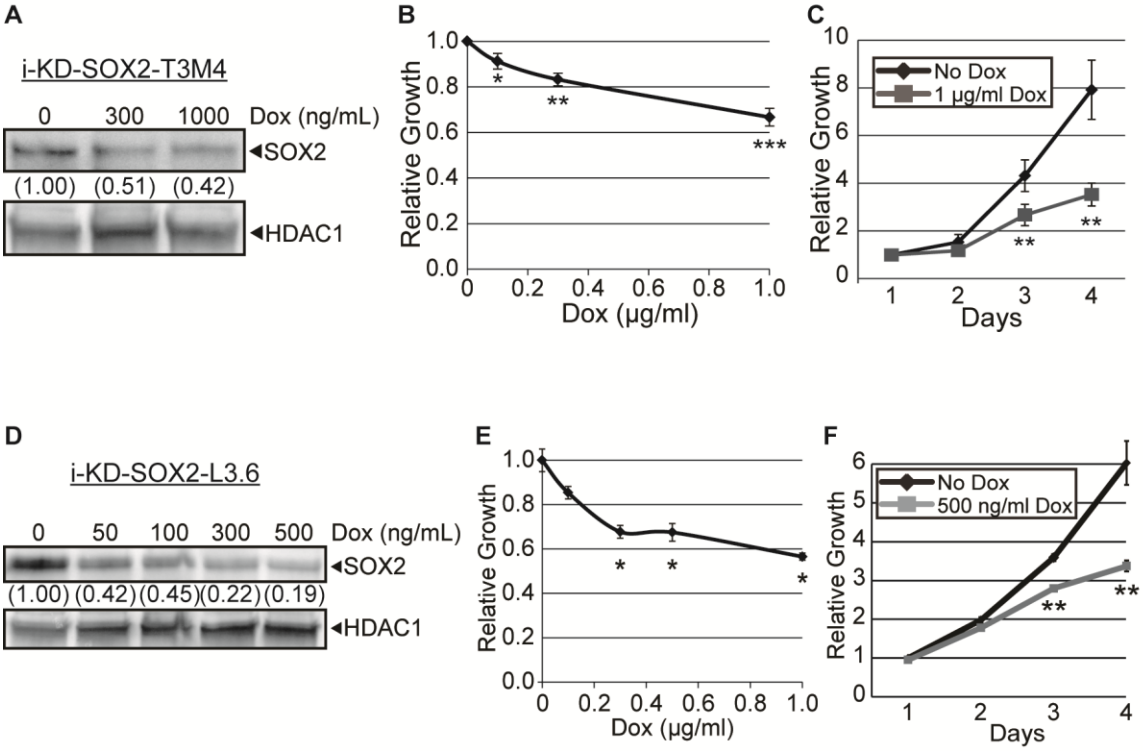

Figure 4.4: Knockdown of SOX2 in i-KdSOX2-T3M4 and i-KdSOX2-L3.6 cells reduces cellular growth. (A) Western blot analysis of SOX2 was performed using whole cell extracts from i-KdSOX2-T3M4 cells following 3 days of Dox-induction of the shRNA targeting SOX2. The level of SOX2 was compared to that in the untreated sample and HDAC1 protein was used as a loading control. **(B)** Cell growth of i-KdSOX2-T3M4 was examined by MTT assay following 4 days growth at the indicated Dox doses. Growth in the absence of Dox was set to 1. **(C)** Proliferation of i-KdSOX2-T3M4 cells in the presence or absence of Dox (1 µg/ml) over a 4-day period was determined by MTT assays. These studies were repeated and similar results were obtained. **(D)** Western blot analysis of SOX2 was performed using whole cell extracts from i-KdSOX2-L3.6 cells following 2 days of Dox-induction of the shRNA targeting SOX2. The knockdown of SOX2 was compared to SOX2 levels in the untreated sample. HDAC1 protein was used as a loading control. **(E)** Proliferation of i-KdSOX2-L3.6 was examined by MTT assay following 4 days growth at the indicated Dox concentrations. **(F)** Growth of i-KdSOX2-L3.6 cells over 4 days was determined by MTT assay following growth in the presence or absence of Dox (500 ng/ml). Error bars represent standard deviation and p values were determined by student's t-test (*$p<0.05$, **$p<0.01$, and ***$p<0.005$). The studies in shown in A, C, D, and F were repeated and similar results were obtained.

Figure 4.5

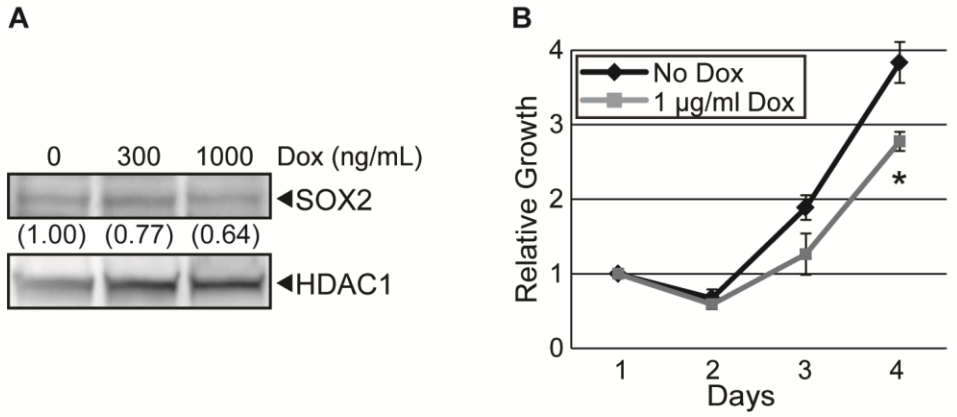

Figure 4.5: Knockdown of SOX2 with a second shRNA vector cells reduces cellular growth. **(A)** Western blot analysis of SOX2 was performed using whole cell extracts from T3M4 cells engineered with a second shRNA targeting SOX2 following 2 days of Dox-induction. The level of SOX2 was compared to that in the untreated sample and HDAC1 protein was used as a loading control. **(B)** Proliferation of T3M4 cells engineered with the second SOX2 shRNA in the presence or absence of Dox (1 µg/ml) over a 4 day period was determined by MTT assays. Error bars represent standard deviation and p values were determined by student's t-test (*$p<0.05$).

mm^3 – a reduction of ~70% (Figure 4.6A). Immunohistochemical staining for the proliferation marker Ki-67 was reduced ~50% in the tumor cell compartment of the Dox-treated tumors compared to untreated tumors ($p<0.05$, Figure 4.6C-E). In a smaller study, i-KdSOX2-T3M4 cells were engrafted subcutaneously into NCr-nu/nu mice. Once palpable tumors had formed by engrafted i-KdSOX2-T3M4 cells, mice with sized-matched tumors were randomly assigned to the control or the Dox-treated group. After an additional 16 days, the tumors in the control group increased from an average of 50 mm^3 to an average of 330 mm^3; whereas tumors in the Dox group increased from an average of 70 mm^3 to an average of 135 mm^3 (Figure 4.7). While this small study was not statistically significant, is does lend credence to the previous study with i-KdSOX2-L3.6 cells in which similar results were seen. Altogether, our studies demonstrate that either increasing SOX2 (Figure 4.3) or decreasing SOX2 reduces the growth of tumors. Thus, the tumorigenicity of these cells is highly dependent on the expression of optimal levels of SOX2.

4.6 Summary

As discussed in the Introduction of this dissertation, SOX2 levels have been studied in many different cancer types, with variable results. In this study, we demonstrate that inducibly either increasing or decreasing levels of SOX2 in PDAC cells reduces growth both *in vitro* and *in vivo*. Prior to the work described here, stable overexpression of SOX2 in a PDAC cell line had been shown to increase cell proliferation *in vitro*. We reexamined the role of SOX2 in PDAC, because we had previously determined that inducible elevation of SOX2 in various types of tumor cells leads to growth inhibition rather than growth promotion [172]. Importantly, the work described here demonstrates that this is also true for PDAC cells. Specifically, we demonstrate that inducible elevation of SOX2 in three different PDAC cell lines *in vitro* leads to growth inhibition, rather than growth stimulation. We also determined that increases in SOX2 lead to a reduction in tumorigenicity. Under no conditions was growth observed to increase when SOX2 levels were elevated from an inducible promoter.

Figure 4.6

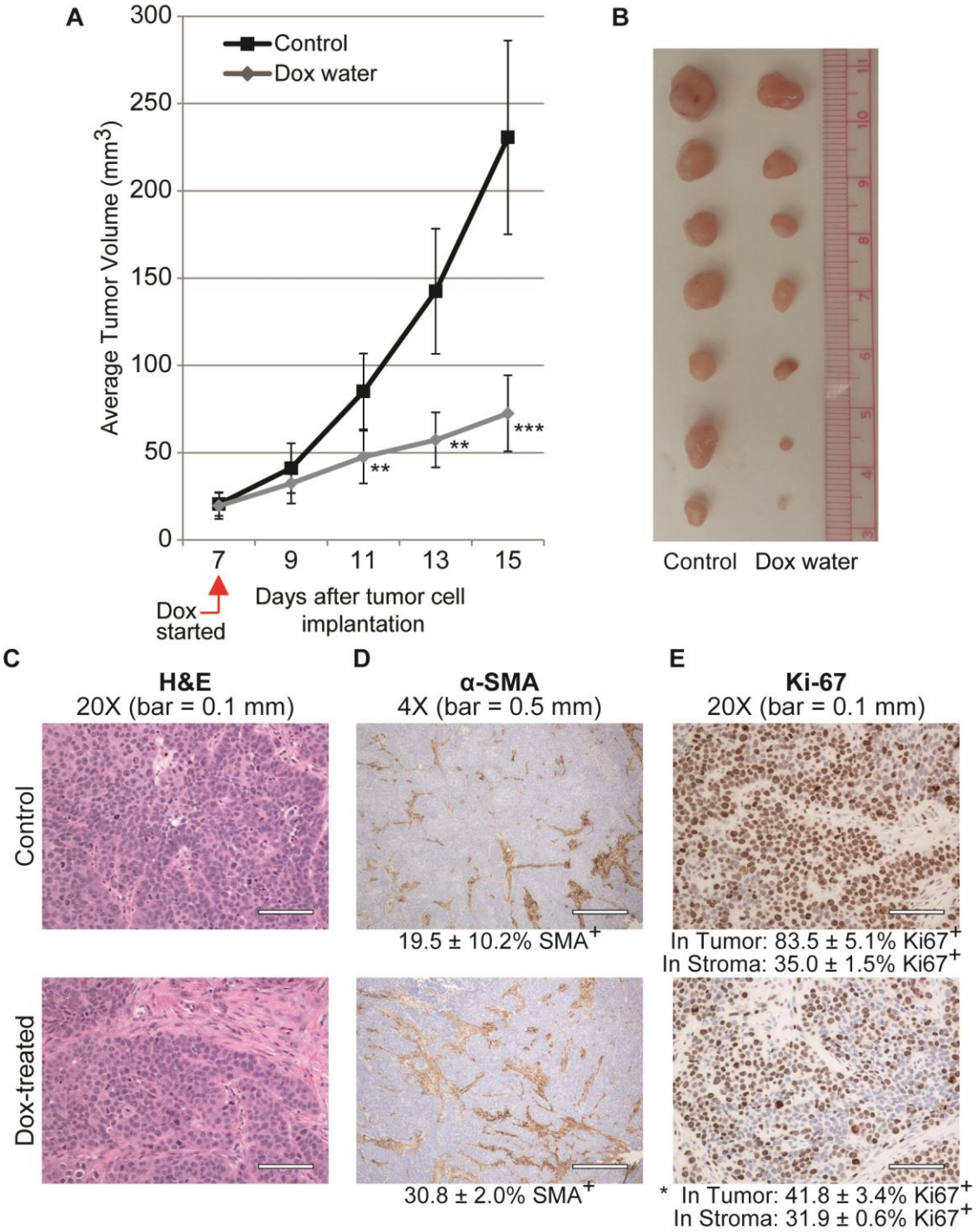

Figure 4.6 Knockdown of SOX2 in i-KdSOX2-L3.6 cells reduces subcutaneous tumor growth. **(A)** Subcutaneous tumor volumes were determined for each group of 7 mice as described in Chapter 2. Average tumor volumes are presented for control and Dox-treated groups. Error bars represent standard error of the mean. **(B)** Tumors were excised after a total 15 days of tumor growth. Subcutaneous tumor sections were stained for **(C)** H&E, **(D)** SMA, and **(E)** Ki-67 as described in Chapter 2. Staining was quantified and statistical significance was determined by student's t-test (*p<0.05, **p <0.01, ***p<0.005).

Figure 4.7

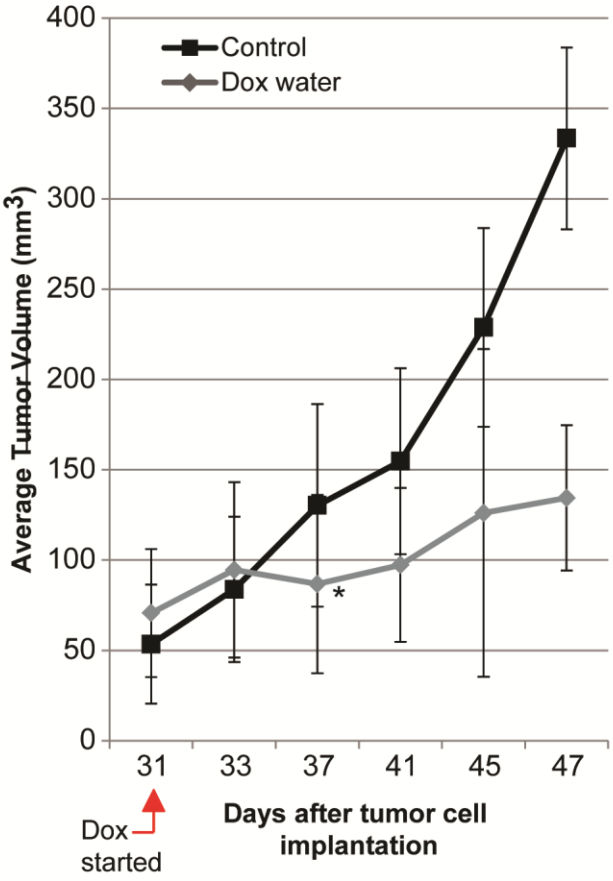

Figure 4.7 Knockdown of SOX2 in i-KdSOX2-T3M4 cells reduces subcutaneous tumor growth. Subcutaneous tumor volumes were determined for each group of 4 mice as described in Chapter 2. Average tumor volumes are presented for control and Dox-treated groups. Error bars represent standard error of the mean and statistical significance was determined by student's t-test (*$p<0.05$).

Although inducible elevation of SOX2 leads to PDAC growth inhibition *in vitro* as well as a substantial reduction in tumor growth, this does not indicate that SOX2 plays little or no role in promoting the growth of PDAC. Previous studies had shown that knockdown of SOX2 in four different PDAC cells lines reduces growth *in vitro* [155]. In the work presented here, we not only demonstrate that knockdown of SOX2 reduces growth *in vitro*; we also demonstrate that tumor growth of i-KdSOX2-L3.6 cells is reduced when SOX2 is knocked down *in vivo*. Thus, SOX2 is clearly required for the growth of PDAC both *in vitro* and *in vivo*. Equally important, our studies indicate that endogenous levels of SOX2 in PDAC cells are optimized for maximum growth, as both increases and decreases in SOX2 reduce PDAC cell growth. Hence, SOX2 functions as a biphasic molecular rheostat in the control of PDAC cell proliferation. Coupled with our demonstration that this is also true for ES cells [17] and four other tumor cell types [172], we suggest that this is a defining feature of SOX2. Going forward, it will be essential to gain a much deeper understanding of how SOX2 influences the growth of PDAC, and the genetically engineered PDAC cell lines described in this dissertation should provide a highly useful platform for addressing this question.

CHAPTER 5: Inducible modulation of SOX2 levels alters the efficacy of drugs used clinically

The studies described in this chapter, which were conducted by Erin Wuebben, and are published in Oncotarget [202].

5.1 Introduction

SOX2 has been implicated in drug resistance in a number of cancers including glioblastoma, bladder, breast, gastric, head and neck, lung, ovarian, and prostate cancers [129, 137, 141, 142, 151, 152, 154, 158, 163, 168, 169]. As described in Chapter 4, SOX2 levels must be tightly regulated to maintain the growth of PDAC cells *in vitro* and *in vivo*, but no studies have examined the effects of SOX2 on drug resistance in pancreatic cancer. The Introduction of this dissertation outlined the desperate need to identify new therapeutic targets for PDAC as current therapeutics have yet to improve the high mortality rate for patients with PDAC. Many genes and signaling pathways have been shown to be aberrantly activated in PDAC; the most common of these is a constitutively activated KRAS mutation. Tumors with KRAS mutations are highly dependent on upregulated AKT and RAF/MEK/ERK signaling, which are downstream of KRAS [179-182], and thus, a variety of PDAC clinical trials have tested AKT inhibitors (AKTi), e.g. MK-2206, and at least five MEK inhibitors (MEKi), e.g. trametinib [183]. Disappointingly, these drugs have not produced significant responses in PDAC clinical trials, which has led to the general belief that PDAC is largely resistant to AKTi and MEKi. Given the association reported for SOX2 and drug resistance in several other cancers, in the following studies we examined how changes in the levels of SOX2 influence the responses of PDAC cells to MEKi and AKTi used in clinical trials.

5.2 Inducible Elevation of SOX2 Alters Cell Cycle Effects in the Presence of MEKi

To begin to understand the impact of altering SOX2 levels on the growth responses of PDAC cells to drugs used in PDAC clinical trials, we initially examined how elevating SOX2 influences the cell cycle of i-SOX2-T3M4 cells when treated with five MEKi that have been, or that are currently, used in PDAC clinical trials. For this purpose, we initially determined the EC50 for each MEKi exhibited by i-SOX2-T3M4 cells based on the reduction in growth over a 4

day period (Table 2.1). Additionally, we confirmed the suppression of ERK1/2 phosphorylation when MEKi are used at their EC50 (Figure 5.1). When used at their respective EC50, each of the MEKi led to a sizable increase in the G1 population of the cells and a sizable decrease in S-phase after 48 hr (Figure 5.2). As expected, elevating SOX2 by treatment with Dox also altered the cell cycle of i-SOX2-T3M4. However, there was only a modest increase in G1 and a modest decrease S-phase. Remarkably, when the cells were treated simultaneously with Dox and any of the five MEKi, we observed a partial reversal of the cell cycle changes observed with each MEKi on its own. More specifically, the increase in G1 and the reduction of S-phase observed with the MEKi was partially reduced when SOX2 levels were elevated in the cells (Figure 5.2A). Interestingly, each of the five MEKi induced pronounced morphological changes exemplified by significant cell spreading, and this effect was also partially reversed when SOX2 was inducibly elevated (Figure 5.2B).

5.3 Elevation of SOX2 Partially Reverses the Reduction in Clonal PDAC Growth Due to Treatment with Trametinib

To more carefully assess the effects of elevating SOX2 on the growth responses of PDAC cells when treated with MEKi, we examined the clonal growth of i-SOX2-T3M4 cells cultured in the presence of one of the MEKi (trametinib) with and without Dox. For this purpose, 24 hr after the cells had been subcultured, trametinib and/or Dox were added to the cells where indicated. During the following 8 days, the cells were refed with fresh medium containing trametinib and/or Dox every other day. After 8 days of treatment, the number of colonies formed when the cells were treated with trametinib at its EC50 was significantly reduced. However, treatment with both trametinib and Dox led to a much smaller reduction in colony number (Figure 5.3). As a control, we determined that treatment of parental T3M4 cells with Dox did not affect the dose response curves of trametinib or a second MEKi, selumetinib (data not shown).

Figure 5.1

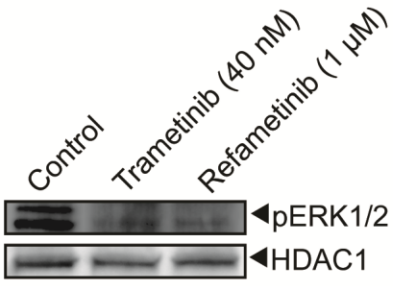

Figure 5.1: Phosphorylation of ERK is suppressed following MEK inhibition.
Western blot analysis of ERK phosphorylation in whole cell extracts from i-SOX2-T3M4 cells treated with the MEK inhibitors trametinib and refametinib for 48 hours at their EC50. HDAC1 protein was used as a loading control.

Figure 5.2

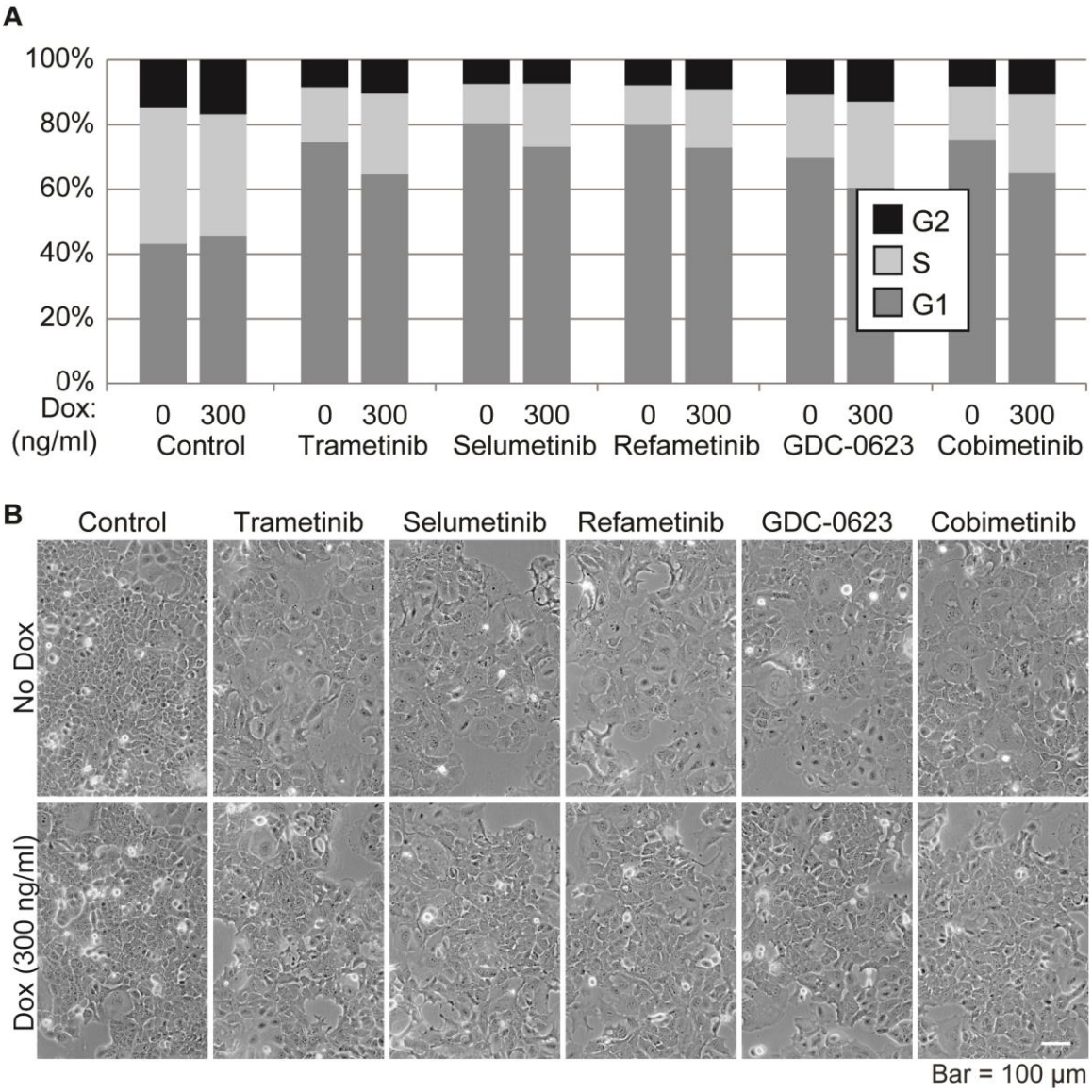

Figure 5.2: Effects of SOX2 overexpression and treatment with MEK inhibitors on the cell cycle of i-SOX2-T3M4 cells. (A) Cell cycle analysis was performed by flow cytometry on i-SOX2-T3M4 cells treated with each MEK inhibitor for 48 hr at their respective EC50 in the presence or absence of Dox (300 ng/ml). (B) Representative photomicrographs of i-SOX2-T3M4 cells following growth with each inhibitor in the presence or absence of Dox (300 ng/ml). Cells in all panels were photographed at the same magnification.

Figure 5.3

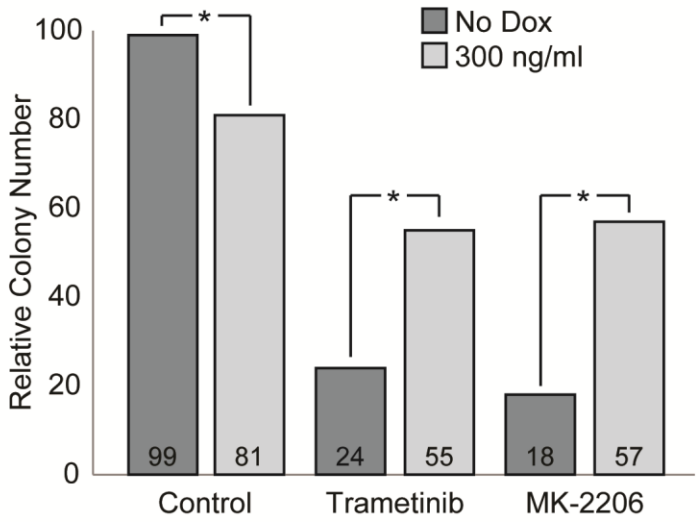

Figure 5.3: Cloning efficiency of i-SOX2-T3M4 cells is reduced by treatment with trametinib or MK-2206 and is partially reversed by overexpression of SOX2. (A) Clonal growth was determined 8 days after plating i-SOX2-T3M4 cells at 80 cells per cm^2. The cells were treated for 8 days with trametinib (40 nM) or MK-2206 (2 μM) in the presence or absence of Dox (300 ng/ml) where indicated. Colony number was determined by an observer unaware of sample designation in 15 random fields. These studies were repeated and similar results were obtained. Statistical significance was determined by student's t-test (*$p<0.05$); studies were repeated multiple times and similar results were obtained.

5.4 Replating Efficiency of Trametinib-treated PDAC Cells is Improved by SOX2 Elevation

To further evaluate the effects of trametinib on i-SOX2-T3M4 cells, the cells were cultured for 6 days at typical cell culture densities ($1.2x10^4/cm^2$) in the presence of trametinib with or without Dox. After 6 days, cells treated with trametinib or trametinib plus Dox both exhibited a change in morphology (Figure 5.4A) relative to untreated i-SOX2-T3M4 cells (Figure 5.2B), but the cells treated with trametinib on its own exhibited the most pronounced morphological change. Next, the trametinib treated and the trametinib plus Dox treated cells were subcultured and replated at clonal densities in the absence of trametinib and Dox. Although the trametinib treated cells and the trametinib plus Dox treated cells were replated at equal cell numbers, the cloning efficiency of the trametinib plus Dox treated cells was substantially higher than those treated with trametinib on its own (Figure 5.4B). As a control, we determined that pre-treatment with Dox on its own does not improve the cloning efficiency of i-SOX2-T3M4 cells when replated. In fact, treatment with Dox on its own for 6 days prior to replating in medium lacking Dox reduces cloning efficiency ~50%. Interestingly, the morphology of the few colonies formed from the trametinib treated cells continued to exhibit a flattened morphology; whereas the colonies formed from the trametinib plus Dox treated cells exhibited morphology much closer to that of untreated i-SOX2-T3M4 cells. In addition, we observed a similar differential in the number of colonies formed when the trametinib, and trametinib plus Dox treated cells were replated and grown under anchorage-independent conditions in serum-free, stem cell medium (Figure 5.4C). Thus, even though elevating SOX2 on its own inhibits the proliferation of i-SOX2-T3M4 cells, elevating SOX2 in these cells reduces the growth inhibitory effects of trametinib under more than one condition.

5.5 Drug Resistant Effects of Elevated SOX2 Are Not Cell Line Specific

To determine whether the protective effects of elevating SOX2 were cell line dependent,

Figure 5.4

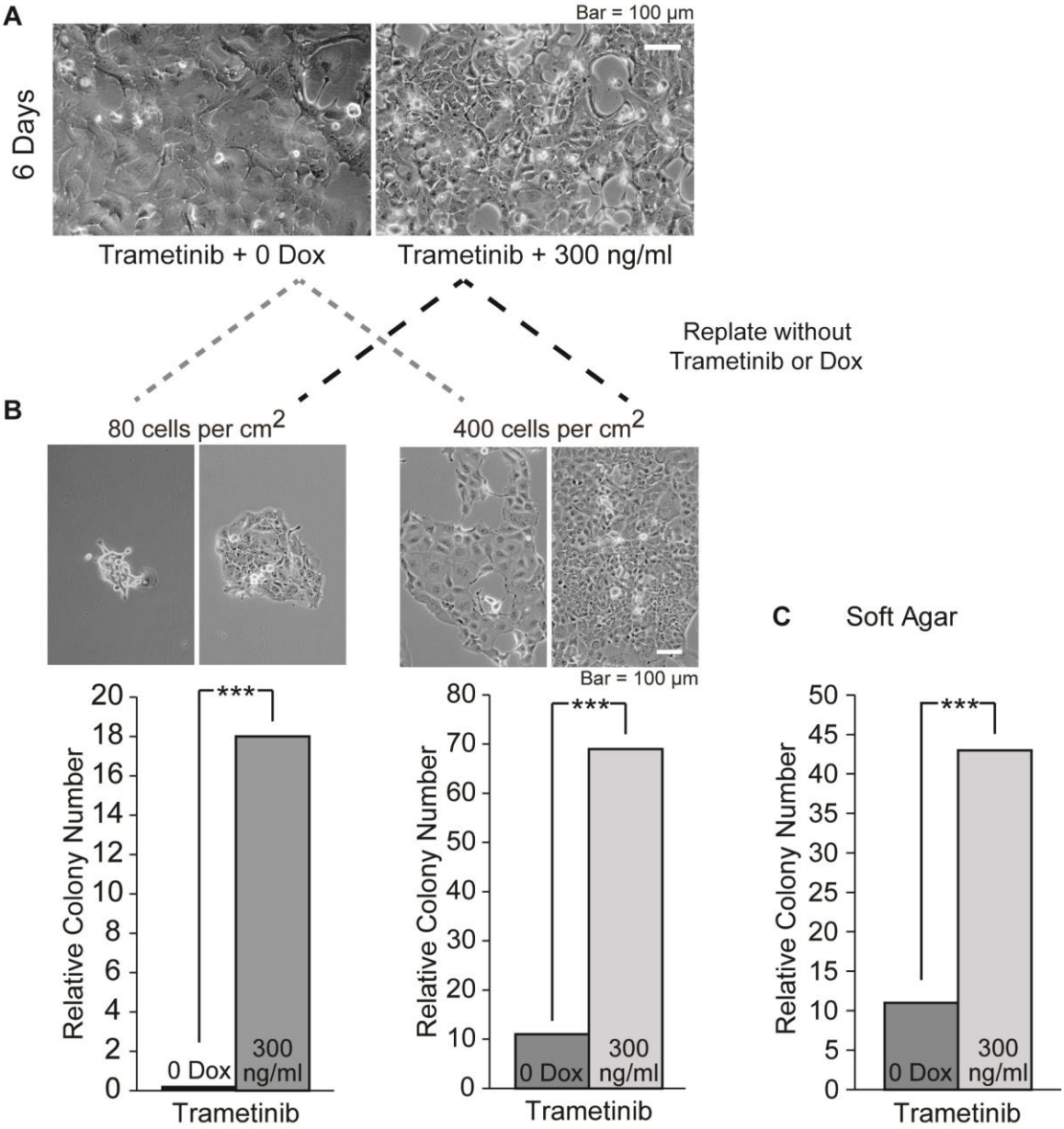

Figure 5.4: Replating efficiency of i-SOX2-T3M4 cells is reduced by treatment with trametinib or MK-2206 and is dramatically improved by overexpression of SOX2.
(A) i-SOX2-T3M4 cells seeded at $1.2x10^4/cm^2$ were treated for 6 days with trametinib (40 nM) in the presence or absence of Dox (300 ng/ml) and representative photomicrographs were taken at the same magnification. **(B)** After 6 days, the pre-treated cells were subcultured and plated in monolayer culture at 400 cells per cm^2 without trametinib or Dox. After 7 days the number of colonies formed was determined as described in Chapter 2 and representative photomicrographs were taken at the same magnification. **(C)** After 6 days, the pre-treated cells were subcultured at 800 cells per cm^2 and grown in soft agar containing serum-free, stem cell medium without trametinib and Dox. After 10 days colony numbers were determined as described in Chapter 2. Statistical significance was determined by student's t-test (*$p<0.05$ and ***$p<0.005$). The studies shown in A were repeated multiple times and similar results were obtained.

we examined how elevation of SOX2 influenced the clonal growth of i-SOX2-BxPC3 cells and i-SOX2-HPAF-II cells. As in the case of i-SOX2-T3M4 cells, inducible elevation of SOX2 also reduced the inhibitory effects of trametinib on the clonal growth of i-SOX2-BxPC3 cells and i-SOX2-HPAF-II cells (Figure 5.5). For these studies, trametinib was used at the EC50 for i-SOX2-BxPC3 cells and i-SOX2-HPAF-II cells (Table 2.1). Thus, the protection afforded by elevating SOX2 was not limited to i-SOX2-T3M4 cells. Equally important, the protective effect of SOX2 was not limited to trametinib. Inducible overexpression of SOX2 in i-SOX2-T3M4, i-SOX2-BxPC3, and i-SOX2-HPAF-II cells also reduced the inhibitory effects of the AKTi, MK-2206 (Figure 5.3, Figure 5.6). Again, MK-2206 was used at the EC50 for each PDAC cell line (Table 2.1). Altogether, our studies show that although inducible elevation of SOX2 on its own reduces the clonal growth of three different PDAC cell lines, elevating SOX2 in these cells partially reverses the growth inhibitory effects of trametinib and MK-2206.

5.6 Knockdown of SOX2 Enhances Growth Inhibitory Effects of MEKi and AKTi

Finally, we examined whether knocking down SOX2 in PDAC cells would lead to further reduction in growth when the cells were treated with trametinib or MK-2206. Initially, we addressed this question using i-KdSOX2-T3M4 cells. As in the case of i-SOX2-T3M4 cells, treatment of i-KdSOX2-T3M4 cells with trametinib or MK-2206 each reduced the number and the sizes of the colonies that formed when the cells were plated at clonal densities (Figure 5.7A). Importantly, knocking down SOX2 in conjunction with trametinib or MK-2206 led to a further reduction in the number of colonies that formed. Like i-KdSOX2-T3M4 cells, growth of i-KdSOX2-L3.6 cells at clonal densities was reduced by trametinib and MK-2206 (Figure 5.7B). Moreover, growth of these cells was reduced even further when SOX2 was knocked down and the cells were treated with drug. Thus, these findings, in conjunction with the SOX2 overexpression studies described earlier, strongly support the conclusion that SOX2 helps protect PDAC cells from the growth inhibitory effects of MEKi and AKTi.

Figure 5.5

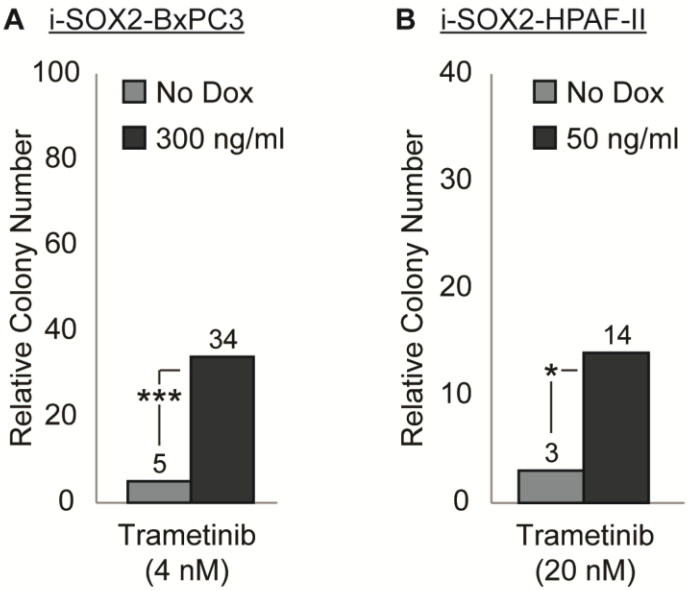

Figure 5.5: Cloning efficiency of PDAC cells is reduced by treatment with trametinib and partially reversed by overexpression of SOX2. I-SOX2-BxPC3 **(A)** and i-SOX2-HPAF-II **(B)** cells were subcultured at 80 cells per cm^2. After 8 days treatment with trametinib (4 nM and 20 nM, respectively) in the presence or absence of Dox (300 ng/ml and 50 ng/ml, respectively) colony numbers were determined. Statistical significance was determined by student's t-test (*$p<0.05$, ***$p<0.005$). The studies shown in A and B were repeated and similar results were obtained.

Figure 5.6

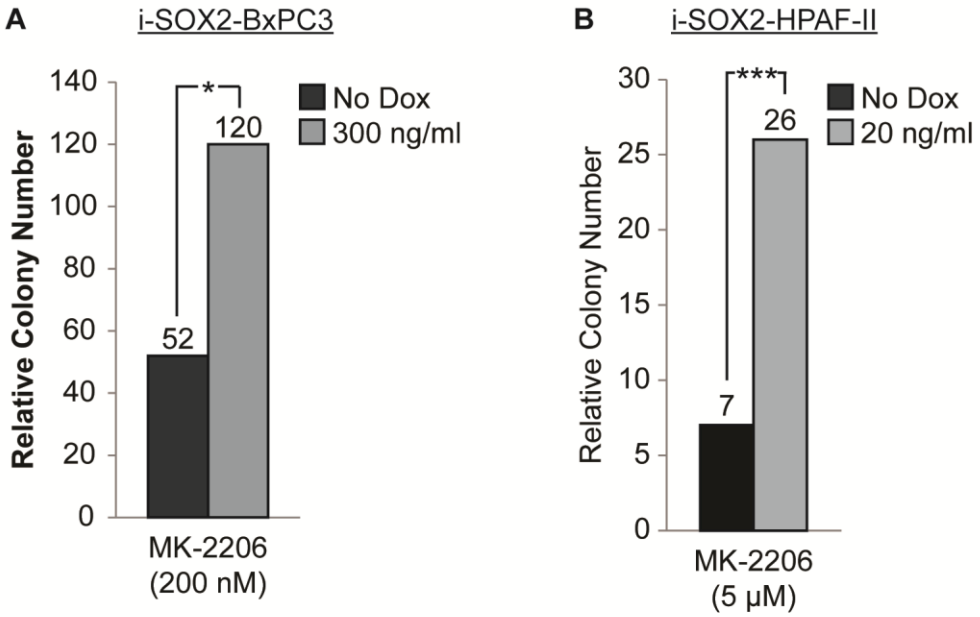

Figure 5.6: Cloning efficiency of PDAC cells is reduced by treatment with MK-2206 and partially reversed by overexpression of SOX2. (A) Clonal growth was determined by subculturing i-SOX2-BxPC3 (A) and i-SOX2-HPAF-II **(B)** cells at 80 cells per cm^2. After 8 days treatment with MK-2206 (200 nM and 5 μM, respectively) in the presence or absence of Dox colony numbers were determined. Statistical significance was determined by student's t-test (*$p<0.05$, ***$p<0.005$). The studies shown in A and B were repeated and similar results were obtained.

Figure 5.7

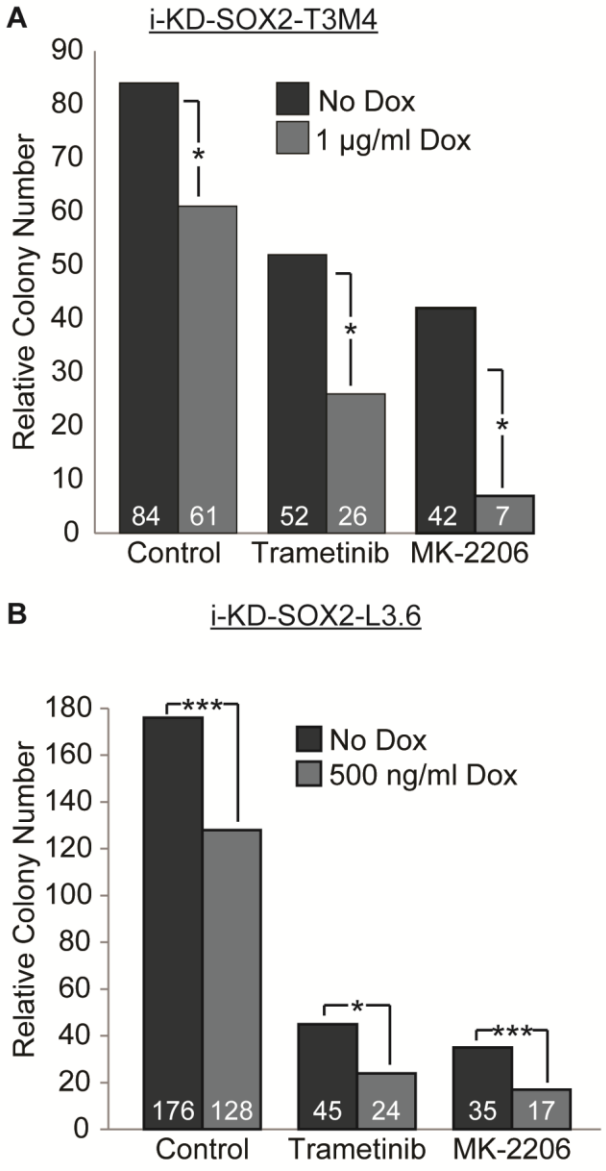

Figure 5.7: Knockdown of SOX2 in i-KdSOX2-T3M4 and i-KdSOX2-L3.6 cells reduces cloning efficiency and enhances the growth inhibiting effects of trametinib and MK-2206. **(A)** Clonal growth was determined by subculturing i-KdSOX2-T3M4 cells at 80 cells per cm^2. After 8 days of treatment with trametinib (40 nM) or MK-2206 (2 µM) in the presence or absence of Dox (1 µg/ml), colony numbers were determined. (B) Cloning efficiency of i-KdSOX2-L3.6 cells was determined after 8 days of treatment with trametinib (20 nM) or MK-2206 (200 nM) in the presence or absence of Dox (500 ng/ml. Statistical significance was determined by student's t-test (*$p<0.05$ and ***$p<0.005$). The studies shown in A and B were repeated and similar results were obtained.

5.7 Summary

Recent work has shown that SOX2 is not only expressed in ~20 different types of human cancer [12, 125, 129, 130, 132, 141, 148, 149, 151-155, 158-160, 162, 163, 167, 168, 170, 209], it also appears to influence drug resistance in at least eight of these cancers [129, 137, 141, 142, 151, 152, 154, 158, 163, 168, 169]. SOX2 had been shown to be expressed in PDAC [125, 155], but its roles in tumor growth and drug resistance had not been examined prior to work described in this dissertation. In this study, we demonstrate that elevating the levels of SOX2 reduces the efficacy of several MEKi, including trametinib, and the AKTi MK-2206, which have thus far yielded disappointing patient responses in PDAC clinical trials. Our studies indicate that the effects of SOX2 on the responses to trametinib and MK-2206 are not dependent on the mutation status of KRAS. Going forward, it will be valuable to determine how SOX2 can reduce the action of MEKi and AKTi. As discussed in Chapter 6, targeting SOX2 or its mode of action could improve the effectiveness of these drugs against PDAC.

CHAPTER 6: Conclusions & Future Directions

6.1 Overview

The studies presented in this dissertation examined the diverse roles of not only SOX2, but also the SOX2-associated protein MSI2 in multiple cell types. Previously, our laboratory performed proteomic screens in ESC, medulloblastoma cells, and glioblastoma cells. Interestingly, MSI2 interacted with SOX2 in each of these cell types, meriting further study of its role in ESC and brain tumor cells. We determined that Msi2 is required for ESC, as knockdown of both Msi2 isoforms induces the differentiation of ESC and reduces their cloning efficiency, which is not rescued by the overexpression of either Msi2 isoform alone. Furthermore, our studies of MSI2 in brain tumor cells determined that MSI2 is required for their continued proliferation, as knockdown of MSI2in DAOY medulloblastoma cells, and in U87 and U118 glioblastoma cells, dramatically reduces cellular growth.

We extended our study of SOX2 by examining its role in PDAC and found that SOX2 is necessary for the growth and proliferation of PDAC cells, and that SOX2 functions as a biphasic molecular rheostat in PDAC, as both small increases and small decreases in SOX2 levels dramatically alter PDAC growth both *in vitro* and *in vivo*. Furthermore, drug resistance of PDAC tumor cells was altered when SOX2 levels were changed. Elevating SOX2 protected PDAC cells from the growth inhibitory effects of MEK and AKT inhibitors, but the knockdown of SOX2 enhanced the growth inhibition in the presence of these drugs.

The need for continued study in both of these areas is well established. In the sections below, the broader outlook and global implications of these studies are examined. Moreover, future perspectives for carrying this research forward are also presented.

6.2 MSI2 is required for Embryonic Stem Cells and Brain Tumor Cells

The studies presented in Chapter 3 of this dissertation demonstrate that the SOX2-associated protein MSI2 is a required protein in both ESC and two types of brain tumor cells. In ESC, we determined that the knockdown of Msi2 disrupts the self-renewal of ESC and induces

their differentiation. Furthermore, the extent of differentiation and the loss of self-renewal capacity correlates with the extent to which Msi2 levels were decreased. Remarkably, the knockdown of Msi2 causes ESC to differentiate despite continued expression of both Sox2 and Oct4. Similar results were observed in a related study from this laboratory, in which barrier to autointegration factor 1 (Banf1) was knocked down in human ESC [97]. In that report, human ESC lost their capacity for self-renewal following Banf1 knockdown even though the localization and nuclear expression of Sox2 and Oct4 did not change. Thus, it would suggest that the knockdown of Msi2, like the loss of Banf1, may alter the critical balance of Sox2 and Oct4 relative to other essential proteins required for the self-renewal and pluripotency of ESC, rather than their absolute levels.

Our studies raise an important question. How does Msi2 regulate the behavior of ESC and brain tumor cells? Previous studies demonstrate that Msi1 binds to target mRNA transcripts to prevent their association with the ribosome and other translation machinery [99]. If Msi2 functions through a similar mechanism to block the translation of several critical RNAs, it will be important to determine which RNAs are targeted. Moreover, it would be interesting to compare which transcripts Msi2 targets in different cellular contexts, including ESC, hematopoietic stem cells, brain tumor cells, and additional cancer cell types. Studies of differential RNA expression following knockdown of MSI2 in brain and other tumor cells could identify critical networks altered by MSI2 expression. Furthermore, RNA-Seq analyses of the different transcriptomes would yield insight into molecular mechanisms necessary for the growth of SOX2- and MSI2-expressing tumors.

In the future, it will be important to determine whether Msi2 plays a role during embryogenesis. Although our studies argue that ESC require Msi2, gene ablation studies argue that Msi2 is not absolutely required for embryogenesis [103, 104]. However, it remains to be determined whether the reduced frequency of null Msi2 mice is the result of minor defects during embryogenesis [103, 104]. Moreover, it is possible that Msi1 can compensate for the absence of

Msi2 during embryogenesis, but is unable to for ESC grown in culture. Thus, much remains to be discovered regarding the functions and interactions of MSI2 across multiple cell types.

6.3 SOX2 Expression must be maintained at Optimal Levels

Work presented in Chapter 4 of this dissertation demonstrates that SOX2 is clearly required for the growth of PDAC, as knockdown of SOX2 reduces PDAC growth both *in vitro* and *in vivo*. Furthermore, our studies indicate that endogenous levels of SOX2 in PDAC cells are optimized for maximum growth, as both increases and decreases in SOX2 reduce PDAC cell growth. Hence, SOX2 functions as a biphasic molecular rheostat to control PDAC cell proliferation.

The finding that SOX2 levels need to be maintained at optimal levels was first described in ES cells, and later in four other tumor types [172]. In ES cells, knockdown of SOX2 or a 2-fold increase in SOX2 disrupts the self-renewal of ES cells and triggers their differentiation [17]. The need to maintain SOX2 levels within narrow limits is not surprising when one examines the SOX2-interactome in different cell types. Proteomic analysis of the SOX2-interactome in ES cells, as well as medulloblastoma cells and glioblastoma cells, indicates that SOX2 associates in high molecular weight protein complexes with a large and diverse set of nuclear proteins [80, 93, 210]. In ES cells, SOX2 is part of a highly integrated transcriptional circuitry that involves multiple master regulators known to control the self-renewal and pluripotency of ESC [93, 95]. Moreover, SOX2 and the other master regulators that it associates with in ES cells each form complexes with many of the same proteins. As a result, a small increase in the level of SOX2 is likely to lead to the formation of incomplete protein complexes that are essential for ES cells. Moreover, the potent biological impact of small changes in SOX2 levels seems all the more likely because SOX2 forms complexes with a wide variety of proteins involved in many critical cellular processes. In addition to transcription, SOX2 forms complexes with proteins involved in signal transduction, DNA repair [80, 93, 210], ubiquitination pathways [80], and RNA binding (MSI2,

as described in Chapter 3 of this dissertation). Thus, even small disturbances in SOX2 levels can lead to waves of change throughout multiple signaling networks.

6.4 Strategies for Experimentally Altering SOX2 Levels

Unfortunately, even with abundant evidence pointing to links between SOX2 levels and tumor growth, there is a lack of consensus regarding the best methods to study changes in SOX2 levels. Some studies outlined in the Introduction report that stable overexpression of SOX2 in PDAC enhances growth, while the studies described in Chapter 4 indicated that inducible overexpression of SOX2 in fact reduces growth. The contrasting results obtained studying SOX2 by stable overexpression and inducible overexpression are likely to result from the fundamental difference in experimental design. Cell lines engineered for inducible overexpression of SOX2 were generated via drug selection of lentiviral transduced cells, which occurred at frequencies greater than 70%, before SOX2 levels were altered. In direct contrast, cell lines engineered to stably overexpress SOX2 are subjected to drug selection while SOX2 levels are ectopically elevated. As a result, any cells that are growth inhibited or grow more slowly due to elevated levels of SOX2, as we have shown is the case for three different PDAC cell lines, will be lost during the drug selection period as the faster proliferating cells expand. Consequently, the cells present in the drug selected population represent only a subpopulation of the parental cells. Importantly, the studies presented in Chapter 4 of this dissertation argue that this subpopulation is likely to represent a very small minority of PDAC cells. This is especially clear in the case of i-SOX2-T3M4 cells. Continual growth of these cells in the presence of Dox for >10 passages failed to lead to the emergence of cells that grow faster due to the elevation of SOX2.

6.5 Increases in SOX2 Expression during Tumor Progression must be accompanied by Compensatory Changes in Other Key Signaling Proteins

It is evident from the studies where SOX2 was elevated from an inducible transgene that

many, if not nearly all, SOX2-expressing tumor cell lines are growth inhibited when SOX2 is initially elevated. However, this does not mean that SOX2 expression cannot rise during cancer. In fact, several lines of evidence argue that increases in the levels of SOX2 undoubtedly occur during oncogenesis. As discussed earlier, the *SOX2* gene is amplified in several cancers [105, 107, 108, 110], and SOX2 has been shown to be expressed in some tumors, but not in their surrounding tissue. Moreover, in some tumors, SOX2 expression has been shown to increase during tumor progression [106, 123, 126, 128, 132-134] and high levels of SOX2 correlate with poor prognosis in many cancers [124, 139, 140, 144, 148]. This raises a fundamental question. If SOX2 levels must be maintained within optimal limits to promote tumor growth, how can SOX2 levels rise during tumor progression? It is likely that SOX2 must function within the constraints of its protein-protein interaction network, and therefore increases in the levels of SOX2 must be accompanied by corresponding changes in the expression of other genes that counterbalance the growth inhibitory effects of elevated SOX2. Or more specifically, accommodating the increases in SOX2 expression would require increased expression of genes required for growth promotion by SOX2 and/or downregulation of genes that interfere with the action of SOX2 when its levels rise during tumor progression. Clearly, changing SOX2 levels in isolation disrupts cell function. SOX2 is by no means unique in this regard. Our studies suggest that the effects of SOX2 are highly context-dependent, similar to other genes, notably TGFβ, which can act as a tumor suppressor or oncogene. As another example, *MAP3K7* and *CHD1* have been shown to be co-deleted in prostate cancer and their co-deletion in ETS rearrangement-negative prostate cancers correlates with poor disease-free survival [211]. In a mouse xenograft model of prostate cancer, knockdown of MAK3K7 on its own had no significant effect on survival, and knockdown of CHD1 on its own enhanced survival. However, combined knockdown of MAK3K7 and CHD1 led to larger tumor volumes and shorter survival [211]. Accordingly, the identification and targeting of genes that must change in concert with increases in SOX2 and permit SOX2 to contribute to tumorigenicity could provide a novel strategy for blocking, or at least, reducing the

growth of tumors dependent on SOX2.

6.6 Tumor Cells do not Exhibit a Single Optimum for SOX2 Expression

In addition to intracellular changes taking place to balance the increased expression of SOX2 within individual cells, there may also be a rise in the number of SOX2-positive cells in the tumor population. Such a shift in the distribution of the tumor cell population may also contribute to the apparent rise in SOX2 during tumor progression. As shown previously, SOX2 protein levels vary considerably between different PDAC cell lines [155]. In the case of T3M4 and L3.6 PDAC cells, SOX2 expression differs by ~15-fold across cell lines [202]. Furthermore, we determined by immunofluorescence that SOX2 expression is not distributed evenly among L3.6 PDAC cells [~10% of the cells endogenously express SOX2 at high levels ($SOX2^{hi}$), ~30% at moderate levels, and ~60% at low/undetectable levels ($SOX2^{low}$), Figure 6.1].Thus, PDAC cells do not exhibit a single optimum for SOX2 expression. If SOX2 is required for the tumor-initiating/cancer stem cell population, which is the case for at some, if not most SOX2-expressing cancers, the proportion of SOX2-positive cells in the tumor may rise as the population of tumor-initiating cells increases during tumor progression. Correspondingly, increases in a SOX2-positive tumor-initiating cell population would account for the decreased survival and worse prognosis seen in many SOX2-expressing patient tumors [124, 133, 137-145].

6.7 SOX2 as a Potential Tumor-Initiating Cell Marker

The close examination of current literature presented in the Introduction revealed that SOX2 is expressed in over 20 different tumors. Importantly, of these SOX2-positive tumors, most studies have concluded from indirect evidence that SOX2 positive cells are likely to represent the tumor-initiating cell population of these tumors. However, the only direct evidence for the role of SOX2 in the tumor-initiating cell population has only been obtained recently using the limiting cell dilution tumor assay [142, 161-166]. The limiting cell dilution tumor assay measures the

Figure 6.1

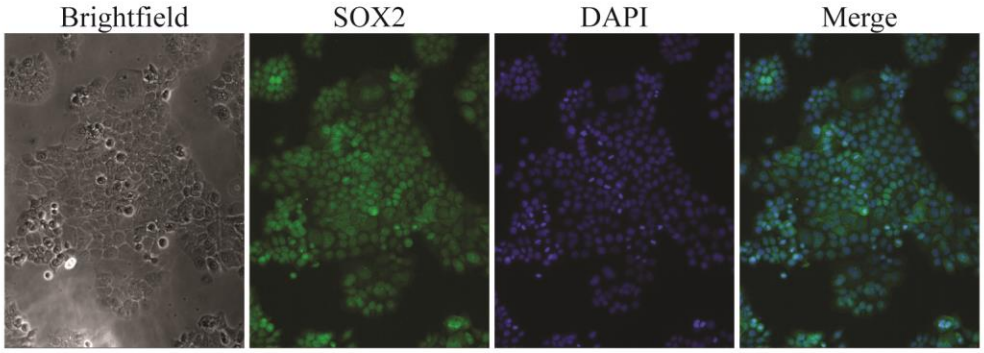

Figure 6.1 SOX2 is expressed heterogeneously in L3.6 PDAC cells. Immunofluorescence of i-KdSOX2-L3.6 cells grown 3 days at subconfluent densities prior to fixation and incubation with SOX2 primary and FITC-conjugated secondary antibodies or DAPI stain.

minimum number of tumor cells needed to reform a tumor when transplanted into a suitable host and, thus, is the gold standard for measuring the frequency of tumor-initiating cells in the tumor. An increase in the percentage of tumor-initiating cells would result in fewer cells necessary to form the tumor. In pancreatic cancer, for example, others have attempted to identify pancreatic tumor-initiating cells using markers such as CD133, CD44, CD24, ESA, and ALDH1 [212-219]. For example, ALDH+ cells have shown enhanced clonigenic growth *in vitro* and *in vivo* [216-219], and studies performed by others argue that 10-15% of pancreatic cancer cells grown in culture are ALDH+ [215]. However, these markers are controversial for PDAC [220]. Studies in our laboratory with CD133 have shown that when isolated CD133- glioblastoma cells are grown in culture, CD133 expression rapidly reappears [172]. In addition, we have observed similar results with the reappearance of ALDH1 positive cells from ALDH1 negative PDAC cells (Wilder and Rizzino, unpublished results). Future studies will need to consider whether SOX2 may also be a potential PDAC tumor-initiating cell marker, and determine whether SOX2 is co-expressed with these other markers previously reported to associate with PDAC tumor-initiating cells.

Although SOX2 has been shown to be essential for the tumor-initiating cells of several other tumors, the roles of SOX2 in the tumor-initiating population of PDAC have not been determined *in vivo*. Knockin of *GFP-SOX2* into the endogenous *SOX2* locus using CRISPR technology may be the best means to study the role of SOX2 in PDAC tumor-initiating cells. Gene editing studies like this would enable the fractionation of PDAC cells on the basis of endogenous SOX2 expression and the isolation of viable $SOX2^{hi}$ and $SOX2^{low}$ cells via differential GFP expression. Using the isolated cells, one could determine whether the $SOX2^{hi}$ PDAC population exhibits a higher percentage of tumor-initiating cells than the unsorted or the $SOX2^{low}$ cell population by performing limiting tumor cell dilution assays. These isolated $SOX2^{hi}$ and $SOX2^{low}$ cells could then be further examined for expression of markers previously shown to be associated with PDAC tumor-initiating cells, as well as critical signaling networks in $SOX2^{hi}$

and $SOX2^{low}$ PDAC cell subpopulations. A recent study has shown that elevating SOX2 in PaTu-8988t cells leads to the expression of CD133, CD44, and AKDH1, all are markers reported by others to be associated with PDAC tumor-initiating cells [155]. However, controversy surrounds these markers in the case of PDAC [220]. Co-expression of SOX2 with one or more of these markers may be a more reliable means of identifying the tumor-initiating cell population. Going forward, a better understanding of the networks used by SOX2 to influence the tumor-initiating cells in PDAC may allow future clinical trials to more accurately and reliably treat PDAC tumors that have elevated SOX2 expression.

6.8 Elevated SOX2 may contribute to a Quiescent Tumor-Initiating Population

In addition to their ability to propagate the tumor, tumor-initiating cells are thought to be responsible for dormant/quiescent population, [217] but this property is rarely examined. Quiescent cells can be identified by thymidine analog label retention; these "label-retaining cells" proceed through the cell cycle at a greatly reduced frequency, resulting in thymidine analog label retention [164, 221, 222]. In medulloblastoma, $SOX2^{+}$ cells have been shown to acquire the labeled thymidine analog more slowly, as well as retain the label following pulse chase experiments [164]. Furthermore, a recent study in bladder cancer has shown that the quiescent label-retaining cancer stem cell population does not respond to cytotoxic therapy and is capable of repopulating the tumor following drug removal [221]. Going forward, it will be important to determine whether elevation of SOX2 during advanced stages of PDAC affects its tumor-initiating population as well as the treatment options for this highly deadly cancer, in particular the response of PDAC to specific classes of drugs currently being tested clinically. It may be possible that the $SOX2^{hi}$ cells of the tumor remain in or enter a quiescent state during chemotherapeutic treatment, which are often designed to target the rapidly dividing cells of the tumor. In this way, upon removal of drug treatment the $SOX2^{hi}$ cells may be the first cells primed and ready to re-enter the cell cycle and begin to repopulate the tumor. Understanding this

interplay between SOX2 and tumor cell quiescence will undoubtedly be important for the improved response of pancreatic tumors to current therapeutics.

6.9 Inducible Modulation of SOX2 Levels Alters the Efficacy of Drugs Used Clinically

Multiple studies have shown that SOX2 also influences responses of tumor cells to other drugs used clinically [129, 130, 141, 154, 158, 163, 168, 169]. Chapter 5 of this dissertation demonstrates for the first time that inducible elevation of SOX2 in three PDAC cell lines leads to a reduction in the efficacy of several MEKi, including trametinib, and the AKTi MK-2206. This is particularly interesting for two reasons. First, elevation of SOX2 on its own inhibits growth, but when SOX2 is elevated it reduces the efficacy of MEKi and AKTi. Thus, the protective effects of SOX2 against these drugs are not coupled mechanistically with the effects of SOX2 on PDAC growth. Going forward, it will be valuable to determine how SOX2 can reduce the action of MEKi and AKTi. Second, knockdown of SOX2 in PDAC cells combined with drug treatment leads to further reductions in PDAC cell growth. Consequently, SOX2 appears to be a potential therapeutic target for improving the treatment of patients with SOX2-positive PDAC. Although it is generally believed that it is very difficult to develop drugs that directly interfere with the action of transcription factors, it may be practical to identify small molecule inhibitors that reduce SOX2 gene expression, block the downstream mechanisms by which SOX2 reduces efficacy of MEKi and AKTi, or, as discussed earlier, target genes that work in concert with SOX2 to promote tumor growth. In this way, targeting SOX2 or its mode of action could improve the effectiveness of these drugs against PDAC.

While the studies presented in Chapter 5 have focused largely on the drug resistance of PDAC cells to MEKi and AKTi, going forward it remains to be determined how SOX2 may or may not protect PDAC cells from additional chemotherapeutics. Current treatment regimens include gemcitabine, a regimen combining fluorouracil, irinotecan, oxaliplatin, and leucovorin (called FOLFIRINOX), and another combining albumin-bound paclitaxel with gemcitabine;

however these strategies have done little to improve patient survival beyond a few months. The possibility remains that SOX2 does not selectively protect PDAC cells from only MEKi and AKTi. It will be important in future studies to evaluate whether SOX2 can also protect PDAC cells from current standards of care. Cells engineered for sorting based on endogenous SOX2 expression as described above would be particularly useful in this regard, as one could examine drug resistance to current therapeutics on cells with different levels endogenous SOX2 expression. These types of experiments could greatly enhance our understanding of SOX2 in drug resistant PDAC cells.

Currently, it is unknown how SOX2 reduces the effectiveness of chemotherapeutics in PDAC cells. However, progress has been made in understanding how SOX2 is regulated in PDAC as well has how SOX2 influences the growth of PDAC cells. A recent study points to an interesting connection between SOX2 and NFATc1. Knockdown of NFATc1, which is often overexpressed in PDAC, leads to a decrease in SOX2 expression, and this appears to be due to a direct effect of NFATc1 on SOX2 transcription [223]. In other studies, stable overexpression of SOX2 in Patu8988t PDAC cells, which do not express detectable levels of endogenous SOX2, has been shown to increase expression of Twist, Snail and Slug, while decreasing the expression of E-Cadherin and ZO-1 [155]. Conversely, knocking down SOX2 in PDAC cells increases the expression of p21Cip1 and p27Kip1 [155]. Thus, under the control of NFATc1, SOX2 appears to regulate the expression of genes involved in epithelial-mesenchymal transition and cell cycle regulation.

6.10 SOX2 may influence Drug Efflux in Tumor-Initiating Cells

In addition to cell cycle control, SOX2 may be using additional means to protect PDAC cells from the growth inhibitory effects of chemotherapeutics used clinically. As outlined in the Introduction of this dissertation, SOX2 may be acting to protect tumor cells through antiapoptotic

signaling or quiescent-like phenotypes [126, 152, 158, 164], or SOX2 may promote drug resistance through various ATP-binding cassette transporters. For example, ABCG2 has been shown to be upregulated in the side population TIC [151, 154], has been considered to be an additional cancer stem cell marker, and additional studies have shown that stable downregulation of SOX2 via shRNAs decreases ABCG2 [142, 159]. An additional study found that induction of SOX2 in glioma stem cells promotes the expression of ABCC3 and ABCC6 transporters [158]. Furthermore, recent RNA-Seq from our laboratory identified 2 additional ATP-binding cassette transporters, ABCB6 and ABCC4, which increased >50% and over 5-fold, respectively, when SOX2 was elevated in PDAC cells. It is possible that SOX2 may be controlling the expression of these cell surface transporters on tumor cells to influence the efflux of drugs. Recognizing and focusing on the role of SOX2 in drug resistance could greatly improve the treatment options for patients with a multitude of cancers, especially those with highly refractory tumors, as the ability to eradicate the TIC population is likely to be the only way to prevent recurrence.

6.11 Summary

In conclusion, SOX2 clearly plays critical roles in multiple cancers, including PDAC. SOX2 not only influences tumor growth in these cases, but also influences the responses of tumors to drugs used clinically. Thus continued study of SOX2 in PDAC and other cancers is clearly warranted, and could lead to major advancements desperately needed for these highly deadly malignancies.

References

1. Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes & development. 1995; 9: 2635-2645.

2. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes & development. 2003; 17: 126-140.

3. Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P, Ng HH. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Molecular and cellular biology. 2005; 25: 6031-6046.

4. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126: 663-676.

5. Okamoto R, Uchikawa M, Kondoh H. Sixteen additional enhancers associated with the chicken Sox2 locus outside the central 50-kb region. Development, growth & differentiation. 2015; 57: 24-39.

6. Wiebe MS, Wilder PJ, Kelly D, Rizzino A. Isolation, characterization, and differential expression of the murine Sox-2 promoter. Gene. 2000; 246: 383-393.

7. Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, Muramatsu M, Okuda A. Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic acids research. 2002; 30: 3202-3213.

8. Zhou HY, Katsman Y, Dhaliwal NK, Davidson S, Macpherson NN, Sakthidevi M, Collura F, Mitchell JA. A Sox2 distal enhancer cluster regulates embryonic stem cell differentiation potential. Genes & development. 2014; 28: 2699-2711.

9. Zappone MV, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK. Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development (Cambridge, England). 2000; 127: 2367-2382.

10. Miyagi S, Nishimoto M, Saito T, Ninomiya M, Sawamoto K, Okano H, Muramatsu M, Oguro H, Iwama A, Okuda A. The Sox2 regulatory region 2 functions as a neural stem cell-specific enhancer in the telencephalon. The Journal of biological chemistry. 2006; 281: 13374-13381.

11. Hotta A, Cheung AY, Farra N, Vijayaragavan K, Seguin CA, Draper JS, Pasceri P, Maksakova IA, Mager DL, Rossant J, Bhatia M, Ellis J. Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. Nature methods. 2009; 6: 370-376.

12. Leis O, Eguiara A, Lopez-Arribillaga E, Alberdi MJ, Hernandez-Garcia S, Elorriaga K, Pandiella A, Rezola R, Martin AG. Sox2 expression in breast tumours and activation in breast cancer stem cells. Oncogene. 2012; 31: 1354-1365.

13. Iglesias JM, Leis O, Perez Ruiz E, Gumuzio Barrie J, Garcia-Garcia F, Aduriz A, Beloqui I, Hernandez-Garcia S, Lopez-Mato MP, Dopazo J, Pandiella A, Menendez JA, Martin AG. The

Activation of the Sox2 RR2 Pluripotency Transcriptional Reporter in Human Breast Cancer Cell Lines is Dynamic and Labels Cells with Higher Tumorigenic Potential. Frontiers in oncology. 2014; 4: 308.

14. Marques-Torrejon MA, Porlan E, Banito A, Gomez-Ibarlucea E, Lopez-Contreras AJ, Fernandez-Capetillo O, Vidal A, Gil J, Torres J, Farinas I. Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression. Cell stem cell. 2013; 12: 88-100.

15. Li H, Collado M, Villasante A, Matheu A, Lynch CJ, Canamero M, Rizzoti K, Carneiro C, Martinez G, Vidal A, Lovell-Badge R, Serrano M. p27(Kip1) directly represses Sox2 during embryonic stem cell differentiation. Cell stem cell. 2012; 11: 845-852.

16. Vilas JM, Ferreiros A, Carneiro C, Morey L, Da Silva-Alvarez S, Fernandes T, Abad M, Di Croce L, Garcia-Caballero T, Serrano M, Rivas C, Vidal A, Collado M. Transcriptional regulation of Sox2 by the retinoblastoma family of pocket proteins. Oncotarget. 2015; 6: 2992-3002.

17. Kopp JL, Ormsbee BD, Desler M, Rizzino A. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem cells (Dayton, Ohio). 2008; 26: 903-911.

18. Ormsbee Golden BD, Wuebben EL, Rizzino A. Sox2 expression is regulated by a negative feedback loop in embryonic stem cells that involves AKT signaling and FoxO1. PloS one. 2013; 8: e76345.

19. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122: 947-956.

20. Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, Loh YH, Yeo HC, Yeo ZX, et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell. 2008; 133: 1106-1117.

21. Fang X, Yoon JG, Li L, Yu W, Shao J, Hua D, Zheng S, Hood L, Goodlett DR, Foltz G, Lin B. The SOX2 response program in glioblastoma multiforme: an integrated ChIP-seq, expression microarray, and microRNA analysis. BMC genomics. 2011; 12: 11-2164-12-11.

22. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 2009; 137: 647-658.

23. Lee SJ, Kim SJ, Seo HH, Shin SP, Kim D, Park CS, Kim KT, Kim YH, Jeong JS, Kim IH. Over-expression of miR-145 enhances the effectiveness of HSVtk gene therapy for malignant glioma. Cancer letters. 2012; 320: 72-80.

24. Ozen M, Karatas OF, Gulluoglu S, Bayrak OF, Sevli S, Guzel E, Ekici ID, Caskurlu T, Solak M, Creighton CJ, Ittmann M. Overexpression of miR-145-5p inhibits proliferation of prostate cancer cells and reduces SOX2 expression. Cancer investigation. 2015; 33: 251-258.

25. Campayo M, Navarro A, Vinolas N, Diaz T, Tejero R, Gimferrer JM, Molins L, Cabanas ML, Ramirez J, Monzo M, Marrades R. Low miR-145 and high miR-367 are associated with unfavourable prognosis in resected nonsmall cell lung cancer. The European respiratory journal. 2013; 41: 1172-1178.

26. Riggi N, Suva ML, De Vito C, Provero P, Stehle JC, Baumer K, Cironi L, Janiszewska M, Petricevic T, Suva D, Tercier S, Joseph JM, Guillou L, et al. EWS-FLI-1 modulates miRNA145 and SOX2 expression to initiate mesenchymal stem cell reprogramming toward Ewing sarcoma cancer stem cells. Genes & development. 2010; 24: 916-932.

27. Jia Y, Liu H, Zhuang Q, Xu S, Yang Z, Li J, Lou J, Zhang W. Tumorigenicity of cancer stem-like cells derived from hepatocarcinoma is regulated by microRNA-145. Oncology reports. 2012; 27: 1865-1872.

28. Sureban SM, May R, Qu D, Weygant N, Chandrakesan P, Ali N, Lightfoot SA, Pantazis P, Rao CV, Postier RG, Houchen CW. DCLK1 regulates pluripotency and angiogenic factors via microRNA-dependent mechanisms in pancreatic cancer. PloS one. 2013; 8: e73940.

29. Fujii T, Shimada K, Tatsumi Y, Hatakeyama K, Obayashi C, Fujimoto K, Konishi N. microRNA-145 promotes differentiation in human urothelial carcinoma through down-regulation of syndecan-1. BMC cancer. 2015; 15: 818-015-1846-0.

30. Lu YX, Yuan L, Xue XL, Zhou M, Liu Y, Zhang C, Li JP, Zheng L, Hong M, Li XN. Regulation of colorectal carcinoma stemness, growth, and metastasis by an miR-200c-Sox2-negative feedback loop mechanism. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014; 20: 2631-2642.

31. Deng Z, Du WW, Fang L, Shan SW, Qian J, Lin J, Qian W, Ma J, Rutnam ZJ, Yang BB. The intermediate filament vimentin mediates microRNA miR-378 function in cellular self-renewal by regulating the expression of the Sox2 transcription factor. The Journal of biological chemistry. 2013; 288: 319-331.

32. Qin J, Ji J, Deng R, Tang J, Yang F, Feng GK, Chen WD, Wu XQ, Qian XJ, Ding K, Zhu XF. DC120, a novel AKT inhibitor, preferentially suppresses nasopharyngeal carcinoma cancer stem-like cells by downregulating Sox2. Oncotarget. 2015; 6: 6944-6958.

33. Forno I, Ferrero S, Russo MV, Gazzano G, Giangiobbe S, Montanari E, Del Nero A, Rocco B, Albo G, Languino LR, Altieri DC, Vaira V, Bosari S. Deregulation of MiR-34b/Sox2 Predicts Prostate Cancer Progression. PloS one. 2015; 10: e0130060.

34. Ma K, Pan X, Fan P, He Y, Gu J, Wang W, Zhang T, Li Z, Luo X. Loss of miR-638 in vitro promotes cell invasion and a mesenchymal-like transition by influencing SOX2 expression in colorectal carcinoma cells. Molecular cancer. 2014; 13: 118-4598-13-118.

35. Jin Y, Jiang Z, Guan X, Chen Y, Tang Q, Wang G, Wang X. miR-450b-5p Suppresses Stemness and the Development of Chemoresistance by Targeting SOX2 in Colorectal Cancer. DNA and cell biology. 2016; 35: 249-256.

36. Li J, Du L, Yang Y, Wang C, Liu H, Wang L, Zhang X, Li W, Zheng G, Dong Z. MiR-429 is an independent prognostic factor in colorectal cancer and exerts its anti-apoptotic function by targeting SOX2. Cancer letters. 2013; 329: 84-90.

37. Lundberg IV, Lofgren Burstrom A, Edin S, Eklof V, Oberg A, Stenling R, Palmqvist R, Wikberg ML. SOX2 expression is regulated by BRAF and contributes to poor patient prognosis in colorectal cancer. PloS one. 2014; 9: e101957.

38. Li YJ, Dong M, Kong FM, Zhou JP, Liang D, Xue HZ. MicroRNA-371-5p targets SOX2 in gastric cancer. Oncotarget. 2016.

39. Otsubo T, Akiyama Y, Hashimoto Y, Shimada S, Goto K, Yuasa Y. MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. PloS one. 2011; 6: e16617.

40. Wang S, Tie J, Wang R, Hu F, Gao L, Wang W, Wang L, Li Z, Hu S, Tang S, Li M, Wang X, Nie Y, et al. SOX2, a predictor of survival in gastric cancer, inhibits cell proliferation and metastasis by regulating PTEN. Cancer letters. 2015; 358: 210-219.

41. Zhang X, Yu H, Yang Y, Zhu R, Bai J, Peng Z, He Y, Chen L, Chen W, Fang D, Bian X, Wang R. SOX2 in gastric carcinoma, but not Hath1, is related to patients' clinicopathological features and prognosis. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2010; 14: 1220-1226.

42. Chen Y, Huang Y, Zhu L, Chen M, Huang Y, Zhang J, He S, Li A, Chen R, Zhou J. SOX2 inhibits metastasis in gastric cancer. Journal of cancer research and clinical oncology. 2016; 142: 1221-1230.

43. Yang C, Hou C, Zhang H, Wang D, Ma Y, Zhang Y, Xu X, Bi Z, Geng S. miR-126 functions as a tumor suppressor in osteosarcoma by targeting Sox2. International journal of molecular sciences. 2013; 15: 423-437.

44. Zhao C, Li Y, Zhang M, Yang Y, Chang L. miR-126 inhibits cell proliferation and induces cell apoptosis of hepatocellular carcinoma cells partially by targeting Sox2. Human cell. 2015; 28: 91-99.

45. Hamada S, Satoh K, Fujibuchi W, Hirota M, Kanno A, Unno J, Masamune A, Kikuta K, Kume K, Shimosegawa T. MiR-126 acts as a tumor suppressor in pancreatic cancer cells via the regulation of ADAM9. Molecular cancer research : MCR. 2012; 10: 3-10.

46. Rutenberg-Schoenberg M, Sexton AN, Simon MD. The Properties of Long Noncoding RNAs that Regulate Chromatin. Annual review of genomics and human genetics. 2016.

47. Fantes J, Ragge NK, Lynch SA, McGill NI, Collin JR, Howard-Peebles PN, Hayward C, Vivian AJ, Williamson K, van Heyningen V, FitzPatrick DR. Mutations in SOX2 cause anophthalmia. Nature genetics. 2003; 33: 461-463.

48. Shahryari A, Jazi MS, Samaei NM, Mowla SJ. Long non-coding RNA SOX2OT: expression signature, splicing patterns, and emerging roles in pluripotency and tumorigenesis. Frontiers in genetics. 2015; 6: 196.

49. Saghaeian Jazi M, Samaei NM, Ghanei M, Shadmehr MB, Mowla SJ. Identification of new SOX2OT transcript variants highly expressed in human cancer cell lines and down regulated in stem cell differentiation. Molecular biology reports. 2016; 43: 65-72.

50. Amaral PP, Neyt C, Wilkins SJ, Askarian-Amiri ME, Sunkin SM, Perkins AC, Mattick JS. Complex architecture and regulated expression of the Sox2ot locus during vertebrate development. RNA (New York, N.Y.). 2009; 15: 2013-2027.

51. Askarian-Amiri ME, Seyfoddin V, Smart CE, Wang J, Kim JE, Hansji H, Baguley BC, Finlay GJ, Leung EY. Emerging role of long non-coding RNA SOX2OT in SOX2 regulation in breast cancer. PloS one. 2014; 9: e102140.

52. Hou Z, Zhao W, Zhou J, Shen L, Zhan P, Xu C, Chang C, Bi H, Zou J, Yao X, Huang R, Yu L, Yan J. A long noncoding RNA Sox2ot regulates lung cancer cell proliferation and is a prognostic indicator of poor survival. The international journal of biochemistry & cell biology. 2014; 53: 380-388.

53. Saghaeian Jazi M, Samaei NM, Ghanei M, Shadmehr MB, Mowla SJ. Overexpression of the non-coding SOX2OT variants 4 and 7 in lung tumors suggests an oncogenic role in lung cancer. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2016.

54. Shahryari A, Rafiee MR, Fouani Y, Oliae NA, Samaei NM, Shafiee M, Semnani S, Vasei M, Mowla SJ. Two novel splice variants of SOX2OT, SOX2OT-S1, and SOX2OT-S2 are coupregulated with SOX2 and OCT4 in esophageal squamous cell carcinoma. Stem cells (Dayton, Ohio). 2014; 32: 126-134.

55. Lin S, Qi W, Han K, Gan Z, Yao Y, Miu D. Prognostic value of SOX2 in digestive tumors: a meta-analysis. Hepato-gastroenterology. 2014; 61: 1274-1278.

56. Shi XM, Teng F. Up-regulation of long non-coding RNA Sox2ot promotes hepatocellular carcinoma cell metastasis and correlates with poor prognosis. International journal of clinical and experimental pathology. 2015; 8: 4008-4014.

57. Shafiee M, Aleyasin SA, Vasei M, Semnani SS, Mowla SJ. Down-Regulatory Effects of miR-211 on Long Non-Coding RNA SOX2OT and SOX2 Genes in Esophageal Squamous Cell Carcinoma. Cell journal. 2016; 17: 593-600.

58. Lin N, Chang KY, Li Z, Gates K, Rana ZA, Dang J, Zhang D, Han T, Yang CS, Cunningham TJ, Head SR, Duester G, Dong PD, et al. An evolutionarily conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. Molecular cell. 2014; 53: 1005-1019.

59. Han Y, Zhou L, Wu T, Huang Y, Cheng Z, Li X, Sun T, Zhou Y, Du Z. Downregulation of lncRNA-MALAT1 Affects Proliferation and the Expression of Stemness Markers in Glioma Stem Cell Line SHG139S. Cellular and molecular neurobiology. 2016; 36: 1097-1107.

60. Jiao F, Hu H, Han T, Yuan C, Wang L, Jin Z, Guo Z, Wang L. Long noncoding RNA MALAT-1 enhances stem cell-like phenotypes in pancreatic cancer cells. International journal of molecular sciences. 2015; 16: 6677-6693.

61. Wang Y, Xu Z, Jiang J, Xu C, Kang J, Xiao L, Wu M, Xiong J, Guo X, Liu H. Endogenous miRNA sponge lincRNA-RoR regulates Oct4, Nanog, and Sox2 in human embryonic stem cell self-renewal. Developmental cell. 2013; 25: 69-80.

62. Ng SY, Bogu GK, Soh BS, Stanton LW. The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. Molecular cell. 2013; 51: 349-359.

63. Zhuang W, Ge X, Yang S, Huang M, Zhuang W, Chen P, Zhang X, Fu J, Qu J, Li B. Upregulation of lncRNA MEG3 Promotes Osteogenic Differentiation of Mesenchymal Stem Cells From Multiple Myeloma Patients By Targeting BMP4 Transcription. Stem cells (Dayton, Ohio). 2015; 33: 1985-1997.

64. Hu D, Su C, Jiang M, Shen Y, Shi A, Zhao F, Chen R, Shen Z, Bao J, Tang W. Fenofibrate inhibited pancreatic cancer cells proliferation via activation of p53 mediated by upregulation of LncRNA MEG3. Biochemical and biophysical research communications. 2016; 471: 290-295.

65. Justilien V, Walsh MP, Ali SA, Thompson EA, Murray NR, Fields AP. The PRKCI and SOX2 oncogenes are coamplified and cooperate to activate Hedgehog signaling in lung squamous cell carcinoma. Cancer cell. 2014; 25: 139-151.

66. Ouyang J, Yu W, Liu J, Zhang N, Florens L, Chen J, Liu H, Washburn M, Pei D, Xie T. Cyclin-dependent kinase-mediated Sox2 phosphorylation enhances the ability of Sox2 to establish the pluripotent state. The Journal of biological chemistry. 2015; 290: 22782-22794.

67. Myers SA, Panning B, Burlingame AL. Polycomb repressive complex 2 is necessary for the normal site-specific O-GlcNAc distribution in mouse embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108: 9490-9495.

68. Jang H, Kim TW, Yoon S, Choi SY, Kang TW, Kim SY, Kwon YW, Cho EJ, Youn HD. O-GlcNAc regulates pluripotency and reprogramming by directly acting on core components of the pluripotency network. Cell stem cell. 2012; 11: 62-74.

69. Van Hoof D, Munoz J, Braam SR, Pinkse MW, Linding R, Heck AJ, Mummery CL, Krijgsveld J. Phosphorylation dynamics during early differentiation of human embryonic stem cells. Cell stem cell. 2009; 5: 214-226.

70. Tsuruzoe S, Ishihara K, Uchimura Y, Watanabe S, Sekita Y, Aoto T, Saitoh H, Yuasa Y, Niwa H, Kawasuji M, Baba H, Nakao M. Inhibition of DNA binding of Sox2 by the SUMO conjugation. Biochemical and biophysical research communications. 2006; 351: 920-926.

71. Jeong CH, Cho YY, Kim MO, Kim SH, Cho EJ, Lee SY, Jeon YJ, Lee KY, Yao K, Keum YS, Bode AM, Dong Z. Phosphorylation of Sox2 cooperates in reprogramming to pluripotent stem cells. Stem cells (Dayton, Ohio). 2010; 28: 2141-2150.

72. Fang L, Zhang L, Wei W, Jin X, Wang P, Tong Y, Li J, Du JX, Wong J. A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. Molecular cell. 2014; 55: 537-551.

73. Wang J, Zhang Y, Hou J, Qian X, Zhang H, Zhang Z, Li M, Wang R, Liao K, Wang Y, Li Z, Zhong D, Wan P, et al. Ube2s regulates Sox2 stability and mouse ES cell maintenance. Cell death and differentiation. 2016; 23: 393-404.

74. Zhao HY, Zhang YJ, Dai H, Zhang Y, Shen YF. CARM1 mediates modulation of Sox2. PloS one. 2011; 6: e27026.

75. Cox JL, Mallanna SK, Luo X, Rizzino A. Sox2 uses multiple domains to associate with proteins present in Sox2-protein complexes. PloS one. 2010; 5: e15486.

76. Baltus GA, Kowalski MP, Zhai H, Tutter AV, Quinn D, Wall D, Kadam S. Acetylation of sox2 induces its nuclear export in embryonic stem cells. Stem cells (Dayton, Ohio). 2009; 27: 2175-2184.

77. Nowling T, Bernadt C, Johnson L, Desler M, Rizzino A. The co-activator p300 associates physically with and can mediate the action of the distal enhancer of the FGF-4 gene. The Journal of biological chemistry. 2003; 278: 13696-13705.

78. Yoon DS, Choi Y, Jang Y, Lee M, Choi WJ, Kim SH, Lee JW. SIRT1 directly regulates SOX2 to maintain self-renewal and multipotency in bone marrow-derived mesenchymal stem cells. Stem cells (Dayton, Ohio). 2014; 32: 3219-3231.

79. Mu WL, Wang YJ, Xu P, Hao DL, Liu XZ, Wang TT, Chen F, Chen HZ, Lv X, Liu DP. Sox2 Deacetylation by Sirt1 Is Involved in Mouse Somatic Reprogramming. Stem cells (Dayton, Ohio). 2015; 33: 2135-2147.

80. Cox JL, Wilder PJ, Gilmore JM, Wuebben EL, Washburn MP, Rizzino A. The SOX2-interactome in brain cancer cells identifies the requirement of MSI2 and USP9X for the growth of brain tumor cells. PloS one. 2013; 8: e62857.

81. Cox JL, Wilder PJ, Wuebben EL, Ouellette MM, Hollingsworth MA, Rizzino A. Context-dependent function of the deubiquitinating enzyme USP9X in pancreatic ductal adenocarcinoma. Cancer biology & therapy. 2014; 15: 1042-1052.

82. Mallanna SK, Ormsbee BD, Iacovino M, Gilmore JM, Cox JL, Kyba M, Washburn MP, Rizzino A. Proteomic analysis of Sox2-associated proteins during early stages of mouse embryonic stem cell differentiation identifies Sox21 as a novel regulator of stem cell fate. Stem cells (Dayton, Ohio). 2010; 28: 1715-1727.

83. Keramari M, Razavi J, Ingman KA, Patsch C, Edenhofer F, Ward CM, Kimber SJ. Sox2 is essential for formation of trophectoderm in the preimplantation embryo. PloS one. 2010; 5: e13952.

84. Wicklow E, Blij S, Frum T, Hirate Y, Lang RA, Sasaki H, Ralston A. HIPPO pathway members restrict SOX2 to the inner cell mass where it promotes ICM fates in the mouse blastocyst. PLoS genetics. 2014; 10: e1004618.

85. Guo G, Huss M, Tong GQ, Wang C, Li Sun L, Clarke ND, Robson P. Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Developmental cell. 2010; 18: 675-685.

86. Bernadt CT, Nowling T, Rizzino A. Transcription factor Sox-2 inhibits co-activator stimulated transcription. Molecular reproduction and development. 2004; 69: 260-267.

87. Boer B, Kopp J, Mallanna S, Desler M, Chakravarthy H, Wilder PJ, Bernadt C, Rizzino A. Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2:Oct-3/4 target genes. Nucleic acids research. 2007; 35: 1773-1786.

88. Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. A protein interaction network for pluripotency of embryonic stem cells. Nature. 2006; 444: 364-368.

89. Liang J, Wan M, Zhang Y, Gu P, Xin H, Jung SY, Qin J, Wong J, Cooney AJ, Liu D, Songyang Z. Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nature cell biology. 2008; 10: 731-739.

90. van den Berg DL, Snoek T, Mullin NP, Yates A, Bezstarosti K, Demmers J, Chambers I, Poot RA. An Oct4-centered protein interaction network in embryonic stem cells. Cell stem cell. 2010; 6: 369-381.

91. Pardo M, Lang B, Yu L, Prosser H, Bradley A, Babu MM, Choudhary J. An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. Cell stem cell. 2010; 6: 382-395.

92. Ding J, Xu H, Faiola F, Ma'ayan A, Wang J. Oct4 links multiple epigenetic pathways to the pluripotency network. Cell research. 2012; 22: 155-167.

93. Gao Z, Cox JL, Gilmore JM, Ormsbee BD, Mallanna SK, Washburn MP, Rizzino A. Determination of protein interactome of transcription factor Sox2 in embryonic stem cells engineered for inducible expression of four reprogramming factors. The Journal of biological chemistry. 2012; 287: 11384-11397.

94. Mallanna SK, Rizzino A. Systems biology provides new insights into the molecular mechanisms that control the fate of embryonic stem cells. Journal of cellular physiology. 2012; 227: 27-34.

95. Rizzino A. Concise review: The Sox2-Oct4 connection: critical players in a much larger interdependent network integrated at multiple levels. Stem cells (Dayton, Ohio). 2013; 31: 1033-1039.

96. Wuebben EL, Mallanna SK, Cox JL, Rizzino A. Musashi2 is required for the self-renewal and pluripotency of embryonic stem cells. PloS one. 2012; 7: e34827.

97. Cox JL, Mallanna SK, Ormsbee BD, Desler M, Wiebe MS, Rizzino A. Banf1 is required to maintain the self-renewal of both mouse and human embryonic stem cells. Journal of cell science. 2011; 124: 2654-2665.

98. Sakakibara S, Nakamura Y, Satoh H, Okano H. Rna-binding protein Musashi2: developmentally regulated expression in neural precursor cells and subpopulations of neurons in mammalian CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2001; 21: 8091-8107.

99. Imai T, Tokunaga A, Yoshida T, Hashimoto M, Mikoshiba K, Weinmaster G, Nakafuku M, Okano H. The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. Molecular and cellular biology. 2001; 21: 3888-3900.

100. Kharas MG, Lengner CJ, Al-Shahrour F, Bullinger L, Ball B, Zaidi S, Morgan K, Tam W, Paktinat M, Okabe R, Gozo M, Einhorn W, Lane SW, et al. Musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia. Nature medicine. 2010; 16: 903-908.

101. Sakakibara S, Nakamura Y, Yoshida T, Shibata S, Koike M, Takano H, Ueda S, Uchiyama Y, Noda T, Okano H. RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99: 15194-15199.

102. Hope KJ, Cellot S, Ting SB, MacRae T, Mayotte N, Iscove NN, Sauvageau G. An RNAi screen identifies Msi2 and Prox1 as having opposite roles in the regulation of hematopoietic stem cell activity. Cell stem cell. 2010; 7: 101-113.

103. Ito T, Kwon HY, Zimdahl B, Congdon KL, Blum J, Lento WE, Zhao C, Lagoo A, Gerrard G, Foroni L, Goldman J, Goh H, Kim SH, et al. Regulation of myeloid leukaemia by the cell-fate determinant Musashi. Nature. 2010; 466: 765-768.

104. de Andres-Aguayo L, Varas F, Kallin EM, Infante JF, Wurst W, Floss T, Graf T. Musashi 2 is a regulator of the HSC compartment identified by a retroviral insertion screen and knockout mice. Blood. 2011; 118: 554-564.

105. Alonso MM, Diez-Valle R, Manterola L, Rubio A, Liu D, Cortes-Santiago N, Urquiza L, Jauregi P, Lopez de Munain A, Sampron N, Aramburu A, Tejada-Solis S, Vicente C, et al. Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. PloS one. 2011; 6: e26740.

106. Annovazzi L, Mellai M, Caldera V, Valente G, Schiffer D. SOX2 expression and amplification in gliomas and glioma cell lines. Cancer genomics & proteomics. 2011; 8: 139-147.

107. Belotte J, Fletcher NM, Alexis M, Morris RT, Munkarah AR, Diamond MP, Saed GM. Sox2 gene amplification significantly impacts overall survival in serous epithelial ovarian cancer. Reproductive sciences (Thousand Oaks, Calif.). 2015; 22: 38-46.

108. Gen Y, Yasui K, Zen Y, Zen K, Dohi O, Endo M, Tsuji K, Wakabayashi N, Itoh Y, Naito Y, Taniwaki M, Nakanuma Y, Okanoue T, et al. SOX2 identified as a target gene for the amplification at 3q26 that is frequently detected in esophageal squamous cell carcinoma. Cancer genetics and cytogenetics. 2010; 202: 82-93.

109. Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, Bergbower EA, Guan Y, Shin J, Guillory J, Rivers CS, Foo CK, Bhatt D, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. Nature genetics. 2012; 44: 1111-1116.

110. Bass AJ, Watanabe H, Mermel CH, Yu S, Perner S, Verhaak RG, Kim SY, Wardwell L, Tamayo P, Gat-Viks I, Ramos AH, Woo MS, Weir BA, et al. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nature genetics. 2009; 41: 1238-1242.

111. Long KB, Hornick JL. SOX2 is highly expressed in squamous cell carcinomas of the gastrointestinal tract. Human pathology. 2009; 40: 1768-1773.

112. Hussenet T, Dali S, Exinger J, Monga B, Jost B, Dembele D, Martinet N, Thibault C, Huelsken J, Brambilla E, du Manoir S. SOX2 is an oncogene activated by recurrent 3q26.3 amplifications in human lung squamous cell carcinomas. PloS one. 2010; 5: e8960.

113. Wilbertz T, Wagner P, Petersen K, Stiedl AC, Scheble VJ, Maier S, Reischl M, Mikut R, Altorki NK, Moch H, Fend F, Staebler A, Bass AJ, et al. SOX2 gene amplification and protein overexpression are associated with better outcome in squamous cell lung cancer. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2011; 24: 944-953.

114. Freier K, Knoepfle K, Flechtenmacher C, Pungs S, Devens F, Toedt G, Hofele C, Joos S, Lichter P, Radlwimmer B. Recurrent copy number gain of transcription factor SOX2 and corresponding high protein expression in oral squamous cell carcinoma. Genes, chromosomes & cancer. 2010; 49: 9-16.

115. Sattler HP, Lensch R, Rohde V, Zimmer E, Meese E, Bonkhoff H, Retz M, Zwergel T, Bex A, Stoeckle M, Wullich B. Novel amplification unit at chromosome 3q25-q27 in human prostate cancer. The Prostate. 2000; 45: 207-215.

116. Schrock A, Goke F, Wagner P, Bode M, Franzen A, Braun M, Huss S, Agaimy A, Ihrler S, Menon R, Kirsten R, Kristiansen G, Bootz F, et al. Sex determining region Y-box 2 (SOX2) amplification is an independent indicator of disease recurrence in sinonasal cancer. PloS one. 2013; 8: e59201.

117. Rodriguez-Pinilla SM, Sarrio D, Moreno-Bueno G, Rodriguez-Gil Y, Martinez MA, Hernandez L, Hardisson D, Reis-Filho JS, Palacios J. Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2007; 20: 474-481.

118. Schmitz M, Temme A, Senner V, Ebner R, Schwind S, Stevanovic S, Wehner R, Schackert G, Schackert HK, Fussel M, Bachmann M, Rieber EP, Weigle B. Identification of SOX2 as a novel glioma-associated antigen and potential target for T cell-based immunotherapy. British journal of cancer. 2007; 96: 1293-1301.

119. Phi JH, Park SH, Kim SK, Paek SH, Kim JH, Lee YJ, Cho BK, Park CK, Lee DH, Wang KC. Sox2 expression in brain tumors: a reflection of the neuroglial differentiation pathway. The American Journal of Surgical Pathology. 2008; 32: 103-112.

120. Sholl LM, Long KB, Hornick JL. Sox2 expression in pulmonary non-small cell and neuroendocrine carcinomas. Applied Immunohistochemistry & Molecular Morphology : AIMM / Official Publication of the Society for Applied Immunohistochemistry. 2010; 18: 55-61.

121. Yuan P, Kadara H, Behrens C, Tang X, Woods D, Solis LM, Huang J, Spinola M, Dong W, Yin G, Fujimoto J, Kim E, Xie Y, et al. Sex determining region Y-Box 2 (SOX2) is a potential cell-lineage gene highly expressed in the pathogenesis of squamous cell carcinomas of the lung. PloS one. 2010; 5: e9112.

122. Laga AC, Zhan Q, Weishaupt C, Ma J, Frank MH, Murphy GF. SOX2 and nestin expression in human melanoma: an immunohistochemical and experimental study. Experimental dermatology. 2011; 20: 339-345.

123. Ye F, Li Y, Hu Y, Zhou C, Hu Y, Chen H. Expression of Sox2 in human ovarian epithelial carcinoma. Journal of cancer research and clinical oncology. 2011; 137: 131-137.

124. Zhang J, Chang DY, Mercado-Uribe I, Liu J. Sex-determining region Y-box 2 expression predicts poor prognosis in human ovarian carcinoma. Human pathology. 2012; 43: 1405-1412.

125. Sanada Y, Yoshida K, Ohara M, Oeda M, Konishi K, Tsutani Y. Histopathologic evaluation of stepwise progression of pancreatic carcinoma with immunohistochemical analysis of gastric epithelial transcription factor SOX2: comparison of expression patterns between invasive components and cancerous or nonneoplastic intraductal components. Pancreas. 2006; 32: 164-170.

126. Jia X, Li X, Xu Y, Zhang S, Mou W, Liu Y, Liu Y, Lv D, Liu CH, Tan X, Xiang R, Li N. SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell. Journal of molecular cell biology. 2011; 3: 230-238.

127. Li XL, Eishi Y, Bai YQ, Sakai H, Akiyama Y, Tani M, Takizawa T, Koike M, Yuasa Y. Expression of the SRY-related HMG box protein SOX2 in human gastric carcinoma. International journal of oncology. 2004; 24: 257-263.

128. Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, Sun L, Yang X, Wang Y, Zhang Y, Shang Y. The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. The Journal of biological chemistry. 2008; 283: 17969-17978.

129. Tian Y, Jia X, Wang S, Li Y, Zhao P, Cai D, Zhou Z, Wang J, Luo Y, Dong M. SOX2 oncogenes amplified and operate to activate AKT signaling in gastric cancer and predict immunotherapy responsiveness. Journal of cancer research and clinical oncology. 2014; 140: 1117-1124.

130. Bourguignon LY, Wong G, Earle C, Chen L. Hyaluronan-CD44v3 interaction with Oct4-Sox2-Nanog promotes miR-302 expression leading to self-renewal, clonal formation, and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma. The Journal of biological chemistry. 2012; 287: 32800-32824.

131. Gure AO, Stockert E, Scanlan MJ, Keresztes RS, Jager D, Altorki NK, Old LJ, Chen YT. Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in

small cell lung cancer. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97: 4198-4203.

132. Ma YH, Mentlein R, Knerlich F, Kruse ML, Mehdorn HM, Held-Feindt J. Expression of stem cell markers in human astrocytomas of different WHO grades. Journal of neuro-oncology. 2008; 86: 31-45.

133. Wang Q, He W, Lu C, Wang Z, Wang J, Giercksky KE, Nesland JM, Suo Z. Oct3/4 and Sox2 are significantly associated with an unfavorable clinical outcome in human esophageal squamous cell carcinoma. Anticancer Research. 2009; 29: 1233-1241.

134. Kregel S, Kiriluk KJ, Rosen AM, Cai Y, Reyes EE, Otto KB, Tom W, Paner GP, Szmulewitz RZ, Vander Griend DJ. Sox2 is an androgen receptor-repressed gene that promotes castration-resistant prostate cancer. PloS one. 2013; 8: e53701.

135. Otsubo T, Akiyama Y, Yanagihara K, Yuasa Y. SOX2 is frequently downregulated in gastric cancers and inhibits cell growth through cell-cycle arrest and apoptosis. British journal of cancer. 2008; 98: 824-831.

136. Tsukamoto T, Mizoshita T, Mihara M, Tanaka H, Takenaka Y, Yamamura Y, Nakamura S, Ushijima T, Tatematsu M. Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes. Histopathology. 2005; 46: 649-658.

137. Piva M, Domenici G, Iriondo O, Rabano M, Simoes BM, Comaills V, Barredo I, Lopez-Ruiz JA, Zabalza I, Kypta R, Vivanco M. Sox2 promotes tamoxifen resistance in breast cancer cells. EMBO molecular medicine. 2014; 6: 66-79.

138. Saigusa S, Tanaka K, Toiyama Y, Yokoe T, Okugawa Y, Ioue Y, Miki C, Kusunoki M. Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy. Annals of surgical oncology. 2009; 16: 3488-3498.

139. Wang X, Liang Y, Chen Q, Xu HM, Ge N, Luo RZ, Shao JY, He Z, Zeng YX, Kang T, Yun JP, Xie F. Prognostic significance of SOX2 expression in nasopharyngeal carcinoma. Cancer investigation. 2012; 30: 79-85.

140. Sholl LM, Barletta JA, Yeap BY, Chirieac LR, Hornick JL. Sox2 protein expression is an independent poor prognostic indicator in stage I lung adenocarcinoma. The American Journal of Surgical Pathology. 2010; 34: 1193-1198.

141. Chou YT, Lee CC, Hsiao SH, Lin SE, Lin SC, Chung CH, Chung CH, Kao YR, Wang YH, Chen CT, Wei YH, Wu CW. The emerging role of SOX2 in cell proliferation and survival and its crosstalk with oncogenic signaling in lung cancer. Stem cells (Dayton, Ohio). 2013; 31: 2607-2619.

142. Lee SH, Oh SY, Do SI, Lee HJ, Kang HJ, Rho YS, Bae WJ, Lim YC. SOX2 regulates self-renewal and tumorigenicity of stem-like cells of head and neck squamous cell carcinoma. British journal of cancer. 2014; 111: 2122-2130.

143. Sun C, Sun L, Li Y, Kang X, Zhang S, Liu Y. Sox2 expression predicts poor survival of hepatocellular carcinoma patients and it promotes liver cancer cell invasion by activating Slug. Medical oncology (Northwood, London, England). 2013; 30: 503-013-0503-1. Epub 2013 Feb 21.

144. Du L, Yang Y, Xiao X, Wang C, Zhang X, Wang L, Zhang X, Li W, Zheng G, Wang S, Dong Z. Sox2 nuclear expression is closely associated with poor prognosis in patients with histologically node-negative oral tongue squamous cell carcinoma. Oral oncology. 2011; 47: 709-713.

145. Wang X, Ji X, Chen J, Yan D, Zhang Z, Wang Q, Xi X, Feng Y. SOX2 enhances the migration and invasion of ovarian cancer cells via Src kinase. PloS one. 2014; 9: e99594.

146. Neumann J, Bahr F, Horst D, Kriegl L, Engel J, Luque RM, Gerhard M, Kirchner T, Jung A. SOX2 expression correlates with lymph-node metastases and distant spread in right-sided colon cancer. BMC cancer. 2011; 11: 518-2407-11-518.

147. Han X, Fang X, Lou X, Hua D, Ding W, Foltz G, Hood L, Yuan Y, Lin B. Silencing SOX2 induced mesenchymal-epithelial transition and its expression predicts liver and lymph node metastasis of CRC patients. PloS one. 2012; 7: e41335.

148. Saigusa S, Mohri Y, Ohi M, Toiyama Y, Ishino Y, Okugawa Y, Tanaka K, Inoue Y, Kusunoki M. Podoplanin and SOX2 expression in esophageal squamous cell carcinoma after neoadjuvant chemo-radiotherapy. Oncology reports. 2011; 26: 1069-1074.

149. Lu Y, Futtner C, Rock JR, Xu X, Whitworth W, Hogan BL, Onaitis MW. Evidence that SOX2 overexpression is oncogenic in the lung. PloS one. 2010; 5: e11022.

150. Bayo P, Jou A, Stenzinger A, Shao C, Gross M, Jensen A, Grabe N, Mende CH, Rados PV, Debus J, Weichert W, Plinkert PK, Lichter P, et al. Loss of SOX2 expression induces cell motility via vimentin up-regulation and is an unfavorable risk factor for survival of head and neck squamous cell carcinoma. Molecular oncology. 2015; 9: 1704-1719.

151. Hepburn AC, Veeratterapillay R, Williamson SC, El-Sherif A, Sahay N, Thomas HD, Mantilla A, Pickard RS, Robson CN, Heer R. Side population in human non-muscle invasive bladder cancer enriches for cancer stem cells that are maintained by MAPK signalling. PloS one. 2012; 7: e50690.

152. Tian T, Zhang Y, Wang S, Zhou J, Xu S. Sox2 enhances the tumorigenicity and chemoresistance of cancer stem-like cells derived from gastric cancer. Journal of biomedical research. 2012; 26: 336-345.

153. Xiang R, Liao D, Cheng T, Zhou H, Shi Q, Chuang TS, Markowitz D, Reisfeld RA, Luo Y. Downregulation of transcription factor SOX2 in cancer stem cells suppresses growth and metastasis of lung cancer. British journal of cancer. 2011; 104: 1410-1417.

154. Yasuda K, Torigoe T, Morita R, Kuroda T, Takahashi A, Matsuzaki J, Kochin V, Asanuma H, Hasegawa T, Saito T, Hirohashi Y, Sato N. Ovarian cancer stem cells are enriched in side population and aldehyde dehydrogenase bright overlapping population. PloS one. 2013; 8: e68187.

155. Herreros-Villanueva M, Zhang JS, Koenig A, Abel EV, Smyrk TC, Bamlet WR, de Narvajas AA, Gomez TS, Simeone DM, Bujanda L, Billadeau DD. SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. Oncogenesis. 2013; 2: e61.

156. Simoes BM, Piva M, Iriondo O, Comaills V, Lopez-Ruiz JA, Zabalza I, Mieza JA, Acinas O, Vivanco MD. Effects of estrogen on the proportion of stem cells in the breast. Breast cancer research and treatment. 2011; 129: 23-35.

157. Lundberg IV, Edin S, Eklof V, Oberg A, Palmqvist R, Wikberg ML. SOX2 expression is associated with a cancer stem cell state and down-regulation of CDX2 in colorectal cancer. BMC cancer. 2016; 16: 471-016-2509-5.

158. Jeon HM, Sohn YW, Oh SY, Kim SH, Beck S, Kim S, Kim H. ID4 imparts chemoresistance and cancer stemness to glioma cells by derepressing miR-9*-mediated suppression of SOX2. Cancer research. 2011; 71: 3410-3421.

159. Singh S, Trevino J, Bora-Singhal N, Coppola D, Haura E, Altiok S, Chellappan SP. EGFR/Src/Akt signaling modulates Sox2 expression and self-renewal of stem-like side-population cells in non-small cell lung cancer. Molecular cancer. 2012; 11: 73-4598-11-73.

160. Rybak AP, Tang D. SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells. Cellular signalling. 2013; 25: 2734-2742.

161. Santini R, Pietrobono S, Pandolfi S, Montagnani V, D'Amico M, Penachioni JY, Vinci MC, Borgognoni L, Stecca B. SOX2 regulates self-renewal and tumorigenicity of human melanoma-initiating cells. Oncogene. 2014; 33: 4697-4708.

162. Nakatsugawa M, Takahashi A, Hirohashi Y, Torigoe T, Inoda S, Murase M, Asanuma H, Tamura Y, Morita R, Michifuri Y, Kondo T, Hasegawa T, Takahashi H, et al. SOX2 is overexpressed in stem-like cells of human lung adenocarcinoma and augments the tumorigenicity. Laboratory investigation; a journal of technical methods and pathology. 2011; 91: 1796-1804.

163. Bareiss PM, Paczulla A, Wang H, Schairer R, Wiehr S, Kohlhofer U, Rothfuss OC, Fischer A, Perner S, Staebler A, Wallwiener D, Fend F, Fehm T, et al. SOX2 expression associates with stem cell state in human ovarian carcinoma. Cancer research. 2013; 73: 5544-5555.

164. Vanner RJ, Remke M, Gallo M, Selvadurai HJ, Coutinho F, Lee L, Kushida M, Head R, Morrissy S, Zhu X, Aviv T, Voisin V, Clarke ID, et al. Quiescent sox2(+) cells drive hierarchical growth and relapse in sonic hedgehog subgroup medulloblastoma. Cancer cell. 2014; 26: 33-47.

165. Boumahdi S, Driessens G, Lapouge G, Rorive S, Nassar D, Le Mercier M, Delatte B, Caauwe A, Lenglez S, Nkusi E, Brohee S, Salmon I, Dubois C, et al. SOX2 controls tumour initiation and cancer stem-cell functions in squamous-cell carcinoma. Nature. 2014; 511: 246-250.

166. Liu XF, Yang WT, Xu R, Liu JT, Zheng PS. Cervical cancer cells with positive Sox2 expression exhibit the properties of cancer stem cells. PloS one. 2014; 9: e87092.

167. Hagerstrand D, He X, Bradic Lindh M, Hoefs S, Hesselager G, Ostman A, Nister M. Identification of a SOX2-dependent subset of tumor- and sphere-forming glioblastoma cells with a distinct tyrosine kinase inhibitor sensitivity profile. Neuro-oncology. 2011; 13: 1178-1191.

168. Ma L, Lai D, Liu T, Cheng W, Guo L. Cancer stem-like cells can be isolated with drug selection in human ovarian cancer cell line SKOV3. Acta biochimica et biophysica Sinica. 2010; 42: 593-602.

169. Li D, Zhao LN, Zheng XL, Lin P, Lin F, Li Y, Zou HF, Cui RJ, Chen H, Yu XG. Sox2 is involved in paclitaxel resistance of the prostate cancer cell line PC-3 via the PI3K/Akt pathway. Molecular medicine reports. 2014; 10: 3169-3176.

170. Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, Ravetti GL, Zona GL, Daga A, Corte G. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem cells (Dayton, Ohio). 2009; 27: 40-48.

171. Basu-Roy U, Seo E, Ramanathapuram L, Rapp TB, Perry JA, Orkin SH, Mansukhani A, Basilico C. Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. Oncogene. 2012; 31: 2270-2282.

172. Cox JL, Wilder PJ, Desler M, Rizzino A. Elevating SOX2 levels deleteriously affects the growth of medulloblastoma and glioblastoma cells. PloS one. 2012; 7: e44087.

173. Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G, German Glioma Network. Long-term survival with glioblastoma multiforme. Brain : a journal of neurology. 2007; 130: 2596-2606.

174. Perek D, Perek-Polnik M, Drogosiewicz M, Dembowska-Baginska B, Roszkowski M, Barszcz S. Risk factors of recurrence in 157 MB/PNET patients treated in one institution. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery. 1998; 14: 582-586.

175. Sutter R, Shakhova O, Bhagat H, Behesti H, Sutter C, Penkar S, Santuccione A, Bernays R, Heppner FL, Schuller U, Grotzer M, Moch H, Schraml P, et al. Cerebellar stem cells act as medulloblastoma-initiating cells in a mouse model and a neural stem cell signature characterizes a subset of human medulloblastomas. Oncogene. 2010; 29: 1845-1856.

176. Kanji ZS, Gallinger S. Diagnosis and management of pancreatic cancer. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne. 2013; 185: 1219-1226.

177. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer research. 2014; 74: 2913-2921.

178. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature. 2012; 491: 399-405.

179. Hatzivassiliou G, Haling JR, Chen H, Song K, Price S, Heald R, Hewitt JF, Zak M, Peck A, Orr C, Merchant M, Hoeflich KP, Chan J, et al. Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers. Nature. 2013; 501: 232-236.

180. Walters DM, Lindberg JM, Adair SJ, Newhook TE, Cowan CR, Stokes JB, Borgman CA, Stelow EB, Lowrey BT, Chopivsky ME, Gilmer TM, Parsons JT, Bauer TW. Inhibition of the growth of patient-derived pancreatic cancer xenografts with the MEK inhibitor trametinib is augmented by combined treatment with the epidermal growth factor receptor/HER2 inhibitor lapatinib. Neoplasia (New York, N.Y.). 2013; 15: 143-155.

181. Hofmann I, Weiss A, Elain G, Schwaederle M, Sterker D, Romanet V, Schmelzle T, Lai A, Brachmann SM, Bentires-Alj M, Roberts TM, Sellers WR, Hofmann F, et al. K-RAS mutant pancreatic tumors show higher sensitivity to MEK than to PI3K inhibition in vivo. PloS one. 2012; 7: e44146.

182. Baer R, Cintas C, Dufresne M, Cassant-Sourdy S, Schonhuber N, Planque L, Lulka H, Couderc B, Bousquet C, Garmy-Susini B, Vanhaesebroeck B, Pyronnet S, Saur D, et al. Pancreatic cell plasticity and cancer initiation induced by oncogenic Kras is completely dependent on wild-type PI 3-kinase p110alpha. Genes & development. 2014; 28: 2621-2635.

183. Anonymous The website: clinicaltrials.gov.

184. Kingsley-Kallesen M, Luster TA, Rizzino A. Transcriptional regulation of the transforming growth factor-beta2 gene in glioblastoma cells. In vitro cellular & developmental biology.Animal. 2001; 37: 684-690.

185. Kim JH, Wilder PJ, Hou J, Nowling T, Rizzino A. Activation of the murine type II transforming growth factor-beta receptor gene: up-regulation and function of the transcription factor Elf-3/Ert/Esx/Ese-1. The Journal of biological chemistry. 2002; 277: 17520-17530.

186. Wiebe MS, Traktman P. Poxviral B1 kinase overcomes barrier to autointegration factor, a host defense against virus replication. Cell host & microbe. 2007; 1: 187-197.

187. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983; 65: 55-63.

188. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of immunological methods. 1989; 119: 203-210.

189. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research. 2009; 37: 1-13.

190. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009; 4: 44-57.

191. Telford WG, King LE, Fraker PJ. Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry. Cell proliferation. 1991; 24: 447-459.

192. Griner LN, Reuther GW. Aggressive myeloid leukemia formation is directed by the Musashi 2/Numb pathway. Cancer biology & therapy. 2010; 10: 979-982.

193. Barbouti A, Hoglund M, Johansson B, Lassen C, Nilsson PG, Hagemeijer A, Mitelman F, Fioretos T. A novel gene, MSI2, encoding a putative RNA-binding protein is recurrently rearranged at disease progression of chronic myeloid leukemia and forms a fusion gene with HOXA9 as a result of the cryptic t(7;17)(p15;q23). Cancer research. 2003; 63: 1202-1206.

194. Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CA,4th, Ramalho-Santos J, Van Houten B, Schatten G. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PloS one. 2011; 6: e20914.

195. Sanchez-Diaz PC, Burton TL, Burns SC, Hung JY, Penalva LO. Musashi1 modulates cell proliferation genes in the medulloblastoma cell line Daoy. BMC cancer. 2008; 8: 280.

196. Muto J, Imai T, Ogawa D, Nishimoto Y, Okada Y, Mabuchi Y, Kawase T, Iwanami A, Mischel PS, Saya H, Yoshida K, Matsuzaki Y, Okano H. RNA-binding protein Musashi1 modulates glioma cell growth through the post-transcriptional regulation of Notch and PI3 kinase/Akt signaling pathways. PloS one. 2012; 7: e33431.

197. Cheng X, Huber TL, Chen VC, Gadue P, Keller GM. Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast. Development (Cambridge, England). 2008; 135: 3447-3458.

198. Katoh M, Katoh M. NUMB is a break of WNT-Notch signaling cycle. International journal of molecular medicine. 2006; 18: 517-521.

199. Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Po A, Sico MA, Alimandi M, Giannini G, Maroder M, Screpanti I, Gulino A. Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. Nature cell biology. 2006; 8: 1415-1423.

200. de Sousa Abreu R, Sanchez-Diaz PC, Vogel C, Burns SC, Ko D, Burton TL, Vo DT, Chennasamudaram S, Le SY, Shapiro BA, Penalva LO. Genomic analyses of musashi1 downstream targets show a strong association with cancer-related processes. The Journal of biological chemistry. 2009; 284: 12125-12135.

201. Euskirchen P, Skaftnesmo KO, Huszthy PC, Brekka N, Bjerkvig R, Jacobs AH, Miletic H. NUMB does not impair growth and differentiation status of experimental gliomas. Experimental cell research. 2011; 317: 2864-2873.

202. Wuebben EL, Wilder PJ, Cox JL, Grunkemeyer JA, Caffrey T, Hollingsworth MA, Rizzino A. SOX2 functions as a molecular rheostat to control the growth, tumorigenicity and drug responses of pancreatic ductal adenocarcinoma cells. Oncotarget. 2016.

203. Nowling TK, Johnson LR, Wiebe MS, Rizzino A. Identification of the transactivation domain of the transcription factor Sox-2 and an associated co-activator. The Journal of biological chemistry. 2000; 275: 3810-3818.

204. Gysin S, Rickert P, Kastury K, McMahon M. Analysis of genomic DNA alterations and mRNA expression patterns in a panel of human pancreatic cancer cell lines. Genes, chromosomes & cancer. 2005; 44: 37-51.

205. Ulivi P, Arienti C, Amadori D, Fabbri F, Carloni S, Tesei A, Vannini I, Silvestrini R, Zoli W. Role of RAF/MEK/ERK pathway, p-STAT-3 and Mcl-1 in sorafenib activity in human pancreatic cancer cell lines. Journal of cellular physiology. 2009; 220: 214-221.

206. Moore PS, Sipos B, Orlandini S, Sorio C, Real FX, Lemoine NR, Gress T, Bassi C, Kloppel G, Kalthoff H, Ungefroren H, Lohr M, Scarpa A. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. Virchows Archiv : an international journal of pathology. 2001; 439: 798-802.

207. Berrozpe G, Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. International journal of cancer. 1994; 58: 185-191.

208. Kalthoff H, Schmiegel W, Roeder C, Kasche D, Schmidt A, Lauer G, Thiele HG, Honold G, Pantel K, Riethmuller G. p53 and K-RAS alterations in pancreatic epithelial cell lesions. Oncogene. 1993; 8: 289-298.

209. Basu-Roy U, Bayin NS, Rattanakorn K, Han E, Placantonakis DG, Mansukhani A, Basilico C. Sox2 antagonizes the Hippo pathway to maintain stemness in cancer cells. Nature communications. 2015; 6: 6411.

210. Fang X, Yoon JG, Li L, Tsai YS, Zheng S, Hood L, Goodlett DR, Foltz G, Lin B. Landscape of the SOX2 protein-protein interactome. Proteomics. 2011; 11: 921-934.

211. Rodrigues LU, Rider L, Nieto C, Romero L, Karimpour-Fard A, Loda M, Lucia MS, Wu M, Shi L, Cimic A, Sirintrapun SJ, Nolley R, Pac C, et al. Coordinate loss of MAP3K7 and CHD1 promotes aggressive prostate cancer. Cancer research. 2015; 75: 1021-1034.

212. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. Cancer research. 2007; 67: 1030-1037.

213. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell stem cell. 2007; 1: 313-323.

214. Jaiswal KR, Xin HW, Anderson A, Wiegand G, Kim B, Miller T, Hari D, Ray S, Koizumi T, Rudloff U, Thorgeirsson SS, Avital I. Comparative testing of various pancreatic cancer stem cells results in a novel class of pancreatic-cancer-initiating cells. Stem cell research. 2012; 9: 249-260.

215. Kim MP, Fleming JB, Wang H, Abbruzzese JL, Choi W, Kopetz S, McConkey DJ, Evans DB, Gallick GE. ALDH activity selectively defines an enhanced tumor-initiating cell population relative to CD133 expression in human pancreatic adenocarcinoma. PloS one. 2011; 6: e20636.

216. Feldmann G, Dhara S, Fendrich V, Bedja D, Beaty R, Mullendore M, Karikari C, Alvarez H, Iacobuzio-Donahue C, Jimeno A, Gabrielson KL, Matsui W, Maitra A. Blockade of hedgehog

signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer research. 2007; 67: 2187-2196.

217. Dembinski JL, Krauss S. Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. Clinical & experimental metastasis. 2009; 26: 611-623.

218. Jimeno A, Feldmann G, Suarez-Gauthier A, Rasheed Z, Solomon A, Zou GM, Rubio-Viqueira B, Garcia-Garcia E, Lopez-Rios F, Matsui W, Maitra A, Hidalgo M. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Molecular cancer therapeutics. 2009; 8: 310-314.

219. Rasheed ZA, Yang J, Wang Q, Kowalski J, Freed I, Murter C, Hong SM, Koorstra JB, Rajeshkumar NV, He X, Goggins M, Iacobuzio-Donahue C, Berman DM, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. Journal of the National Cancer Institute. 2010; 102: 340-351.

220. Dosch JS, Ziemke EK, Shettigar A, Rehemtulla A, Sebolt-Leopold JS. Cancer Stem Cell Marker Phenotypes Are Reversible and Functionally Homogeneous in a Preclinical Model of Pancreatic Cancer. Cancer research. 2015.

221. Kurtova AV, Xiao J, Mo Q, Pazhanisamy S, Krasnow R, Lerner SP, Chen F, Roh TT, Lay E, Ho PL, Chan KS. Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. Nature. 2015; 517: 209-213.

222. Chen J, Li Y, Yu TS, McKay RM, Burns DK, Kernie SG, Parada LF. A restricted cell population propagates glioblastoma growth after chemotherapy. Nature. 2012; 488: 522-526.

223. Singh SK, Chen NM, Hessmann E, Siveke J, Lahmann M, Singh G, Voelker N, Vogt S, Esposito I, Schmidt A, Brendel C, Stiewe T, Gaedcke J, et al. Antithetical NFATc1-Sox2 and p53-miR200 signaling networks govern pancreatic cancer cell plasticity. The EMBO journal. 2015; 34: 517-530.