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Oct3 and Sox2 Expression in Mammary Carcinoma Cells

By

Yvette Marie Soignier

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor G. Steven Martin, Chair
Professor Kunxin Luo
Associate Professor David Bilder
Professor David Schaffer

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Abstract

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by

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor G. Steven Martin, Chair

A complex transcriptional circuitry is essential in maintaining the self-renewal capacity and the undifferentiated state of embryonic stem cells (ESCs). Key factors in this pluripotency network include Sox2, Nanog, and Oct3/4, which form multiple positive and negative feedback loops to regulate an array of downstream genes. Although it was previously thought that pluripotency factors are downregulated upon differentiation, the phenotypic similarities between ESCs and cancer cells have led to speculation that common underlying regulatory mechanisms may exist. For example, the cancer phenotype includes a block in differentiation, limitless replicative potential and increased proliferation, characteristics that are also observed in pluripotent embryonic stem cells. Taken together, these observations regarding similarities in tumor cells and embryonic stem cells suggest regulatory mechanisms that may be shared in development and cancer. In the studies presented here, I investigated the expression, localization, and function of Nanog, Sox2, Oct4 and particularly the Oct3 splice variant protein in cancer.

I performed a variety of biochemical, genetic and pharmacological experiments to elucidate potential roles of these pluripotency factors in maintaining the cancer phenotype, using a panel of breast cancer cells as a model system. Expression of Sox2 and the Oct3 isoform was detected in all breast cancer cell lines investigated, whereas Nanog and Oct4 expression were not detected. Moreover, Sox2 was localized to both the cytoplasm and nucleus, but Oct3 expression was detected exclusively the cytoplasm of breast cancer cells. This study also shows that Oct3 and Sox2 are upregulated in a number of breast cancer cell lines when compared to expression levels in mammary epithelial cells. I also determined that Src is an upstream regulator of Oct3 and Sox2 expression.

Given the known function of Sox2 as a regulator of the cell cycle, I focused subsequent studies on the characterization and function of Oct3. Results showed that the Oct3 N-terminal domain contains an auto-inhibitory sequence that blocks its nuclear translocation despite the presence of a functional nuclear localization signal. Additional experiments to investigate Oct3 function suggested that Oct3 may be involved in regulating the cell cycle and that this regulation may be in concert with Sox2 and β-catenin to modulate cyclin D1 expression. However, studies of Oct3 function in cancer

remain inconclusive due to technical difficulties in Oct3 RNAi that precluded achieving consistent Oct3 knockdown. Despite these difficulties, the data presented here suggest that Oct3 may play a role in maintaining the cancer phenotype, warranting further functional studies.

To my wonderful, supportive, patient, and dedicated partner
(and should the law allow us someday, Wife), Caroline

and

To my loving father, Elmo
who is greatly missed.

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Other scientific inspiration began at a young age, although I didn't even realize it at the time. I came to academic science relatively late after pursuing other interests, but an early influence and support was certainly my high school freshman biology teacher, Peggy Kersh, who offered me understanding and friendship, and taught me that science was pretty cool even though my interests were generally about poetry and art at that age. I would also like to thank Dr. Jeffery Miller from the University of Minnesota Cancer Center. Jeff was my P.I. at my first lab job upon graduating with my biology degree, and it is certainly largely because of him that I came to Berkeley. He gave me so much encouragement and so many opportunities, and his influence steered me to pursue cancer biology. I am forever grateful.

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rooted culture based on intense family ties. It made me realize how much a part of me my extended family and my upbringing are, despite my pursuit of a different sort of life in a very different place. I would especially like to thank my mom, Ginger, and my "little" sister, Holly, for their love and unconditional support. I am thankful for how our relationships have deepened and evolved over time, and I am thankful for their embrace of my differences. I also take great comfort in the fact that I can head home to the South at any time and be assured of receiving that famous Southern hospitality and delicious home-cooked Cajun/Creole meals, since my mom loves nothing more than cooking for her family. My mom's diagnosis of breast cancer last year, which fortunately turned out to be fully treatable, was another motivating reminder that the basic research I have pursued during my graduate studies has at its core a goal of advancing cancer therapies.

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Chapter 1

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass of the pre-implantation embryo and are characterized by their unlimited capacity for self-renewal and their pluripotency, or capacity to differentiate into all cell lineages. A complex transcriptional circuitry comprised of multiple feedback loops is essential in maintaining the self-renewal capacity and the undifferentiated state of ESCs. Core factors in this pluripotency network include the transcription factor Nanog and its upstream regulators, the HMG-binding protein Sox2 and the homeodomain protein Oct3/4 (Boyer et al., 2005; Rodda et al., 2005). The human *oct-3/4* gene encodes two alternatively spliced protein isoforms, Oct3 (also known as Oct3b or POU5F1b) and Oct4 (also known as Oct3a or POU5F1a) (Takeda et al., 1992). Members of this pluripotency network exhibit distinct, highly regulated expression patterns during embryonic development.

Although it was previously thought that ESC factors are downregulated upon differentiation, the striking phenotypic similarities between ESCs and cancer cells have led to speculation that common underlying regulatory mechanisms may exist (Greenburg et al., 1982; Thiery et al., 2002). Many parallels are found between stem cells and cancer cells, including unlimited self-renewal, increased proliferation, and an undifferentiated phenotype (Sell and Pierce, 1994; Reya et al., 2001). Because it has been shown that stem cells and tumor cells have common activated signaling pathways, including the Wnt, Shh, and Notch pathways (Taipale and Beachy, 2001; Varnum-Finney et al., 2000), and because they share phenotypic characteristics, it has been suggested that similar regulatory mechanisms might be involved in maintaining stem cell pluripotency and in promoting cancer. Thus, I endeavored to investigate mechanisms that contribute to breast cancer progression, applying paradigms of stem cell regulation to the biology of breast tumors.

The objective of these studies was to better understand the role that pluripotency transcriptional regulators might play in cancer, using breast cancer as a model. I initially hypothesized that the ectopic expression of transcription factors that maintain pluripotency in ESCs contributes to the continued proliferation and the block in differentiation observed in many cancers. Preliminary expression studies demonstrated that Nanog or Oct4 were not expressed in breast cancer cells, whereas Sox2 and the Oct3 splice variant were expressed. I therefore focused on the roles of Sox2 and the Oct3 splice variant in breast cancer progression. Since a role for Sox2 in the regulation of proliferation in breast cancer cells was recently published (Chen et al., 2008), I focused in particular on Oct3, whose functions and potential roles in cancer development remain largely unknown. I sought to clarify the functions of Oct3 and its possible role in tumor progression using a panel of breast carcinoma cell lines as a model system. Additionally, I endeavored to further characterize Oct3 and how its regulation by investigating the mechanisms of its cellular localization.

The core pluripotency transcription network: Nanog, Sox2, and Oct3/4

High-throughput screening methods such as global expression profiling using ChIP and ChIP-seq data have facilitated the identification of important components of the pluripotency network. In 2005, Boyer et al. investigated the transcriptional circuitry responsible for regulating mammalian cell pluripotency and differentiation. Using ChIP experiments coupled with microarrays, they determined that the Oct3/4, Sox2, and Nanog transcription factors collaborate to form a network that consists of feedforward and

autoregulatory loops *(Figure 1-1)*. These proteins have critical roles in early development and are considered central in a hierarchy of transcription factors that maintain pluripotency and specify ESC identity (Avilion et al., 2003; Chambers et al., 2003; Hart et al., 2004; Lee et al., 2004; Mitsui et al., 2003; Nichols et al., 1998; Schöler et al., 1990). They form multiprotein complexes on DNA and orchestrate expression of hundreds of downstream proteins. Taken together, Nanog, Sox2 and Oct3/4 regulate approximately 10% of the human genome (Boyer et al., 2005; Babaie et al., 2007).

In the complex Oct3/4-Sox2-Nanog regulatory circuit, all three transcription factors bind to their own promoters and regulate themselves as well as regulating each other in both positive and negative feedback loops (Pan et al., 2006). Additionally, they co-occupy a number of promoters of downstream genes as well as independently regulating other genes (Boyer et al., 2005; Chen et al., 2008). Among Oct3/4 bound genes, half are also bound by Sox2. More than 90% of promoters co-occupied by Oct3/4 and Sox2 are also bound by Nanog. ChIP-Seq data have shown that combinatorial binding of pluripotency transcription factors drives ESC-specific gene expression, and Sox2-Oct3/4 co-occupancy on canonical sox-octamer composite elements is a common regulatory motif (Chew et al., 2005; Nishimoto et al., 1999). Notably, components of the TGF-β and Wnt signaling pathways, known to regulate pluripotency, are downstream targets of the Oct3/4-Sox2-Nanog network (Boyer et al., 2005).

Nanog

Nanog is a homeodomain protein that is a central regulator of embryonic stem cell pluripotency. It was first identified by screening mouse ESCs for genes specifically expressed in that cell type and not in adult cells, and was found to be capable of maintaining pluripotency in the absence of LIF (leukemia inhibitory factor) (Mitsui et al., 2003). Structurally, Nanog comprises a C-terminal domain, a conserved homeobox domain that facilitates DNA binding, and an N-terminal domain. A nuclear localization signal (NLS) is found in the homeobox domain and is required for the complete nuclear localization of Nanog, critical to its DNA binding and transcriptional regulatory functions (Do et al., 2007). Oct3/4 and Sox2 are critical regulators of Nanog expression, and it has been shown that the presence of canonical octamer and sox elements are essential for transcriptional cis regulation of Nanog gene expression (Rodda et al., 2005; Kuroda et al., 2005). Constitutive expression of Nanog is sufficient to maintain pluripotency of ESCs in the absence of LIF (Leukemia inhibitory factor), although the presence of endogenous levels of Sox2 and Oct3/4 is required. Constitutive expression of Sox2 or Oct3/4 is not sufficient to maintain pluripotency in the same context (Pan and Thomson, 2007; Chambers et al., 2003). Loss of Nanog induces differentiation of ESCs into endoderm lineages (Lin et al., 2005); therefore, deletion of Nanog causes early embryonic lethality (Mitsui et al., 2003b).

Sox2

Sox2 (Sex-determining region-Y box 2), which was initially identified as playing a role in neural development (Uwanogho et al., 1995), is a pluripotency transcription factor that contains an HMG (High Mobility Group) domain and is coexpressed with Oct3/4 in the early embryo (Wegner, 1999). Sox2 is localized to both the nuclei and cytoplasm of pre-implantation embryos, and has been shown to shuttle between these

cellular compartments in a mechanism mediated by its two nuclear localization signals and in cooperation with Oct3/4 (Avilion et al., 2003, Li et al., 2007). This shuttling function is critical for maintaining the pluripotent state (Li et al., 2007). Sox2 knockout mice have an embryonic lethal phenotype, as lack of Sox2 induces trophectoderm differentiation and polyploidy (Li et al, 2007). Sox2 is necessary for regulating a number of transcription factors that affect Oct3/4 expression. Forced expression of Oct3/4 rescues the pluripotency of Sox2-null ESCs, indicating that the essential function of Sox2 is to stabilize ESCs in a pluripotent state by maintaining a steady-state level of Oct3/4 expression (Masui et al., 2007). The cooperative interaction of Sox2 with Oct3/4, an important aspect of regulation of their downstream genes, is discussed below.

Oct3/4

The transcription factor Oct3/4 is an essential regulator of pluripotency in the mammalian embryo. It is expressed in unfertilized oocytes, in the inner cell mass and in epiblasts of pre-implantation embryos as well as in primordial germ cells (Abdel-Rahman et al., 1995; Pesce et al., 1998; Adjaye et al., 1999; Goto et al., 1999). Oct3/4 is rapidly downregulated upon differentiation (Okamoto et al., 1990). An Oct3/4 deficiency causes embryonic lethality in mice, since the inner cell mass does not form and embryonic cells exclusively differentiate into trophoblasts (Niwa, 2001; Pesce and Scholer, 2001; Nichols et al., 2001). *oct3/4* gene regulation is accomplished by two enhancers linked to a single promoter (Ovitt et al, 1998). One of these enhancers is active only in undifferentiated cell types, whereas the second enhancer drives post-implantation Oct3/4 expression in the embryo (Okazawa et al., 1991; Yeom et al., 1996; Minucci et al., 1996).

Oct3/4, which is a member of the POU (PIT/Oct/UNC) class of homeodomain proteins, can heterodimerize with Sox2 via interactions between the Oct3/4 POU homeodomain and the Sox2 HMG box. The POU domain consists of two separate DNA binding regions, an approximately 75 amino acid N-terminal POU-specific (POU_S) domain and a 60-amino acid C-terminal homeo (POU_H) domain (Herr et al., 1988). These two domains are joined by a flexible linker of variable length, allowing for a range of DNA binding opportunities (Herr and Cleary, 1995; Vigano et al., 1996; Remenyi et al., 2001). The domain structure of Oct3 is depicted in *Figure 1-2*. Expression profiling of Oct3/4-manipulated ESCs has determined that Oct3/4 can have a positive or negative effect on expression of downstream genes, and that the activation or repression is dependent on the level of Oct3/4 (Matoba et al., 2006).

Oct3/4-Sox2 interactions, either by heterodimerization or by their combined binding to octamer-sox elements of downstream promoters, have been shown to affect the expression of a number of genes in ESCs (Botquin et al., 1998; Nishimoto et al., 1999; Yuan et al., 1995). Structural studies have found that Oct3/4 and Sox2 can dimerize onto DNA in distinct conformational arrangements, and that the DNA enhancer region of their target genes is responsible for the spatial alignment of the interaction domains (Remenyi et al., 2003). One example of Sox2-Oct3/4 synergistic activation is their cooperative binding to the *fgf4* enhancer that is dependent on the spatial arrangement of Oct3/4 and Sox2 on their binding sites (Ambrosetti et al., 1995). In fact, cooperative interaction between POU domain transcription factors and HMG-box factors is considered to be an essential mechanism for the developmental control of gene expression (Dailey and Basilico, 2001).

Pluripotency, the Epithelial-Mesenchymal Transition, and Cancer

During metazoan embryogenesis, the Epithelial to Mesenchymal Transition (EMT) is a dynamic and essential process that induces migration and morphogenesis via epithelial cell plasticity. Beyond embryonic developments, an example of the importance of normal epithelial plasticity and the acquisition of invasive properties without resulting in a mesenchymal phenotype occurs during mammalian puberty and pregnancy with branching morphogenesis in the mammary gland (Hens and Wysolmerski, 2005). Outside of developmental contexts, the EMT is recapitulated in the deregulated signaling pathways of tumor cells, conferring these cells with invasive and metastatic ability (Thiery and Chopin, 1999). Precise kinetics and regulatory mechanisms of the EMT are not well understood. There are numerous parallels between EMT and normal development. In both normal embryogenesis and cancer progression, the EMT is characterized by a dedifferentiation to a disorganized, fibroblastoid phenotype; loss of E-cadherin expression; gain of vimentin expression; loss of epithelial cell polarity and cytoskeletal organization; and cell scattering, which is defined by a loss of intercellular junctions and gain of motility (Lee et al., 2006; Micalizzi et al., 2010).

It has been shown that cancer cells with an EMT phenotype also exhibit stem cell-like characteristics (Kong et al., 2010). One theory purports that cancer is, in fact, a disease of stem cells, postulating that cancers arise in progenitor cells or stem cells, an idea supported by the block in differentiation exhibited in tumor cells (Reya et al., 2001). While it may be true that a subpopulation of cancer cells are so-called "cancer stem cells," our studies focused on the possibility that global expression of factors in carcinoma cells may maintain the pluripotency phenotype.

Potential roles of Nanog, Sox2 and Oct3/4 in cancer progression

The possible significance of ESC transcription factors in tumor progression first came to light in germ cell tumors and leukemias (Reya et al., 2001; Hart et al., 2005; Looijenga et al., 2003; Korkola et al., 2006), and recently their potential role in solid tumor progression has garnered attention. A series of reports have claimed that Nanog and Oct3/4, previously thought to be expressed exclusively in pluripotent cells and rapidly dowregulated upon differentiation, are expressed in various cancer cell lines and primary tumors. Sox2, in contrast, has been shown by a number of studies to be expressed in a variety of normal and cancerous adult tissue types, although overexpression has been shown to correlate with a cancer phenotype (Rodriguez-Pinilla et al., 2007; Lu et al., 2010; Tani et al., 2007).

Nanog expression has been reported in retinoblastoma, breast cancer cells and germ cell tumors by immunocytochemisty, immunohistochemistry and RT-PCR. (Seigel et al., 2007; Santagata et al., 2007; Ezeh et al., 2005). Of particular significance are two recent studies demonstrating Nanog's ability to transform NIH3T3 mouse fibroblast cells and promote proliferation when overexpressed (Piestun et al, 2006; Zhang et al., 2005).

Oct3/4 expression has been reported in pancreatic, liver, breast, cervical, bladder and prostate cancer cell-lines, in solid tumor samples and in peripheral blood mononuclear cells (Jin et al., 1999; Monk and Holding, 2001; Ezeh et al., 2005; Tai et al., 2005, Zangrossi et al., 2007; Chang et al., 2008; Bae et al., 2010). It is unclear in these reports whether the observed expression is of the Oct3 or Oct4 isoform, and the methods were primarily non-quantitative. The oncogenic capacity of Oct3/4 was investigated in a

study in which ectopic expression of Oct3/4 in Swiss3T3 cells resulted in transformation and tumor growth in nude mice. The oncogenic potential of Oct3/4 was also found to be dose-dependent, and inactivation of Oct3/4 induced tumor regression (Gidekel et al., 2003). Oct3/4 has also been shown to promote epithelial dysplasia by inhibiting progenitor cell differentiation (Hochedlinger et al., 2005). Gene expression profiling data has identified several broad categories of genes that are correlated to Oct3/4 expression, including genes regulating chromatin structure, nuclear architecture, DNA repair, apoptosis, and cell cycle control (Campbell et al., 2007). Oct3/4 has also been shown to be a potential transcriptional activator of FGF-4 in breast cancer cells (Wang et al., 2003). While the aforementioned studies have explored the possibility of the oncogenic potential of Oct3/4 without discriminating between the alternatively spliced variants, limited data have yet been published regarding the expression or specific functions of Oct3 or Oct4 in normal or cancerous cells.

Sox2, which is notably located at chromosome 3q, a region frequently gained in basal-like and BRCA1 germline mutated breast cancer (Wessels et al., 2002), has been reported to be expressed in a number of tumor types. Expression of Sox2 has been demonstrated in prostate, breast, gastric, colorectal, glioma, lung and germ cell tumors by immunohistochemistry and immunoblotting. (Tani et al., 2007; Gu et al., 2007; Schmitz et al., 2007; Rodriguez-Pinilla et al., 2007; Bae et al., 2010; Lu et al., 2010). Sox2 expression has also been shown to correlate with vimentin expression in cells with an invasive or basal-like phenotype and may play a role in defining their less differentiated phenotypic characteristics (Rodriguez-Pinilla et al., 2007). Overexpression of Sox2 in the epithelial cells of the lung in a mouse model led to extensive hyperplasia and eventual carcinoma (Lu et al., 2010). One recent study suggested that ectopic expression of Sox2 may be associated with abnormal differentiation of colorectal cancer cells (Tani et al., 2007).

A report published in June of 2008 entitled "The Molecular Mechanism Governing the Oncogenic Potential of Sox2 in Breast Cancer" by Chen et al. detailed several aspects of our originally proposed research investigations with respect to Sox2, demonstrating that Sox2 is overexpressed in human breast tumors and that expression level correlates with tumor grade. This study also showed that Sox2 promotes proliferation of breast cancer cells by binding to the cyclin D1 promoter in complex with β-catenin and that Sox2 directly binds to β-catenin and does not stably bind DNA on its own.

The above observations suggest potential roles for the Oct3/4-Sox2-Nanog regulatory network in tumor progression. In a clinical study, it was shown that Oct3/4 and Sox2 are associated with an unfavorable outcome in patients with esophageal squamous cell carcinoma (Wang et al., 2009), highlighting the significance of the studies presented here.

I was unable to detect Nanog or Oct4 expression in breast cancer cells by either immunoblot or quantitative-RT-PCR methods; however, I did detect expression of Oct3 and Sox2 in all of the breast cancer cell lines tested. The discrepancy between our results and those of previously published reports might be attributed to several factors, including the existence of a number of Nanog pseudogenes, the existence of Oct4 pseudogenes, or due to the homology of the Oct3 and Oct4 alternatively spliced transcripts. There are 11 known Nanog pseudogenes, one of which, NanogP8, has been identified as a retrogene

that is highly homologous to Nanog and has been shown to be expressed in cancer cell lines and cancer tissues (Booth and Holland, 2004; Zhang et al., 2006). The methods used in previously published reports demonstrating Nanog expression in breast cancer may have detected NanogP8 expression or other Nanog pseudogenes. Additionally, there are six known pseudogenes of the Oct4 isoform with potential to generate artifacts in PCR experiments (Liedtke et al., 2007, Kotoula et al., 2008). In reports that describe "Oct4" or "Oct3/4" expression, the existence of the two isoforms is not explained and the Oct3 variant is not distinguished from Oct4. Therefore, previous demonstrations of "Oct4" expression may have mistakenly identified Oct3 as Oct4. A single recent report investigating Oct3 and Oct4 expression in prostate cancer cells (Monsef et al., 2009) explored this issue and determined that the observed expression of "Oct3/4" in prostate cancer was only of the Oct3 isoform.

Given that I detected Oct3 and Sox2 expression in breast cancer cells and because of the results presented by Chen et al. detailing a mechanism for Sox2 regulation of the cell cycle in breast cancer cells, I pursued further investigations into the role of Oct3 in regulating cyclin D1, β-catenin, and breast cancer cell proliferation. Several authors have suggested that OCT3/4 and β-catenin might both be involved in the same oncogenic pathway, since both genes are master regulators of cell differentiation and overexpression of either gene may result in transformation (Palma et al., 2008). Significantly, β-catenin can physically interact with Oct3/4 to upregulate Nanog expression (Takao et al., 2007). The fact that Oct3/4 and Sox2 cooperate in maintaining ES cell pluripotency and are able to heterodimerize to elicit downstream effects presents a strong case for the idea that Oct3 may also be involved in the regulation of the cell cycle in cooperation with Sox2.

Oct3 structure, function, and regulation

Oct3 and Oct4 are protein isoforms arising from alternatively spliced transcripts belonging to a family of transcription factors that contain the conserved POU DNA-binding domain. POU domains bind to the consensus octamer motif (ATG-CAAAT) in DNA (Parslow et al., 1984; Flakner and Zachau, 1984). Both Oct3 and Oct4 comprise three domains *(Figure 1-2)*: the N-terminal domain (NTD) which is unique to each isoform, the central POU domain that is two amino acids shorter in Oct3 but otherwise identical to Oct4, and identical C-terminal domains (CTD) (Scholer et al., 1990; Okamato et al., 1990; Rosner et al., 1990; Goto et al., 1999; Hansis et al., 2000; Burdon et al, 2002). Oct4 activates transcription via octamer motifs located proximal or distal to transcriptional start sites. Importantly, the unique Oct4 N-terminal domain includes a tranactivation motif that is not present in Oct3. While the CTD of Oct3/4 serves a redundant transactivation function (Niwa et al., 2002), it is not clear whether the CTD can compensate for the lack of a transactivation motif in the Oct3 NTD. The Oct3/4 CTD Oct4 binding sites have been found in a number of genes, including such cancer-relevant genes as *fgf4* and *pdgfr* (Ambrosetti et al, 2000; Kraft et al, 1996). Compared with Oct4, little is known about the properties of Oct3; however, it has been shown that in human ESCs, Oct3, unlike Oct4, is located in the cytoplasm, does not stimulate transcription and does not bind to DNA (Lee et al, 2006; Cauffman et al 2006). Additionally, the report from Lee et al. demonstrated that the Oct3 NTD contains two separate sequences inhibitory to DNA binding. These observations have called into question whether Oct3 is a regulator of pluripotency, or whether it might serve other cytoplasmic functions.

While both Oct3 and Oct4 encode a conserved NLS within their POU domains (Pan et al., 2004), a unique feature of the Oct 4 NTD is a conserved NLS that is not present in Oct3. However, the N terminal domain of Oct3 does contain several potential serine, threonine and tyrosine phosphorylation sites (Saxe et al., 2009), which suggests the possibility that the extranuclear Oct3 may be modified by signaling molecules. Consistent with this, Oct3/4 has been reported to exist as a phosphoprotein in embryonic carcinoma cells (Brehm et al), although the specific isoform is again unclear.

Phosphorylation of Oct3/4 may be one post-translational modification that impacts Oct3 expression levels and/or localization; another modification that may be at work to regulate Oct3 is ubiquitination. Xu et al. (2004) demonstrated that the Oct3/4 NTD and CTD can both bind ubiquitin and that this modification dramatically suppresses its transcriptional activity. In addition, they found that Wwp2, a novel ubiquitin ligase, promotes Oct3/4 ubiquitination and degradation by the proteosome pathway.

Beyond the known oct-sox composite motifs to which Oct3/4 itself and Sox2 bind and regulate Oct3/4, other data regarding Oct3/4 regulation is limited. It has been shown that a specific nuclear receptor, Gcnf (germ cell nuclear factor) is involved in regulating Oct3/4 expression (Donovan, 2001; Fuhrmann et al., 2001), and that the Src family non-receptor tyrosine kinase c-Yes is also involved in regulating Oct3/4 expression levels (Anneren et al., 2004).

Very limited data have yet been published regarding the function of Oct3 in normal or cancerous cells. Reports of Oct3/4 in cancer cells have, for the most part, been limited to non-quantititative expression studies that do not distinguish between the Oct3 and Oct4 variants. One recent report clarified that Oct3 is expressed in the cytoplasm of prostate cancer cells and that Oct4 is not expressed in prostate cancer (Monsef et al., 2009). These observations are in agreement with our observations of Oct3 expression in the cytoplasm of breast cancer cells.

Oct3 localization

Our experiments demonstrated that endogenous Oct3 is found exclusively in the cytoplasm of breast cancer cells. These data are consistent with reports that have described cytoplasmic Oct3 localization in ESCs (Lee et al., 2006) and in prostate cancer cells (Monsef et al., 2009). Interestingly, when I exogenously overexpressed Oct3 by transient transfection of cells with an Oct3 expression plasmid, the protein is localized to both nuclear and cytosolic compartments, albeit maintaining a predominantly cytoplasmic localization. This suggested that a modification of Oct3, such as phosphorylation, may be involved in maintaining Oct3 in the cytoplasm.

I performed a series of mutagenesis experiments to pinpoint the critical amino acids involved in regulating Oct3 cellular localization.

Regulation of Oct3 expression by Src family kinases

The Src family of tyrosine kinases is critical for ESC self-renewal and maintaining stem cells in an undifferentiated state (Anneren et al., 2004). Src functions at the hub of diverse networks modulating proliferation, apoptosis, angiogenesis, migration, and adhesion, all of which impact on cancer progression when dysregulated (Summy and Gallick, 2003). c-Src is the cellular counterpart of the highly activated v-Src oncogene of the avian Rous sarcoma virus. While the constitutively active v-Src protein is highly

transforming and has been shown to promote metastasis in vivo (Stoker and Sieweke, 1989; Boyer et al., 2002), the role of c-Src and other Src family members such as c-Yes and c-Fyn in transformation and disease progression is unclear. c-Src is only weakly transforming (Irby et al., 1997); however, it is known to promote an array of functions implicated in cancer progression when overexpressed or activated in primary tumors and cell lines (Boyer et al., 2002). c-Src is commonly overexpressed and/or activated in cancers, including breast, lung, and colon carcinomas (Summy and Gallick, 2003; Irby et al., 1997; Talamonti et al., 1993). Moreover, increases in c-Src activity and levels have been correlated with disease progression both in cell lines and clinical samples (Summy and Gallick, 2003, Boyer et al., 2002; Anneren et al., 2004).

A recent report demonstrated that Src family kinases are necessary for maintaining the undifferentiated state in ESCs (Anneren et al., 2004). This study showed that Src inhibition reduced cell growth, induced differentiation, and reduced expression of Oct3/4 in mouse & human ESCs; additionally, RNAi against the Src family member c-Yes induced differentiation. Chemical inhibition of Src family kinases was also shown to inhibit expression of Oct3/4 and Nanog as well as other stem cell markers such as FGF-4 and alkaline phosphatase. I selectively inhibited Src activity in MCF7 breast carcinoma cells using PP2, a small molecule chemical inhibitor and observed concurrent downregulation of Sox2 and Oct3 expression, suggesting that Src is a regulator of Sox2 and Oct3 in breast cancer cells.

Summary of findings

The data presented here demonstrate that Sox-2 and Oct3 are overexpressed in breast cancer cells, while Nanog and Oct4 are not expressed. Known functions of Sox2 in maintaining pluripotency and in promoting breast cancer development, as well as its cooperative interactions with Oct3/4, suggest that Oct3 may play a role in the proliferation, differentiation, survival or invasive properties of breast cancer cells. Additionally, I have determined that Oct3 is localized exclusively in the cytoplasm of all cell lines tested, and that Sox2 is localized to both the nuclei and cytoplasm, with predominant localization in the cytoplasm. Further experiments to characterize Oct3 by mutagenesis of its unique NTD have shown us that the Oct3 NTD contains an autoinhibitory domain that blocks nuclear transport, perhaps by blocking a nuclear localization signal present in the Oct3 POU domain. Given the upregulation of expression and the unexpected localization of embryonic pluripotency factors in the cytoplasm of breast carcinoma cells, it is possible that these proteins perform novel functions that are involved in the etiology of breast cancer.

Chapter 1
Figure legends

Figure 1-1. Core Transcriptional network of the embryonic stem cell

Shown is a model of the pluripotency transcriptional circuitry taken from Chickarmane et al., 2006.

Figure 1-2. Schematic representation of Oct3/4 domain structure

From Lee et al., 2006. Shown is a schematic representation of the protein domains of the human Oct-4 isoforms, Oct-4A (Oct4) and Oct-4B (Oct3). The N-terminal domains (NTD), POU DNA binding domains (POU), and C-terminal domains (CTD) of the two splice variants are depicted with the corresponding amino acid positions. The unique 133 amino acids at the N terminus of Oct4 are shown in black and the unique 40 amino acids at the N terminus of Oct3 are shown in dark gray.

Chapter 1
Figures

Figure 1-1

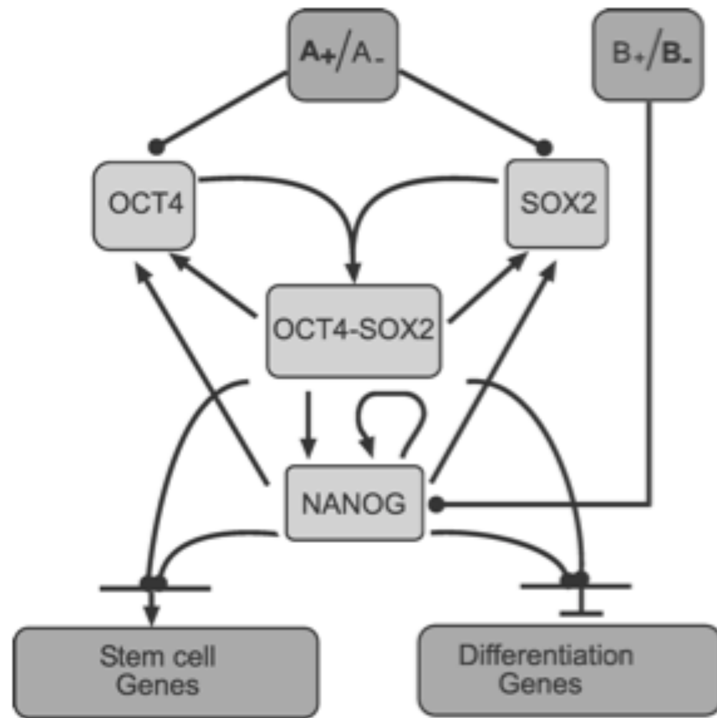
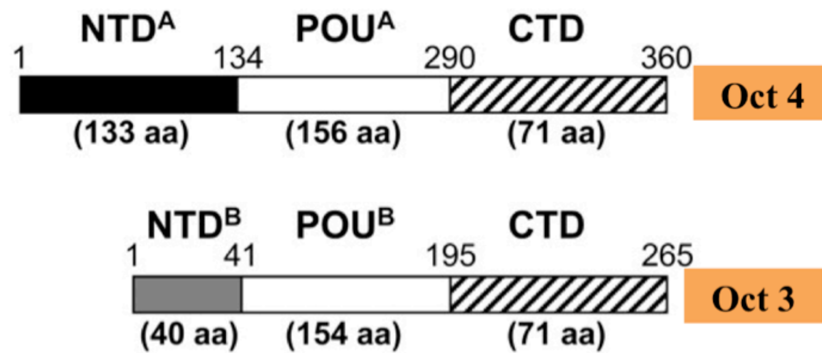


Figure 1-2



Chapter 2

Materials and Methods

Reagents and DNA constructs

pCMV5α-Oct4-3xFlag, pCMV5α-Sox2-3xFlag and pCMV5α-Nanog-3xFlag were kind gifts from Robert Tjian (UC Berkeley). pMax-GFP was purchased from Lonza as a component of the Solution V nucleofection kit.

All mutations were generated by PCR using the Phusion site directed mutagenesis kit (Finnzymes) with primers designed and products amplified according to the manufacturer's instructions. HPLC- purified phosphorylated primers used to generate the mutations were all purchased from IDT, and are shown in *Table 2-1*. The following cycling conditions were used: (1) 98° C for 30 s; (2) 98° C for 10 s; (3) annealing at primer-specific temperature for 30 s; (4) 72° C for 30 s/1 kb; (5) repeat steps 2-4 24 times (6) 72°C for 10 min; (7) 4° C hold. PCR products were purified by agarose gel electrophoresis and isolated using a gel purification kit (Qiagen). DNA was then ligated with T4 quick ligase (Finnzymes) and used to transform E. coli (Top-10, Invitrogen) by chemical transformation as per the manufacturer's instructions. Bacteria were spread on sterile agarose plates containing the appropriate selection antibody and grown overnight at 37°C. Colonies were screened for the presence of the desired mutation(s) by extracting DNA from cultures using a miniprep kit (Qiagen) followed by DNA sequencing.

The Oct3 overexpression construct pCMV6-Oct3 (POU5F1 transcript variant 2 Human cDNA clone) was purchased from Origene Technologies. The ORF of Oct3 was excised via its flanking Not1 sites. A Kozak sequence to maximize expression and flanking 5' SacI and 3' Hind III restriction sites were added by PCR mutagenesis (Forward primer, (5'GCCGCCATGCACTTCTACAGACTATTCCTTGG; Reverse primer, 5'GAGCTCGGATCCGAGCTCGGTACCAAGCTTAA3'). This cassette was then subcloned into SacI and HindIII sites of the pcDNA 3.1 MCS. 5'HindIII and 3' NotI sites were added to a TEV-3xFlag tag (provided by Don Rio, UC Berkeley) by PCR (Forward primer, (5'GGTATTAAGCTTCACCACAAAACCGCGGCTCTTGCCAA3'; Reverse primer, (5'GCGTTAAGCGGCCCGCATTACTTGTCATCGTCATCCTTGTA3'), which was then cloned into pcDNA3.1-Oct3 at the 3' end of the Oct3 ORF via HindIII and NotI sites. The final resulting expression construct is as follows: pcDNA 3.1-(5'-Sac1–Kozak-Oct3-HindIII-TEV-3xFlag-Not1-3').

pmCherry-N1-Oct3 was engineered by subcloning the Oct3 ORF from pcDNA3.1 into SacI / HindII sites of the pmCherry-N1 MCS.

Sources for primary antibodies used in this research are shown in *Table 2-2*. Secondary antibodies used in these studies were HRP-conjugated anti-mouse IgG, anti-rabbit IgG (Cell Signaling) and anti-goat IgG (Santa Cruz Biotechnology); and anti-goat IgG Alexa-fluor 546 (Invitrogen) for immunofluorescence staining.

The Src-specific inhibitor PP2 was purchased from Calbiochem.

Cell culture

Cell lines were obtained from ATCC and cultured according to recommendations from ATCC. Briefly, the mammary epithelial cell lines MCF10A and 184a1 were maintained in MEGM complete medium (Lonza); all other mammary carcinoma cell lines were maintained in DMEM basal medium containing 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin (PS) (Invitrogen). Medium was replaced 2-3 times a week. Cells were propagated in a 37°C incubator with 5% CO_2 and were passaged upon reaching 80-90% confluency by treating with 0.25% trypsin containing 0.2 mg/l EDTA (Invitrogen) for 5 minutes at 37°C. When necessary, cells were counted using a Coulter counter (Beckman Coulter) and plated at appropriate densities according to ATCC guidelines for propagation or according to experimental requirements. A table describing the properties of the cell lines used in these studies is provided below (*Table 2-3*).

Transfections

Cells were transfected via electroporation using the Amaxa Nucleofection instrument (Lonza) as per the manufacturer's instructions. Electroporation programs, transfection reagents, and culture conditions were selected based on the optimized conditions for each cell line as described by the manufacturer. Briefly, for the MCF7 cell line, cells were subcultured at a density of $2x10^5$ cells/cm^2 3 days before transfection. Prior to harvesting for transfection, cells were examined under a light microscope and determined by eye to be ~75-80% confluent to ensure optimum survival and transfection efficiency. Cells were then harvested using 0.25% trypsin-EDTA, washed with DMEM+10% FBS followed by phosphate-buffered saline (PBS) and then counted. For each transfection, $2x10^6$ cells were suspended in 100 μl Nucleofector Solution V, and 2 μg total plasmid DNA and/or 30-300 nM siRNA was added. The entire sample was transferred to a cuvette, placed in the Amaxa Nucleofector Apparatus II, and pulsed using pre-set program "P-020". Following electroporation, samples were immediately suspended in 500 ul complete medium, transferred to a single well of a 6 well tissue culture plate containing 1.5 ml complete medium, then placed in a humidified incubator. Transfection efficiency was assessed after 24 hours and further analysis was performed after 48-96 hours. Similar procedures were used for other cells but were modified using cell-line specific conditions suggested by Amaxa.

Cell Lysis

Whole-cell extracts for immunoblotting were prepared in RIPA lysis buffer (20 mM Tris-HCL (pH 7.5), 1% sodium deoxycholate, 2 mM EDTA, 1% Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 0.2 mM Na_3VO_4, and 1 Mini Complete Protease Inhibitor Cocktail tablet (Roche) per 10 ml).

Subcellular fractionation

To separate cell homogenates into nuclear and cytosolic fractions, cells were grown until nearly confluent on 10 cm plates. All protocol steps were performed using ice-cold buffers, tubes were kept on ice and centrifugation steps were performed at 4°C. Plates were washed twice with PBS, then 500 μl Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl 0.1 mM EDTA, and 1 Mini Complete Protease Inhibitor Cocktail tablet (Roche) per 10 ml) was added and plates were incubated at 4° C with gentle rocking for 10 min. Cells were detached by scraping with a rubber policeman, cell clumps were disrupted by pipetting and then were

transferred to a microcentrifuge tube. Cells were lysed on ice using a dounce homogenizer and centrifuged at 15,000 x g for 3 min. The supernatant, comprising the cytosolic fraction, was removed to a fresh tube. Pellets were washed briefly with 500 µl Buffer A, then resuspended in 150 µl Buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10% glycerol, and 1 Mini Complete Protease Inhibitor Cocktail tablet (Roche) per 10 ml). Samples were sonicated for 20 sec on ice at 30 kHz and incubated with gentle rocking at 4°C for 15 min. Membranes were pelleted by centrifugation at 15,000 x g for 5 min and the nuclear fraction was removed using a Hamilton syringe. Protein concentration was determined by Bradford assay and samples were either immediately subjected to further analysis or flash-frozen in liquid nitrogen and stored at
-80°C.

Immunoprecipitation

Lysate samples for immunoprecipitation were prepared from whole-cell extracts, or in some experiments, cells were first fractionated into nuclear and cytosolic components prior to immunoprecipitation. In all cases, samples were resuspended to a final volume of 1 ml in IP lysis buffer (50mM Tris-HCl (pH 8), 150 ml NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.2 mM Na_3VO_4, 50 mM NaF, and 1 Mini Complete Protease Inhibitor Cocktail tablet (Roche) per 10 ml). Samples were kept cold throughout the protocol. Lysates were precleared by gently rotating for 30 min at 4° C with a 50% slurry of 100 µl lysis buffer-equilibrated Ultralink Protein A/G agarose beads (Pierce) and 0.25 µg of the appropriate control IgG (corresponding to the host species of the primary antibody). Beads were pelleted by centrifugation at 2500 x g at 4° C, and then the supernatant was transferred to a new microcentrifuge tube using a Hamilton syringe to completely dry the bead pellet. This transfer method was used in all subsequent wash steps. The recommended amount of primary antibody was added to the sample, which was gently rotated overnight at 4° C. For each sample, 50 µl lysis buffer-equilibrated bead slurry was added per 10 µg primary antibody. Samples were incubated with gentle mixing for 2 hrs at 4° C. Beads were washed 6 times with 500 µl IP lysis buffer, then 2x sample buffer (5% β-mercaptoethanol, 0.004% bromophenol blue solution, 19% glycerol, 4% sodium dodecyl sulfate, 120 mM Tris-HCl (pH 6.8)) was added and samples were boiled for 5 min before loading on SDS-PAGE gels for further analysis.

Immunoblot analysis

For immunoblot analysis, proteins were resolved with SDS-PAGE using NuPage 10% or 12% precast gels (Invitrogen) and transferred to Immobilon PVDF membranes (Millipore). Blots were incubated for 30 min in PBS + 0.1% Tween-20 (PBS-T) containing a blocking solution of 3-5% nonfat dry milk or 3% bovine serum albumin (BSA). Blots were briefly washed with PBS-T and incubated with primary antibody overnight at 4°C on a rocking platform. Primary antibodies were diluted to empirically determined concentrations in blocking solution. At the end of the incubation time, blots were washed for 3 times, 5 minutes each, with PBS-T then placed in blocking solution containing horseradish peroxidase-conjugated secondary antibody against the appropriate species, diluted 1:10,000 (mouse)-1:20,000 (rabbit or goat). Blots were then treated to a final series of 3x 5 min washes in PBS-T, and proteins were visualized using Western Lighting ECL reagent (Perkin-Elmer) and BioMax Chemiluminescence film (Kodak). When necessary, blots were stripped

of bound antibody by incubation with stripping buffer (100 mM β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.7, 2% SDS) at 55°C for 30 min. Stripped blots were tested for complete removal of bound antibody by treatment with ECL reagent and visualization as above, then blocked and re-probed with additional antibodies as above.

Immunofluorescence Microscopy and Immunohistochemistry

For cells transfected with plasmids expressing fluorescent tags, immunofluorescence microscopy was performed and cells were visualized on a confocal microscope system (510 LSM Meta, Carl Zeiss MicroImaging, Inc; analysis on Zeiss 510 LSM software) for fixed cells or on an inverted microscope (Axiovert, Carl Zeiss MicroImaging, Inc) for live cells. The procedure for fixing and and mounting cells for confocal microscopy was as follows: cells were initially plated on coverslips in a 6-well plate immediately following transfection. Cells on coverslips were washed with PBS then fixed by incubation at room temperature in PBS-buffered 4% formaldehyde for 15 min. The coverslips were then washed twice with PBS and mounted on slides with ProLong Gold Antifade reagent (Invitrogen) containing 4'-6-Diamidino-2-phenylidole (DAPI) for visualization of nuclei.

To determine endogenous protein expression, cells were prepared for immunohistochemical analysis. Cells were washed with PBS then fixed as described above. Fixed cells were washed 3 times for 10 minutes each in quenching solution (13.2 mM Na_2HPO_4, 3 mM NaH_2PO_4 H_20, 130 mM NaCl, 100 mM glycine). Cells were permeabilized in permeabilization buffer (13.2 mM Na_2HPO_4, 3 mM NaH_2PO_4 130 mM NaCl, 0.05% NaN_3, 10% BSA, 2% Triton X-100, 0.05% Tween-20) for 10 min. To block background staining, cells were incubated in a solution of PBS + 10% goat serum for 1 hr at room temperature. Cells were washed 3 times with PBS then incubated overnight at 4°C with primary antibody diluted to the appropriate concentration in PBS. Unbound antibody was removed by three washes in PBS and cells were subsequently incubated with fluorophore-conjugated secondary antibody at the appropriate dilution for 1 hr in the dark at room temperature. Cells were then washed three times in the dark at room temperature and then mounted on slides with ProLong Gold Antfade reagent (with DAPI) (Invitrogen). Confocal microscopy was used to visualize the cells as described above.

MTT cell proliferation assays

MCF7 cell proliferation rates were determined using an MTT cell proliferation assay kit (ATCC). Cells transfected with either Oct3 knockdown shRNAs or an Oct3 overexpression construct were passaged into a 96-well plate 24 hours post-transfection. Cells were seeded at 1000, 3000, 10,000 or 20,000 cells/well and each condition was plated in quadruplicate. The MTT assay was performed 48 hours after passage (72 hours post-transfection). Briefly, 10 uL MTT reagent was added to each well, cells were returned to the incubator until a purple precipitate was visible (approximately 3 hours), then 100 uL of detergent reagent was added. Cells were then incubated in the dark at room temperature for 2 hours, then the absorbance at 570 nm was recorded by a microplate reader.

Semi-quantitative RT-PCR

RNA was extracted from cells using the Qiagen RNeasy kit as per the manufacturers instructions, and was quantified on a spectrophotometer using the A260/280 method. Single-

strand cDNA was synthesized from 500 ng total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo dT (12-18) primers (Invitrogen). 1 μl cDNA was used with Phusion Hot Start polymerase (Finnzymes) in PCR amplification reactions set up according the manufacturer's protocol. The appropriate number of cycles for each primer pair was empirically determined in a series of experiments to determine the linear range of amplification. Primer pairs and the cycle number and annealing temperature for each pair used to achieve amplification in the linear range were as follows:

Oct3: (Forward 5' ATG CAT GAG TCA GTG AAC AG 3', Reverse 5'CTC CTG GAG GGC CAG GAA TC 3'), 28 cycles, annealing temperature 55°C.

Cyclin D1 (Forward 5'CAC TTC CTC TCC AAA ATG CCA3' , Reverse 5'CCT GGC GCA GGC TTG ACT C3'), 25 cycles, annealing temperature 56°C.

Sox2 (Forward 5'CACCTACAGCATGTGCTACTC3', Reverse 5'CATGCTGTTTCTTACTCTCCTC3'), 29 cycles, annealing temperature 55°C.

GAPDH (Standard primer pair purchased from R&D), 25 cycles, annealing temperature 56°C.

PCR Cycling conditions for all primer pairs were as follows: (1) 98° C for 10 sec; (2) 98°C for 2 sec; (3) (annealing temperature) for 5 sec; (4) 72° C for 20 sec; (5) repeat steps 2-4 for appropriate number of cycles (6) 4°C hold.

RNAi

shRNA plasmids against Oct3 and Sox2 were purchased from Origene Technologies (HuSH 29mer system). siRNAs against Oct3 were purchased from Invitrogen (STEALTH siRNA system). For shRNA knockdown, cells were cultured and transfected as per the transfection protocol described above, using 2 μg shRNA plasmid or 100-300 nM siRNA per 2×10^6 cells. Cells were harvested at 72 h post-transfection and lysates or homogenates were prepared or RNA was extracted for protein assays or RT-PCR as described above.

Table 2.1 Mutagenesis Primers

Mutation	Forward Primer	Reverse Primer	Annealing Temperature (° C)
ΔNTD	5- /5Phos/-CAG GAC ATC AAA GCT CTG CAT AAA G-3	5- /5Phos/CAT CCA TAC TGG ATC CCGA GCT CGG TAC C-3	65
ΔPOU	5- /5Phos/-GGA TAT ACA CAG GCC GAT GTG GGG CTC A-3	5- /5Phos/-AGC GAC TAT GCA CAA CGA GAG GAT TTT GAG-3	65
ΔCTD	5- /5Phos/AAG CTT CAC CAC AAA ACC GCG GCT CTT-3	5- /5Phos/GCT TGA TCG CTT GCC CTT CTG GCG-3	66
Δ1-8	5-/5Phos/GCC ACA CGT AGG TTC TTG AAT CCC GA -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	67
Δ1-16	5- /5Phos/GAA TGG AAA GGG GAG ATT GAT AAC TGG TGT -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	67
Δ1-24	5- /5Phos/TGG TGT GTT TAT GTT CTT ACA AGT CTT -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	67
Δ1-32	5- /5Phos/CTT CTG CCT TTT AAA ATC CAG TCC CAG GAC -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	69
Δ1-34	5- /5Phos/CCT TTT AAA ATC CAG TCC CAG GAC ATC AAA -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	67
Δ1-38	5- /5Phos/CAG TCC CAG GAC ATC AAA GCT CTG CAG AAA -3	5- /5Phos/CAT GGC GGC GGA TCC GAG CTC GAG AT -3	67
GSRVD	5- /5Phos/AGA GTA GAT ACC AGT ATC GAG AAC CGA GTG AGA G -3	5- /5Phos/ACT TCC GGC CTG CAC GAG GGT TTC TGC TTT -3	67
S141A	5- /5Phos/AGA AAG CGA ACC GCA ATC GAG AAC CGA GT -3	5- /5Phos/CTT TCG GGC CTG CAC GAG GGT TTC TG -3	67
S141D	5- /5Phos/AGA AAG CGA ACC GAC ATC GAG AAC CGA GT -3	5- /5Phos/CTT TCG GGC CTG CAC GAG GGT TTC TG -3	67

Table 2-2. Primary Antibodies used in these studies.

Cyclin D1	mouse	Cell signaling Technology
FGF-4 C-18	goat	Santa Cruz Biotechnology
FLAG-M2	mouse	Sigma
Histone H4	rabbit	Cell Signaling Technology
Nanog monoclonal	mouse	eBiosciences
Nanog N-17	goat	Santa Cruz Biotechnology
Oct-3/4 C-20 (and blocking peptide)	goat	Santa Cruz Biotechnology
Oct-3/4 H-65	rabbit	Santa Cruz Biotechnology
phospho-β-catenin (S33/S37/Thr41)	rabbit	Cell Signaling Technology
Sox-2 D-17 (and blocking peptide)	goat	Santa Cruz Biotechnology
Sox-2 H-65	rabbit	Santa Cruz Biotechnology
α-tubulin (TU-02)	mouse	Santa Cruz Biotechnology
β-catenin (6D209)	mouse	Santa Cruz Biotechnology
β-tubulin	mouse	Sigma

Table 2-3. Cell lines used in these studies

MCF10a	Non-tumorigenic mammary epithelial cells	Non-invasive
184a1	Non-tumorigenic mammary epithelial cells	Non-invasive
BT-474	Ductal carcinoma- from duct,	Non-invasive
MDA-MB-231	Mammary adenocarcinoma, metastatic- from pleural effusion	Highly invasive
MDA-MB-468	Mammary adenocarcinoma (from breast)	Low-moderately invasive
MCF7	Mammary adenocarcinoma cells from metastatic site- from pleural effusion	Low-moderately invasive
SK-BR-3	Mammary adenocarcinoma cells from metastatic site- from pleural effusion	Low-moderately invasive
T47D	Ductal carcinoma (from duct)	Low-moderately invasive
Zr75	Ductal carcinoma cells from metastatic site- ascites fluid	Low-moderately invasive
NTera-2 (NT-2)	pluripotent human testicular embryonal carcinoma	N/A
COS-7	African green monkey kidney	N/A

Chapter 3

Experimental Results

Introduction

Although there have been a number of studies focused on the expression of pluripotency factors Nanog, Sox2, and Oct3/4 in embryonic stem cells, tumor cells, and in subpopulations of cancer stem cells that may exist within a tumor cell population, our understanding of how these factors may regulate tumor progression is limited. The idea that critical embryonic pluripotency factors may be re-expressed in cancer cells has been considered and tested by a number of groups; however, the methodologies used have only rarely taken into consideration the existence of pseudogenes of Nanog and Oct4 or the existence of the Oct3 and Oct4 alternatively spliced isoforms. This latter oversight is of particular relevance here, as I sought to clarify the specific isoform expressed in breast cancer cells as well as to further characterize elements of the isoform-specific domain structure that are responsible for localization and ultimately, function. While expression of "Oct3/4" has been shown in a number of cancer cell lines and primary tumors, the details of the expression levels, regulation of expression and localization, and possible downstream effects have not been explored. Although a mechanism of Sox2 regulation of the cell cycle in cancer has been determined in a previous study (Chen et al., 2008), the relationship between Sox2 and Oct3 in cancer cells has not been considered in detail, nor has the potential role of Oct3 in the cell cycle been studied.

By studying the details of expression and localization of embryonic stem cell pluripotency factors in breast cancer, I hoped to increase our understanding of the processes that drive cancer progression. I sought first to determine whether Nanog, Sox2, and/or Oct3/4 are expressed in breast carcinoma cells by utilizing a panel of breast cancer cell lines of varying phenotypes with carefully chosen positive controls. These controls allowed us to determine that Nanog is not expressed, or is not expressed in sufficient levels to be detected, and that Sox2 and Oct3, but not Oct4, are expressed in breast cancer cells. These initial results led us to the hypothesis that Sox2 and Oct3 may play a role in tumor cell maintenance or cancer progression.

Our results suggest that previous reports may have mistakenly identified pseudogenes of Oct4 and Nanog as being expressed as functional protein in breast cancer cells, or may have identified the Oct4 isoform rather than Oct3. Significantly, the results presented here show that Oct3 is expressed exclusively in the cytoplasm of breast cancer cells. This localization appears to preclude any transcriptional activity, at least under the conditions of in vitro culture used here.

In order to better understand the role of embryonic stem cell factors in tumor cell maintenance or cancer progression, I primarily focused on Oct3 since it had not previously been well characterized and nothing was yet known about its function. Oct3 may act independently of Sox2, but it is likely that there is an interaction since the extensive interactions between the conserved POU domain of Oct3/4 and the homeodomain of Sox2 play an important role in embryonic stem cell maintenance. Since Sox2 is known to play a role in the cell cycle of cancer cells, Oct3 may be involved in this same pathway. I used biochemical, pharmacological and genetic methods in an attempt to better understand the function of Oct3 and its possible interactions with Sox2. I primarily chose to use the MCF7 cell line for studies on Sox2 and Oct3 function and Oct3 characterization, since these cells are easy to transfect and also exhibit relatively high levels of endogenous Sox2 and Oct3 expression. I also performed extensive mutagenesis experiments to better characterize the Oct3 protein and determine the roles

of its protein domains in its cellular localization. Our results indicate that the N-terminal domain of Oct3 prevents its nuclear localization. Preliminary results suggest a possible role for Oct3 in regulating proliferation of breast cancer cells, but the variability of the results precluded making definitive conclusions. In sum, these studies further the knowledge of how re-expressed embryonic stem cell factors are involved in maintaining a stem-cell-like phenotype in cancer cells.

Results

Pluripotency factor expression in breast carcinoma cells

Determination of Oct3/4, Sox2 and Nanog expression in breast cancer cells.

Others have reported that Nanog, Sox2 and Oct3/4 are expressed in cancer cells. I demonstrated by immunoblotting whole cell lysates from a panel of breast cancer cell lines and two non-tumorigenc breast epithelial cell lines (MCF10a and 184a1) that Oct3 and Sox2 are expressed in breast carcinoma cells *(Figure 3-1a, b and c),* whereas the Oct4 isoform and Nanog *(Figure 3-1 e)* are not expressed or are expressed at very low levels undetectable by immunoblot analysis. Results for Nanog and Sox2 were confirmed using Quantitative Real-Time PCR by another member of our laboratory (data not shown); however, Oct3/4 data could not be confirmed by Q-RT-PCR due to the difficulty in designing effective isoform-specific probesets.

Previous reports may have mistakenly identified Oct3 as Oct4 in immunoblot experiments, especially since the two proteins are close in size, with Oct3 having a molecular mass of 33 kDa and Oct4 having a molecular mass of 43 kDa. I determined that the observed band in immunoblots of breast carcinoma cells was Oct3 rather than Oct4 with the use of several positive controls, including Ntera-2 (NT-2) embryonic carcinoma cells which express high levels of both Oct4 and Oct3, and Cos7 cells transfected with an Oct4 expression construct, pCMV5a-Oct4-3xFLAG *(Figure 3-1a).* These experiments clearly show that Oct3 is expressed in mammary epithelial carcinoma lines, whereas Oct4 is not. Additionally, the data suggest that protein expression levels may correlate with cancer progression, since the mammary epithelial lines MCF10a and 184a1 exhibited lower expression than in a number of cancer lines *(Figure 3-1a, b).*

Oct3 expression may be regulated at the post-translational level.

I investigated the basis of regulation of Oct3 expression levels by simultaneously isolating total RNA and cytoplasmic fractions from a panel of breast cancer cells and performing semi-quantitative RT-PCR *(Figure 3-2a)* and immunoblots *(Figure 3-2b)* on these samples. This strategy allowed comparison of mRNA levels and protein levels from populations of cells at a particular timepoint. Results suggested that the relative mRNA levels do not directly correlate with the relative protein levels. For example, MCF7 and T47D cells exhibit relatively high levels of Oct3 protein, but relatively low levels of Oct3mRNA, whereas the MDA-MB-468 cells express low levels of Oct3 protein but high levels of Oct3 mRNA. These results may indicate post-translational regulation of Oct3. In an attempt to further quantify protein levels, quantitative immunoblots were attempted using the Odyssey Infrared Imaging System (LI-COR Biosciences); however, these experiments did not yield meaningful results due to high background fluorescence. Real-time q-PCR experiments were likewise inconclusive, despite our attempts at amplifications using several different TaqMan primer/probesets

(Applied Biosystems).

Src may regulate Oct3 and Sox2 expression levels.

Since Src family members are known to regulate expression of Oct3/4 in embryonic stem cells, I endeavored to determine whether Src regulates Oct3 and Sox2 expression levels in breast cancer cells. MCF7 cells were treated with 10uM of the Src-specific inhibitor PP2 or DMSO (vehicle) control. Cells were harvested by subcellular fractionation and analyzed by immunoblot. Results showed a significant reduction of Oct3 and Sox2 expression in cells treated with PP2 as compared to controls *(Figure 3-3).* No changes in localization patterns of Sox2 or Oct3 were observed.

Localization of Oct3 and Sox2 in breast carcinoma cells

Oct3 is expressed exclusively in the cytoplasm of breast carcinoma cells.

To determine the cellular localization of Oct3, I performed subcellular fractionation on a panel of breast cancer cell lines and non-tumorigenic breast cancer cells and analyzed the samples by immunoblotting *(Figure 3-4a,c).* The results showed that Oct3 expression is restricted to the cytoplasm of breast carcinoma cells and confirmed that Oct3 expression is upregulated in breast cancer cells when compared to non-tumorigenic breast cells.

In order to ensure that these results from immunoblot experiments were not an artifact of the subcellular fractionation procedure, MCF10a and MCF7 cells were fixed and stained with fluorescent Oct3/4 antibodies and visualized by confocal microscopy *(Figure 3-4d).* It appears that low levels of Oct3 are present in the cytoplasm of both MCF10a and MCF7 cells, although expression levels could not be compared with this method. To confirm that results were not due to non-specific staining, an isotype control, a background staining control without primary antibody, and a control of cells blocked with a specific peptide against the antibody were also fixed, stained and visualized. NT-2 cells were also used as a positive antibody control in this experiment, but since there are no currently available commercial antibodies that discriminate between Oct3 and Oct4 epitopes, the staining in the control NT2 cells may primarily represent Oct4 expression.

The observed pattern of Oct3 localization and lack of Oct4 expression in breast cancer cells is consistent with several published reports on Oct3/4 expression in different cell types. Lee et al. reported in 2006 that the expression pattern for the Oct3 and Oct4 isoforms in human embryonic stem cells is that Oct3 is localized to the cytoplasm whereas Oct4 expression is nuclear. Another recent report demonstrated that Oct3 is expressed exclusively in the cytoplasm of prostate cancer cells, whereas Oct4 is not expressed (Monsef et al., 2009). These reports, taken together with the data presented here, call into question whether Oct3 is a marker of pluripotency. It is also apparent that Oct3 cannot act as a transcription factor due to its cytoplasmic localization, unless it undergoes nuclear translocation under other circumstances.

Sox2 is expressed in both the cytoplasm and nucleus of breast carcinoma cells, and Sox2 localization changes may correlate with tumor progression.

Cellular localization of Sox2 was investigated using the same methods as with Oct3, above (*Figure 3-4b, c*). While it appears that the majority of Sox2 protein is localized to the cytoplasm, several cell lines exhibit nuclear localization of Sox2, including MCF7, Zr75, and 231 cells. The highest level of nuclear Sox2 was observed in 231 cells, which are the most highly invasive of the cell lines tested. This suggests that a change in localization of Sox2 from the cytoplasm to the nucleus may correlate with tumor progression. Correlating with Oct3 expression patterns, low levels of Sox2 were detected in the cytoplasm of 184a1 and MCF7 cells when compared with the mammary carcinoma cell lines, supporting the hypothesis that pluripotency factors are upregulated in breast cancer cells.

Sox2 expression and localization were confirmed in intact MCF10a and MCF7 cells by immunofluorescent staining in an experiment performed as described for Oct3 immunostaining, above *(Figure 3-4e).* Results confirm that Sox2 is expressed in the cytoplasm of MCF10a and MCF7 cells, and show that nuclear expression of Sox2 is present in both cell lines. Although expression of Sox2 was not observed in immunoblots of MCF10a nuclear fractions, this discrepancy may be because the low levels of nuclear Sox2 were not detected by immunoblot. Relative expression levels cannot be determined from immunostaining experiments, so although the Sox2 expression is present in MCF10a nuclei, the levels may be quite low.

Overexpressed Oct3 is both cytoplasmic and nuclear.

Interestingly, overexpressing Oct3 in breast carcinoma cells by transfecting a commercially available pCMV6-Oct3 expression construct led to significant nuclear localization *(Figure 3-5a),* which I never observed in numerous experiments on cells expressing endogenous levels of Oct3. This was true for the breast cancer lines MDA-MB-468, MDA-MB-231, and MCF7 as well as in HEK 293 and Cos7 cells. Oct3 has a predicted nuclear localization, with one identified NLS (Pan et al., 2004), two putative NLS's (as per sequence prediction algorithm, PredictNLS, Rost et al., 2004)) and no predicted or identified Nuclear Export Signal (NES) (NetNES 1.1 software, la Cour et al., 2004). The fact that endogenous Oct3 is cytoplasmic combined with the result that overexpression leads to nuclear expression suggests that there may be a mechanism preventing Oct3 from shuttling to the nucleus that is overwhelmed by high levels of the protein.

Oct3 may localize to chromosomes in dividing cells, suggesting regulation at the level of the nuclear membrane.

Although it has been shown that the N-terminal domain of Oct3 hinders DNA binding, and that Oct3 does not efficiently bind DNA, an inverted fluorescence microscope was utilized to visualize live MCF7 cells transfected with mCherry-N1 Oct3 WT. A dividing cell that showed Oct3 colocalization with DNA *(Figure 3-6)* was observed. This suggests that regulation may be at the level of the nuclear membrane. This result was seen in a single dividing cell and therefore needs further confirmation. Synchronizing cells with colchicine treatment may provide more conclusive results in future experiments.

Oct3 appears to be post-translationally modified.

Immunoblot analysis reveals a mobility shift upon Oct3 overexpression.

The hypothesis that Oct3 may be retained in the cytoplasm by a mechanism that is overwhelmed upon saturation with high levels of the Oct3 protein is strengthened by the observation that in cells that do not express endogenous Oct3 or express low levels of endogenous Oct3, the mobility shift pattern and localization patterns that occur upon forced Oct3 overexpression differs from that of cells with higher levels of endogenous Oct3 expression *(Figure 3-5a)*. This experiment was performed only once, so results presented in *Figure 3-5a* need further confirmation. It is also important to note that endogenous levels of Oct3 cannot be compared in this experiment, since a percentage of the total homogenate was loaded for each lane on the immunoblot rather than a specific amount of total protein. For an accurate analysis of endogenous protein levels in the panel of breast cancer cell lines, see *Figure 3-1b*.

In the experiment shown in *Figure 3-5a*, the negative control HEK 293 cells do not express endogenous Oct3. Upon overexpression, the cytoplasmic Oct3 appears on the immunoblot as a single mobility shifted band, and there is an approximately equal amount of Oct3 localized to the nucleus. In MDA-MB 231 cells, which have low levels of endogenous Oct3, the overexpressed Oct3 appears to be predominantly cytoplasmic and is mobility shifted in the blot. Assuming that the mobility-retarded species is modified, this result indicates that all of the cytoplasmic Oct3 is modified. Alternatively, although less likely, nuclear Oct3 could be post-translationally modified, resulting in a change in charge or another change that would cause a downward shift. MDA-MB 231 cells also exhibit a small amount of nuclear Oct3 upon overexpression

The above results from transfections of cells that have no or low levels of Oct3 expression are in contrast with those of cells that express intermediate or relatively high levels of endogenous Oct3. In MDA-MB 468 cells, which express intermediate levels of endogenous Oct3, forced overexpression results in a doublet on the immunoblot in the cytoplasmic fraction, indicating the presence of both modified Oct3 and unmodified Oct3, with a larger amount localized to the nucleus. It is possible that in cells already expressing a moderate amount of Oct3, a modification mechanism such as phosphorylation becomes overwhelmed upon overexpression of large amounts of the Oct3 protein. This putative modification may be the critical mechanism that retains Oct3 in the cytoplasm, perhaps by blocking the Oct3 NLS or by facilitating interactions with Oct3 binding partners. This theory is further strengthened by the results obtained when MCF7 cells expressing relatively high amounts of Oct3 are transfected with the overexpression construct. In these cells, the mobility-shifted band disappears when Oct3 is exogenously overexpressed, which suggests that when high levels of Oct3 are present the modification mechanism fails, and concurrently increased expression in the nucleus is observed.

In order to determine whether exogenously overexpressed Oct3 is localized entirely to the nucleus or whether it is expressed in both compartments of breast cancer cells, I engineered a FLAG tagged expression construct, pcDNA3.1 TEV-3xFLAG. Transfecting MCF7 cells with this plasmid and analyzing expression levels by an immunoblot probed with anti-FLAG allowed discrimination between exogenous expression and endogenous expression *(Figure 3-5b)*. Results of this experiment show

that exogenously overexpressed Oct3 is expressed in both the cytoplasm and the nucleus of breast cancer cells. The addition of the FLAG tag, however, masks the mobility shift that was observed in *Figure 3-5a*.

Post-translational modification(s) of Oct3 may regulate its localization.

Exogenous overexpression of Oct3 resulted in a mobility shift of cytoplasmic Oct3 on immunoblots when compared to nuclear Oct3. This mobility shift might be indicative of phosphorylation or perhaps another modification. Sequence analysis of Oct3 using NetPhos 2.0 software (Blom et al., 1999) revealed a number of potential phosphoylation sites, and others have reported that the Oct3/4 POU domain has a critical phosphorylation site located at S229 in the murine Oct4 amino acid sequence, which corresponds to S141 in the POU domain of human Oct3. In mouse teratocarcinoma cells, this site is regulated by the Protein Kinase A and EPAC signaling pathways and regulates Oct4 transactivation (Saxe et al., 2009).

Several attempts were made to identify the mobility shift as attributable to phosphorylation by treating cell lysates with phosphatase; however, this treatment resulted in protein degradation despite protocol troubleshooting (data not shown). Therefore, I was unable to determine whether the observed mobility shift is due to phosphorylation or some other modification.

Mutational analysis

Oct3 mutagenesis experiments elucidate the functions of its regulatory sequences

To further characterize Oct3 and to determine the sequences that play a role in its localization, I generated a series of Oct3 mutants via PCR-based site directed mutagenesis. These included domain mutants, an NLS mutant, phosphorylation mutants, and truncation mutants, with results discussed below. All were cloned into the pmCherry-N1 expression plasmid and were separately transiently transfected into MCF7 cells for analysis using confocal microscropy. *Table 3-1* lists the mutants and summarizes their localization patterns when transiently transfected into MCF7 cells.

Wild type Oct3 localization patterns

Transfecting MCF7 cells with pmCherry-N1 Oct3 WT resulted in its predominant localization to the cytoplasm *(Figure 3-7a, upper panel);* however, a rare minority of cells exhibited nuclear expression of overexpressed Oct3. Interestingly, when Oct3 did localize to the nucleus, the expression levels were much higher than when cytoplasmic *(Figure 3-4a, lower panel).*This change in localization could be due to variations in expression levels in individual cells: infrequently, individual transfected cells might exhibit particularly high expression levels of the transfected plasmid, which could then result in nuclear localization due to saturation of a cytoplasmic retention mechanism as previously discussed for *Figure 3-5*.

In an attempt to determine whether changes in localization are due to the amount of Oct3 expression plasmid that is transfected I performed a titration experiment, transfecting varying amounts of pmCherry-N1 Oct3 WT into MCF7 cells ranging from 50 ng-2μg DNA per million cells. There was no change in the localization pattern of Oct3 regardless of the amount of DNA transfected (data not shown). This result indicates

that differences in the expression levels of pmCherry Oct3 between individual cells are significant regardless of the amount of DNA transfected.

To determine if Oct3 WT localization changes over time, I also perfomed a timecourse experiment in which pmCherry-Oct3 WT was transfected into MCF7 cells and localization patterns were monitored over time by fixing cells at specific post-transfection timepoints (24h, 48h, 72h, 96h, and 118h) and observing expression by confocal microscopy. By 118h, expression levels were very low. There were no changes in expression patterns at any of the experimental timepoints (data not shown).

Deletion of the Oct3 N-terminal domain allows nuclear localization of Oct3.

Deletion of the NTD resulted in significant nuclear localization of pmCherry-N1 Oct3ΔNTD *(Figure 3-7b),* suggesting that the NTD may inhibit Oct3 nuclear localization *(Figure 3-7b).* One published report that demonstrated that two separate regions of the NTD of the Oct3 isoform inhibit DNA binding (Lee et al, 2006), thus the NTD may inhibit both nuclear localization and DNA binding.

The POU domain of Oct3, which includes DNA binding regions and two predicted NLS's, is required for nuclear localization.

Transfecting MCF7 cells with the pmCherry-N1 Oct3ΔPOU resulted in distinct cytoplasmic localization of the POU deletion mutant without any of the rare, strong nuclear expression that was observed for Oct3 WT, indicating that the POU domain is required for nuclear localization *(Figure 3-7c).* The POU domain may therefore play a role in the endogenous cytoplasmic localization of Oct3. The POU domain contains the putative NLS's and the previously identified NLS (RKRKR) found in the Oct3 sequence. It is possible that these sequences may be blocked in endogenous Oct3 by the NTD, by binding partners, or by phosphorylation or other modifications. Blocking of the NLS by any of these mechanisms could lead to the observed cytoplasmic localization of endogenous Oct3.

The CTD of Oct3 is required for nuclear localization of Oct3

MCF7 cells transfected with pmCherry-N1 Oct3ΔCTD exhibited Oct3ΔCTD expression only in the cytoplasm, identical to the localization pattern of Oct3ΔPOU *(Figure 3-7d).* This result indicates that the Oct3 CTD is required for its nuclear localization.

Truncation mutants reveal a critical localization sequence at the 3' end of the Oct3 NTD.

MCF7 cells were transiently transfected with a series of sequential Oct3 truncation mutants in the pmCherry-N1 expression vector *(Figure 3-8).* The mutations included: Oct3Δ1-8, Oct3Δ1-16, Oct3Δ1-24, Oct3Δ1-32, Oct3Δ1-34, and Oct3Δ1-38. Of these, only Oct3Δ1-38 produced a change in phenotype from the predominantly cytoplasmic Oct3 WT. While the other truncation mutants remained predominantly cytoplasmic upon expression in MCF7 cells, the Oct3Δ1-38 mutant localized almost entirely to the nucleus *(Figure 3-8e).* This pinpoints the critical localization residues as being within the amino acid sequence between position 35 and the end of the NTD at position 40.

Cellular Localization of the Oct3ΔNTD-NLS mutant

Previous studies have shown that there is an NLS in the POU domain of Oct4 which is critical for its transcriptional activity, and that its ablation blocks nuclear localization (Pan et al., 2004). To investigate whether this NLS might also be important in the mechanism of cytoplasmic retention of Oct3, MCF7 cells were transfected with pmCherry-N1 Oct3 WT *(Figure 3-9a)* and pmCherry-N1 Oct3ΔNTD *(Figure3-9b)* as controls for the transfection of an Oct3 double mutant, pmCherry-N1 Oct3ΔNTD-GSRVD *(Figure 3-9d).* As in previous experiments, Oct3 WT localized predominantly to the cytoplasm with occasional strong nuclear expression, whereas Oct3ΔNTD was primarily expressed in the nucleus. Oct3ΔNTD-GSRVD, which lacks the Oct3 NTD and whose POU domain nuclear localization signal (RKRKR) has been mutated to GSRVD (as per Pan et al., 2004), restores cytoplasmic localization of Oct3ΔNTD. Therefore, a functional NLS is required for Oct3 nuclear localization, and I can conclude that the presence of the Oct3 NTD somehow blocks the Oct3 NLS. This blocking could be due to a protein folding conformation in which the NTD physically blocks the NLS, or due to interactions of the NTD with other cytoplasmic proteins that block the NLS, or due to phosphorylation or other modifications of the NTD that block the NLS in the POU domain.

Cellular Localization of Oct3 phosphorylation mutants

To determine whether phosphorylation of serine 141 in the Oct3 POU domain plays a role in the retention of Oct3 in the cytoplasm, I generated point mutations in the Oct3ΔNTD mutants that mimic (S141D) or prevent (S141A) phosphorylation (as per Saxe et al., 2009). These double mutants were cloned into the pmCherry-N1 expression vector and transfected into MCF7 cells. Controls included pmCherry-N1 Oct3 WT and pmCherry-N1 Oct3ΔNTD *(shown in Figure 3-9a&b).* Both Oct3ΔNTD-S141A *(Figure 3-10a)* and Oct3ΔNTD-S141D *(Figure 3-10b)* localized to the cytoplasm, "rescuing" the ΔNTD nuclear localization phenotype. These results indicate that the presence of the serine residue at position 141 in the Oct3 sequence is critical for Oct3 nuclear localization, but that phosphorylation of this residue may not be a regulatory mechanism.

Investigations into the potential role of Oct3 in cell cycle regulation

Oct3 regulation of cellular proliferation

MTT assays were utilized to investigate whether cellular proliferation might be regulated by Oct3. Since a previous study determined that Sox2 regulates proliferation of breast cancer cells by regulation of β-catenin and cyclin D1 (Chen et al., 2008), it is possible that Oct3 may also be involved in this pathway. Oct3 is able to heterodimerize with Sox2 and might thus play a cytoplasmic role in regulating downstream pathways via Sox2, which shuttles between the cytoplasm and nucleus. In Figure *3-11a*, I performed three separate experiments in which Oct3 was knocked down by shRNA plasmids by transfecting MCF7 cells with the RNAi constructs. In each of the experiments, effective knockdown was achieved by shRNA#3 and/or shRNA#4. Controls included pMax-gfp alone (transfection control) and a scrambled RNAi construct. While the effective shRNA's against Oct3 appeared to cause a significant reduction in proliferation, the scrambled construct also negatively affected proliferation *(Figure 3-11a).* Therefore, it is

difficult to ascertain whether the reduction in proliferation is a specific effect of Oct3 knockdown or is due to non-specific effects.

When Oct3 was overexpressed by transient transfection of pCMV6-Oct3, the effect on proliferation of MCF7 cells was clear. An MTT assay showed that overexpression of Oct3 resulted in significantly increased proliferation of MCF7 cells when compared with mock-transfected controls *(Figure 3-11b).* This result indicates that Oct3 may be involved in regulation of the cell cycle, and that the increased expression levels of Oct3 in breast cancer cells may play a role in driving tumor expansion. One caveat to this experiment is our observation that overexpressed Oct3 can localize the nucleus in a rare minority of cells, which could potentially have an effect on cell proliferation that would be an artifact of overexpression rather than a refection of physiological conditions. However, the frequency of cells within the total population that exhibit this nuclear expression of Oct3 upon overexpression is low, and these cells are therefore unlikely to have an effect on the proliferation rate of the entire cell population.

Oct3 regulation of β-catenin

Following the model of Sox2 regulation of cell cycle in breast cancer cells (Chen et al., 2008), I pursued further investigations into Oct3 regulation of β-catenin expression levels and localization. I also sought to determine if Oct3 interacts with β-catenin.

Knocking down Oct3 expression by transfection of MCF7 cells with an effective shRNA against Oct3 (shRNA#4) resulted in complete abrogation of the nuclear pool of β-catenin *(Figure 3-12a, top left)* as well as a slight reduction in the cytoplasmic fraction of β-catenin *(Figure 3-12a, top right).* This result suggests that Oct3 may be involved with maintaining the nuclear pool of β-catenin and may also play a role in regulation of total cellular β-catenin levels. Since Oct3 is localized to the cytoplasm and does not act as a transcription factor, these regulatory roles must be via other proteins, perhaps Sox2, which can shuttle between the nucleus and the cytoplasm and binds directly to β-catenin. In complex with β-catenin, Sox2 binds to the cyclin D1 promoter and thereby regulates the cell cycle.

To determine whether Oct3 binds β-catenin, immunoprecipitation experiments were performed in which Oct3 was immunoprecipitated from cytoplasmic extracts of MCF7 cells followed by immunoblot analyses that were performed using an antibody against total β-catenin (*Figure 3-12b)* or a phospho-specific β-catenin (Ser33/Ser37/Thr41) antibody *(Figure 3-12c).* Small amounts of total β-catenin immunoprecipitate with Oct3, although the majority remains in the supernatant fraction.

In contrast, it appears as though the predominant form of β-catenin that immunoprecipitates with Oct3 is the phosphorylated form, which is almost entirely found in the immunoprecipitation pellet. Interestingly, this phosphorylated form of β-catenin would normally be targeted for degradation. β-catenin, like Sox2, shuttles between the cytoplasm and the nucleus, and in the cytoplasm a fraction of the β-catenin binds and is stabilized by E-cadherin. In the absence of Wnt signaling, unbound cytoplasmic β-catenin is phosphorylated on S33/S37 and T41 by gsk3β or PKC, and is then targeted for ubiqitination and degradation. Non-phosphorylated β-catenin enters the nucleus where it accumulates and binds a number of target promoters (Daugherty and Gottardi, 2007). The presence of stabilized phosphorylated β-catenin in the cytoplasm of breast cancer cells

that is specifically associated with Oct3 could be due to activation of Wnt signaling, which is known to occur in cancer, or because of its stabilization by binding to Oct3 or other factors.

To confirm Sox2 binding to β-catenin as was reported by Chen et al., I performed immunoprecipitation of Sox2 from cytoplasmic and nuclear extracts of MCF7 cells followed by immunoblot analysis using antibodies against phospho-β-catenin and total β-catenin *(Figure 3-13).* Results suggest that Sox2 binds β-catenin in both the cytoplasm and the nucleus of breast cancer cells, with preferential binding to the phosphorylated form. I was unable to confirm efficiency of Sox2 immunoprecipitation in this experiment because the Sox2 antibody produced too much background upon stripping and re-probing the PVDF membrane.

These results are in agreement with published results and are similar to results shown in *Figure 3-12b & c* showing co-immunoprecipitation of Oct3 and β-catenin. While the paper by Chen et al. described Sox2 binding to B-catenin and synergistically activating the cyclin D1 promoter, Figure 3-14 represents some new information, since they did not mention the phosphorylation state of β-catenin in their report. Since phosphorylated β-catenin is typically rapidly degraded in the cytoplasm, it may be protected from degradation when in complex with Sox2 and /or Oct3.

The above observations lend support to the hypothesis that Oct3 may be involved in the cyclinD1 regulatory pathway with Sox2 and β-catenin. However, results from the experiments shown in *Figure 3-12b&c* and *Figure 13* are not conclusive due to lack of available pre-immune serum controls for the commercial antibodies I used, which would clarify whether the co-immunoprecipitation results are due to non-specific protein pulldown.

Oct3 regulation of cyclin D1

Since the study by Chen et al. (2008) determined that Sox2 binds the cyclin D1 promoter in complex with β-catenin, and Oct3/4 is known to bind Sox2, I also investigated whether Oct3 regulates cyclinD1 expression. In the majority of experiments in which Oct3 knockdown was achieved, cyclin D1 expression was also reduced at the RNA level *(Figure 3-14b)* and a single experiment showed that cyclin D1 nuclear protein expression was absent following Oct3 knockdown *(Figure 3-14a).* However, results were variable in other experiments due to difficulties in achieving consistent Oct3 knockdown (data not shown).

Sox2 interaction with Oct3 in breast cancer cells

Numerous reports have confirmed that Oct3/4 interacts extensively with Sox2 in ESCs to regulate an array of downstream genes. To investigate whether the Oct3 isoform interacts with Sox2 in breast carcinoma cells, I performed immunoprecipitation experiments in which I immunoprecipitated Sox2 from cytoplasmic extracts of MCF7 cells and tested for co-immunoprecipitation by immunoblotting with an Oct3 antibody *(Figure 3-15).* Results show that Sox2 does appear to bind Oct3 in the cytoplasm of breast cancer cells, although this experiment is not conclusive due to the lack of availability of pre-immune serum controls.

Oct3 regulation of Sox2

Because Oct3/4 is known to regulate Sox2 expression in ESCs, I sought to determine if Oct3 regulates Sox2 expression in breast cancer cells. Oct3 shRNA knockdown experiments did not give consistent results, as shown in the Figure 3-15 semi-quantitative RT-PCR results. In some experiments, knockdown of Oct3 resulted in concurrent reduction in Sox2 mRNA expression levels *(Figure 3-16, Experiment #1),* whereas in other experiments, Oct3 knockdown was inefficient and/or when effective knockdown was achieved, a reduction in Sox2 mRNA expression was not observed *(Figure 3-16, experiment #2).* If further experiments confirm Oct3 regulation of Sox2 in breast carcinoma cells it would strengthen the hypothesis that Oct3 is involved in cell cycle regulation, since Sox2 regulates cyclin D1 in breast cancer cells.

Chapter 3
Tables

Table 3-1. Oct3 Mutants

Mutation	**Localization** Predominant (*Minor)*
*Oct3ΔNTD	N*(C)*
Oct3ΔPOU	C
Oct3ΔCTD	C
*Oct3ΔNTD GSRVD (NLS mutant)	C *(N)*
*Oct3 ΔNTD S141A	C *(N)*
*Oct3 ΔNTD S141D	C *(N)*
Oct3Δ1-8	C
Oct3Δ1-16	C
Oct3Δ1-24	C *(N)*
Oct3Δ1-32	C *(N)*
Oct3Δ1-34	C *(N)*
*Oct3Δ1-38	N *(C)*

*mutations that resulted in a significant phenotype

Chapter 3
Figure legends

Figure 3-1. Immunoblot analysis of Oct3, Oct4, Sox2 and Nanog expression in mammary epithelial and mammary carcinoma cells

Whole cell lysates (WCL) of various breast cancer and breast epithelial cell lines were prepared. 184a1 and MCF10a are breast epithelial cell lines; all others are breast carcinoma cells. 20 μg or 40 μg of whole cell lysate per sample was loaded onto polyacrylamide gels and SDS-PAGE was performed to resolve the proteins. Proteins were transferred onto PVDF membrane. **a)** Initial expression level immunoblots with 20 μg total protein per lane, probed with Oct3/4 C-20 antibody (1:250 dilution). These blots also demonstrate antibody specificity. In the upper panel, COS-7 African green monkey kidney cells serve as a negative control and COS-7 cells transfected with an Oct4-expressing plasmid construct, pCMV5a-Oct4-3xFLAG, serve as a positive control for Oct4 isoform expression. In the lower panel, NT-2 cells serve as a positive control for both Oct3 and Oct4 expression. **b)** Immunoblot prepared with 40 μg whole cell lysate per lane and probed with anti-Oct-3/4 C-20 (1:250 dilution). **c)** Immunoblot prepared with 40 μg whole cell lysate per lane and probed with anti-Sox2 H-65 (1:250 dilution). Controls included COS-7 whole cell lysate (cos ∅) as a negative control and COS-7 cells transfected with pCMV5a-Sox2-3xFlag (cos-Sox2), as a positive control. **d)** Loading control blot with 40 μg total protein per lane, probed with anti-β-tubulin (1:1000 dilution). Samples are representative of all lysates in **a, b, and c** (prepared from the same harvest). **e)** Immunoblot prepared with varying amounts of nuclear extract from MCF7 cells, COS-7 cells, or NT-2 cells. Controls are nuclear extracts from COS-7 cells with and without transfected pCMV-5a-Nanog, and NT-2 nuclear extracts.

Figure 3-2. Comparison of Oct3 mRNA expression levels with Oct3 protein levels

Mammary cell lines were grown to near confluency, and cells were harvested by trypsinization then divided into two portions. Total RNA was prepared from one portion of the sample for semi-quantitative RT-PCR analysis. Subcellular fractionation and immunoblot analysis of the cytoplasmic fraction were performed on the other portion of cells from the same harvest. **a)** Semi-quantitative RT-PCR analysis of Oct3 mRNA expression levels. 500 ng of total RNA was used to prepare a final volume of 20 μL cDNA, which was subsequently treated with RNAse H. 2μL cDNA was then amplified in a PCR reaction. Cycle number was empirically determined for each primer pair to ensure that the amplification was in the linear range and the signal was not saturated. Top panel, amplification of Oct3. Bottom panel, loading control amplification of GAPDH. **b)** Immunoblot analysis of cytoplasmic Oct3 expression levels, top panel. Blot was stripped and re-probed with anti-α-tubulin (1:1000 dilution) as a loading control, shown in bottom panel.

Figure 3-3. Effect of Src inhibition on Oct3 and Sox2 expression levels

MCF7 cells growing on 10 cm plastic tissue culture dishes in DMEM + 10% FBS +1% PS were treated with 10 μM PP2 or DMSO (vehicle) control for 24 h at 37°C + 5% CO_2. Cells were harvested by subcellular fractionation, and 20 μg of each nuclear (nuc)

and cytoplasmic (cyt) fraction were loaded into separate lanes of a 10% polyacrylamide gel. Proteins were resolved by SDS-PAGE and transferred onto a PVDF membrane for immunoblot analysis. Shown in the left top panel, the blot was probed with anti-Sox2 H-65 (1:250 dilution). In the right top panel, the blot was probed with anti- Oct-3/4 C-20 (1:250 dilution). The bottom panels show the top blots after being stripped and re-probed with anti-α-tubulin (1:1000 dilution) to demonstrate fraction purity as well as serving as a loading control.

Figure 3-4. Localization patterns of Oct3 and Sox2 in breast carcinoma cells

Various mammary epithelial and mammary carcinoma cell lines were grown to near confluency and nuclear and cytoplasmic fractions were prepared by subcellular fractionation. Immunoblot analysis was performed by loading 2% of the total volume of each cytoplasmic fraction and 2% of the total volume of each nuclear fraction onto polyacrylamide gels, performing SDS-PAGE to resolve the proteins, transferring the proteins onto a PVDF membrane, and probing the membrane with appropriate antibodies. **a)** Immunoblot analysis of Oct3 expression in cytoplasmic (c) and nuclear (N) fractions of mammary cell lines, probed with Oct-3/4 C-20 (1:250 dilution). **b)** Immunoblot analysis of Sox2 expression in cytoplasmic fractions (top panel) and nuclear fractions (bottom panel) of mammary cell lines. Primary antibody, Sox2 H-65 (1:250 dilution). **c)** Loading controls and fraction purity controls for samples shown in **a** and **b**. Top panel, probed with anti-histone H4 (1:1000 dilution). Bottom panel, the blot was stripped and re-probed with anti-α-tubulin (1:1000 dilution). **d) and e)** MCF10a, MCF7 and NT-2 (positive control) cells were plated on glass coverslips and grown to near-confluency in DMEM medium + 10% Fetal Bovine Serum (FBS) + 1% Penicillin-Streptomycin (PS), fixed and immunostained with **(d)** anti-Oct 3/4 C-20 (1:100 dilution), or **(e)** anti-Sox2 D17 (1:100 dilution) primary antibody and anti-goat Alexa-fluor 546 conjugated secondary immunofluorescent antibody and mounted with DAPI mounting medium as described in Methods. Cells were visualized via confocal microscopy with **(d)** Oct3 and **(e)** Sox2 seen in the green channel and DAPI seen in the blue channel. Negative controls (shown in right panels) included no primary antibody, cells blocked with **(c)** Oct-3/4 C-20 peptide or **(d)** Sox2 D-17 peptide, and anti-goat igG isotype controls.

Figure 3-5. Overexpression of Oct3 results in changes in localization patterns.

a) HEK 293 cells, MDA-MB-231 cells, MDA-MB-468 cells and MCF7 cells were co-transfected with with pCMV6-Oct3 and pMax-GFP plasmids or pMax-GFP alone for the negative controls. Cells were harvested by the subcellular fractionation procedure 72 h post-transfection. 40 μg of each cytoplasmic fraction and 20 μg of each nuclear fraction were subjected to SDS-PAGE and subsequently transferred onto a PVDF membrane. Immunoblot analysis was performed using anti-Oct-3/4 C-20 (1:250 dilution) as the primary antibody. **b)** pcDNA3.1-Oct3-TEV-3xFLAG was transfected into MCF7 cells. Cells were harvested 72 h post-transfection by subcellular fractionation and 40 μg of each cytoplasmic fraction and 20 μg of each nuclear fraction were analyzed by SDS-PAGE and immunoblot. The blot was first probed with anti-FLAG M2 (1:1000 dilution), then stripped and re-probed with anti-Oct 3/4 C-20 (1:250 dilution).

Figure 3-6. Oct3 may localize to chromosomes in dividing cells.

MCF7 cells were transfected with pmCherry-N1 Oct3 constructs expressing wild type mCherry-tagged Oct3. Live cells were visualized 72 hours post transfection using an inverted microscope. Left panel, red channel; right panel, phase contrast view of the same field.

Figure 3-7. Cellular localization of Oct3 wild type and domain mutants

MCF7 cells were transfected with pmCherry-N1 plasmids expressing mCherry-tagged Oct3 wild type **(a)**, Oct3ΔNTD **(b)**, Oct3ΔPOU **(c)**, or Oct3ΔCTD **(d)**, plated on glass coverslips, and fixed and mounted in DAPI mounting medium 72 hours post-transfection. Cells were visualized by confocal microscopy at 40x or 63x magnification with Oct3 visualized in the red channel and DAPI visualized in the blue channel.

Figure 3-8. Cellular localization of Oct3 truncation mutants

MCF7 cells were transfected with pmCherry-N1 plasmids expressing mCherry-tagged Oct3 with sequential truncations in the NTD. Cells were plated on glass coverslips and were fixed and mounted in DAPI mounting medium 72 h post-transfection. Cells were visualized by confocal microscopy at 40x magnification with Oct3 visualized in the red channel and DAPI visualized in the blue channel. Shown are MCF7 cells expressing **a)** pmCherry-N1 Oct3Δ1-8, **b)** pmCherry-N1 Δ1-16 **c)** pmCherry-N1 Oct3Δ1-24 **d)** pmCherry-N1 Oct3Δ1-32, **e)** pmCherry-N1 Oct3Δ1-34, **f)** pmCherry-N1 Oct3Δ1-38.

Figure 3-9. Cellular localization of Oct3 ΔNTD – GSRVD mutant

MCF7 cells were transfected with pmCherry-N1 Oct3 constructs expressing mCherry-tagged wild type or mutant Oct3. Cells were plated on glass coverslips and were fixed and mounted in DAPI mounting medium 72 h post transfection. Cells were visualized by confocal microscopy at 40x magnification with Oct3 visualized in the red channel and DAPI visualized in the blue channel. Shown are cells expressing **a)** pmCherry-N1 Oct3 WT; **b)** pmCherry-N1 Oct3ΔNTD; **c)** pmCherry-N1 Oct3ΔNTD-GSRVD

Figure 3-10. Cellular localization of Oct3 phosphorylation mutants

MCF7 cells were transfected with pmCherry-N1 Oct3 constructs expressing mCherry-tagged wild type or mutant Oct3. Cells were plated on glass coverslips and were fixed and mounted in DAPI mounting medium 72 hours post transfection. Cells were visualized by confocal microscopy at 40x magnification with Oct3 visualized in the red channel and DAPI in the blue channel. Shown are cells expressing **a)** pmCherry-N1 Oct3ΔNTD-S141A; **b)** pmCherry-N1 Oct3ΔNTD-S141D; **c)** pmCherry-N1 Oct3-S141A; and **d)** pmCherry-N1 Oct3-S141D.

Figure 3-11. Effect of Oct3 on the proliferation rate of breast cancer cells

a) Oct3 knockdown. MCF7 cells were co-transfected with shRNAs against Oct3 and pMax-GFP to visualize transfection efficiency. MTT assays were performed as described in the Methods section, with cells plated in quadruplicate and outliers removed from the analysis. In the three separate experiments shown here, shRNA#3 and shRNA#4 represent the effective knockdown constructs, scr represents the scrambled

control shRNA, and pMax represents the empty vector pMax-GFP control. **b)** Oct3 overexpression. MCF7 cells were co-transfected with pCMV6-Oct3, an Oct3 overexpression construct, and pMax-GFP to visualize transfection efficiency. MTT assays were performed as above. "Mock" is the control sample transfected with pMax-GFP alone.

Figure 3-12. Investigations into Oct3 regulation of β-catenin expression and localization

a) MCF7 cells were transfected with pMax –GFP + an ineffective shRNA construct against Oct3 (shRNA#1), pMax-GFP + an effective shRNA construct against Oct3 (shRNA#4), or pMax-GFP alone. Cells were harvested 72 h post-transfection by the subcellular fractionation method. Cytosolic (40 μg) and nuclear (20 μg) fractions of each sample were analyzed by SDS-PAGE followed by immunobloting. Blots were initially probed with anti-β-catenin (1:500 dilution, top left), then were stripped and re-probed with anti-Oct3/4 C-20 (bottom right). Finally, the blots were stripped once more and probed with anti-histone H4 (1:1000 dilution, bottom left) for the nuclear fractions or anti-α-tubulin (1:1000 dilution, middle right) for the cytoplasmic fractions. **b)** MCF7 cells were grown to near confluency and harvested by the subcellular fractionation method. 1 mg, 3 mg, and 5 mg MCF7 cytoplasmic extracts were prepared. A whole cell lysate of NT2 cells was also prepared as a control. Immunoprecipitation of Oct 3/4 was performed using Oct-3/4 H-65 antibody and protein A/G agarose beads as described in the Methods section. The bead pellet and 2% of the final supernatant volume were boiled in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-phopho-β-catenin (1:1000 dilution), shown in top panel. The blot was then stripped and re-probed with anti-β-catenin (1:500 dilution) (middle panel), and finally stripped and re-probed with Oct-3/4 C-20 (1:250 dilution) (bottom panel). **c)** Serial immunoblots on the same lysates from **(b)** probed with anti phopho-β-catenin Ser33/37/Thr41 (1:1000 dilution) and Oct3/4 C-20 (1:250 dilution) antibodies. Blots were stripped of bound antibody before each subsequent primary antibody incubation.

Figure 3-13. Sox2 interaction with β-catenin

MCF7 cells were grown to near confluency and harvested by the subcellular fractionation method. MCF7 cytoplasmic extracts were prepared. Immunoprecipitation of Oct 3/4 was performed using Oct-3/4 H-65 antibody and protein A/G agarose beads as described in the Methods section. The bead pellet and 2% of the final supernatant volume were boiled in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-phopho-β-catenin (1:1000 dilution), shown in left panel. The blot was then stripped and re-probed with anti-β-catenin (1:500 dilution) (right panel).

Figure 3-14. Investigations into Oct3 regulation of cyclin D1

a) MCF7 cells were transfected with pMax-GFP + an ineffective shRNA construct against Oct3 (shRNA#1), pMax-GFP + an effective shRNA construct against Oct3 (shRNA#4), or pMax-GFP+ a scrambled shRNA control. Cells were harvested 72 h post-transfection by the subcellular fractionation method. Cytosolic (40 μg) and nuclear (20 μg) fractions of each sample were analyzed by SDS-PAGE followed by

immunoblotting with anti-cyclin D1 (1:1000 dilution) (top), anti-histone H4 (1:1000 dilution) (middle) and anti-Oct3/4 (1:250 dilution) (bottom). **b)** Knockdown efficiency of shRNA#4 was confirmed with semi-quantitative RT-PCR using Oct3-specific primers (top) and GAPDH primers as a loading control (bottom). **c)** MCF7 cells were transfected with siRNA's against Oct3 (siRNA 112, siRNA 119, or siRNA 145) or control siRNA's specific for each. Cells were harvested as in (**a**), and semi-quantitative RT-PCR was performed as in **(b).**

Figure 3-15. Oct3 may be a binding partner of Sox2 in breast cancer cells.

MCF7 cells were grown to near confluency and harvested by the subcellular fractionation method. Immunoprecipitation of Oct 3/4 was performed on cytoplasmic extracts using Sox2 D-17 antibody and protein A/G agarose beads as described in the Methods section. The bead pellet and 2% of the final supernatant volume were boiled in SDS sample buffer and analyzed by SDS-PAGE followed by immunoblotting with anti-Oct3/4 C-20 (1:250 dilution), shown in left panel. The blot was then stripped and re-probed with anti-Sox-2 H-65 (1:250 dilution) (right panel).

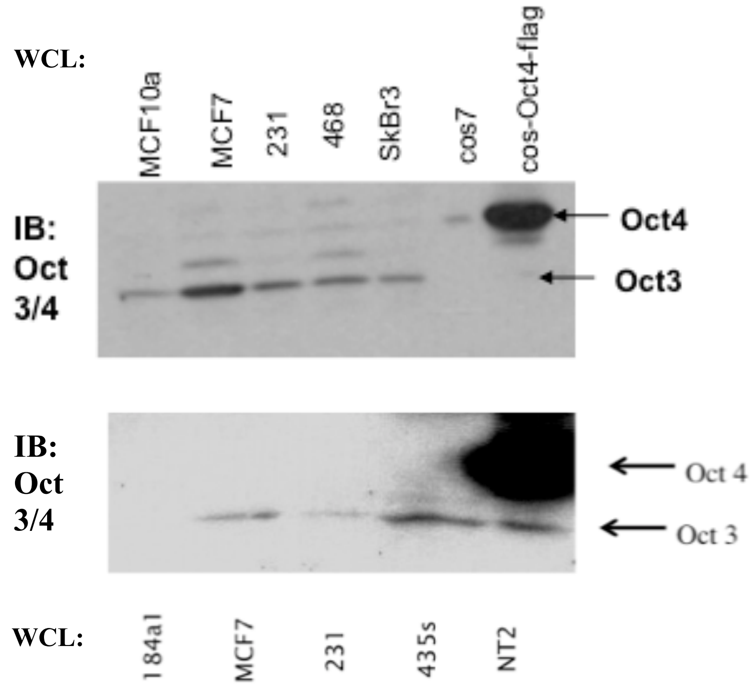
Figure 3-16. Investigations into Oct3 regulation of Sox2

a) Results are representative of two separate experiments that were performed using identical conditions. For both experiments, MCF7 cells were transfected with pMax-GFP + an effective shRNA construct against Oct3 (shRNA#3, shRNA#4) or pMax-GFP + a scrambled Oct3 shRNA construct. Cells were harvested 72h post transfection by trysinization and total RNA was isolated. cDNA was prepared by reverse transcription and semi-quantitative RT-PCR was performed using an Oct3 specific primer pair (top panels) or a Sox2 specific primer pair.

Chapter 3
Figures

Figure 3-1

a)



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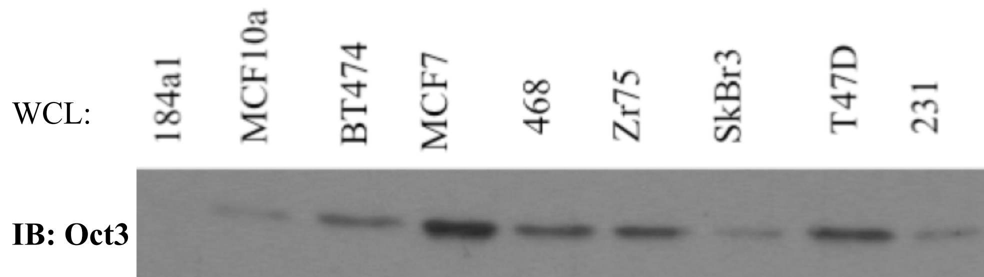

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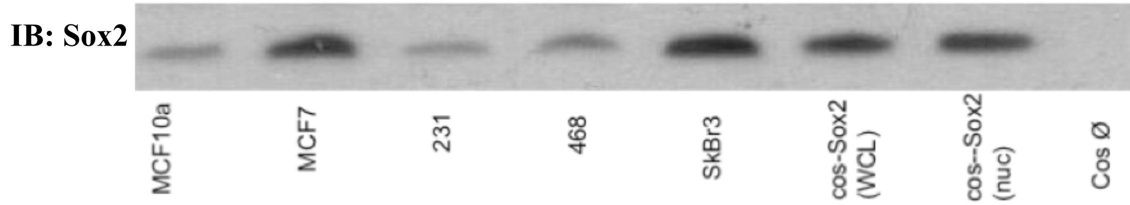

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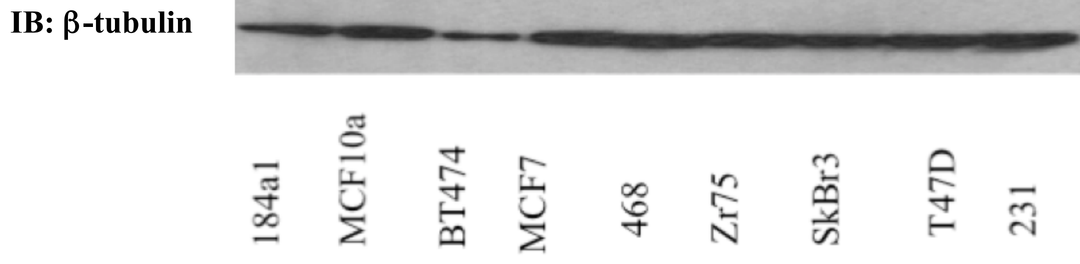
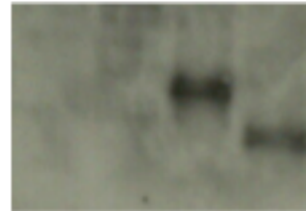


e)

IB:Nanog

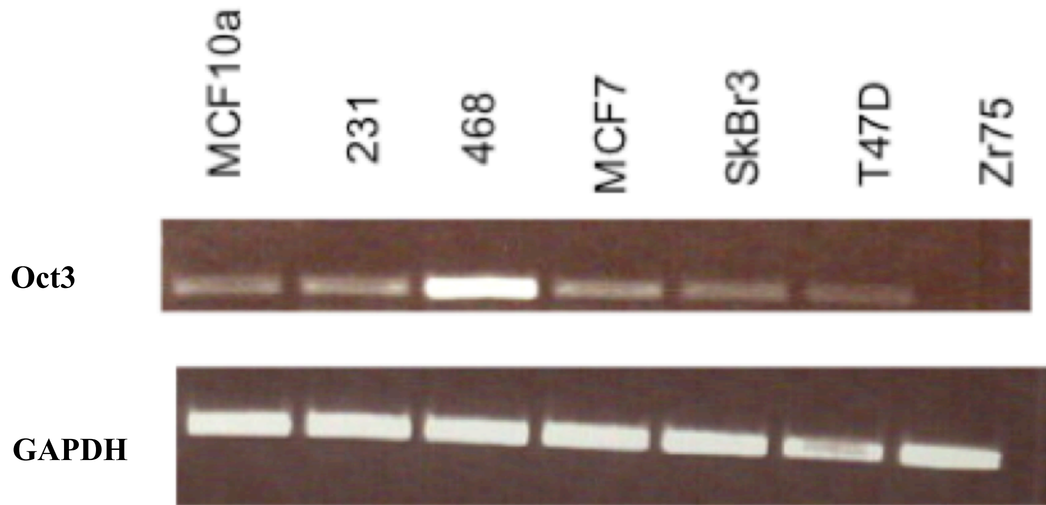


Nuclear Extracts:

200 μg MCF7
100 μg cosø
100 μg cos pCMV-Nanog
20 μg NT2

Figure 3-2

a)



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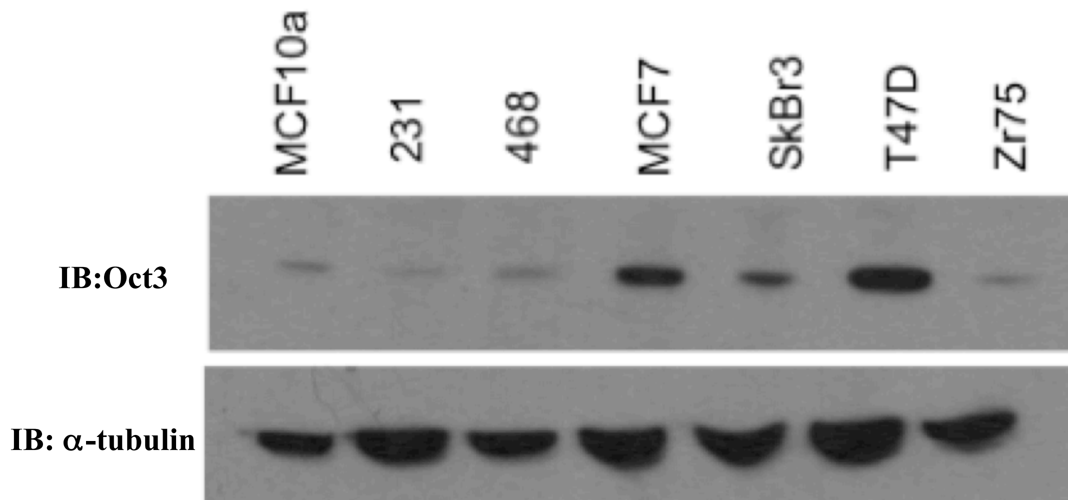


Figure 3-3

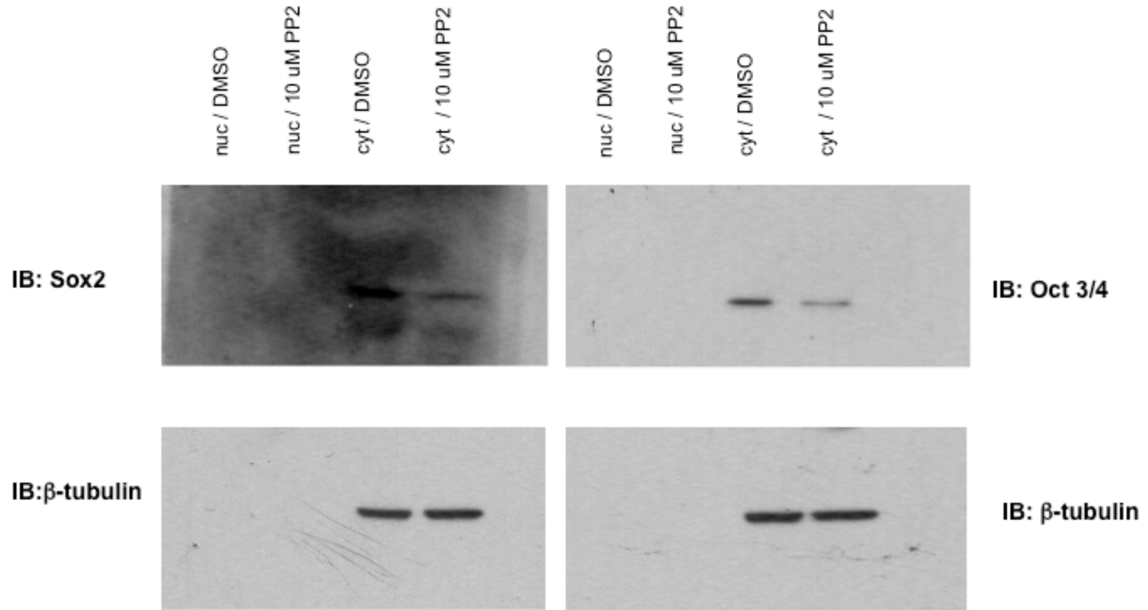
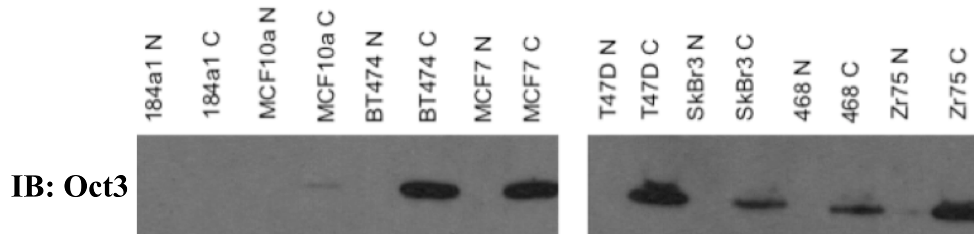


Figure 3-4

a)



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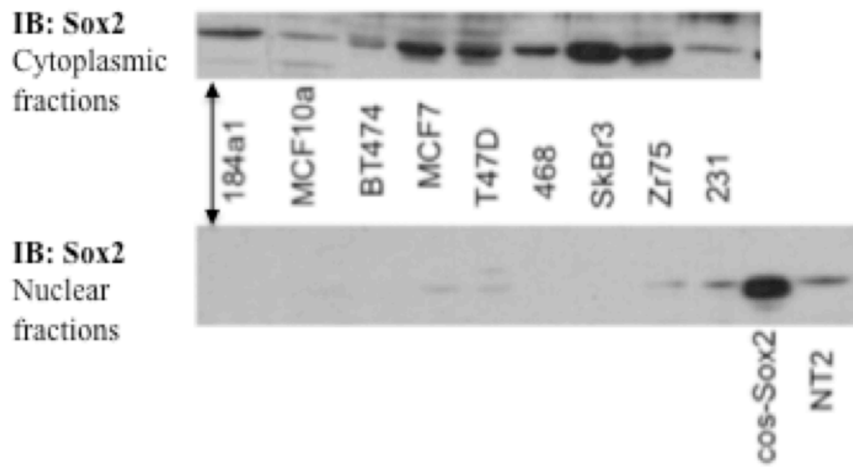

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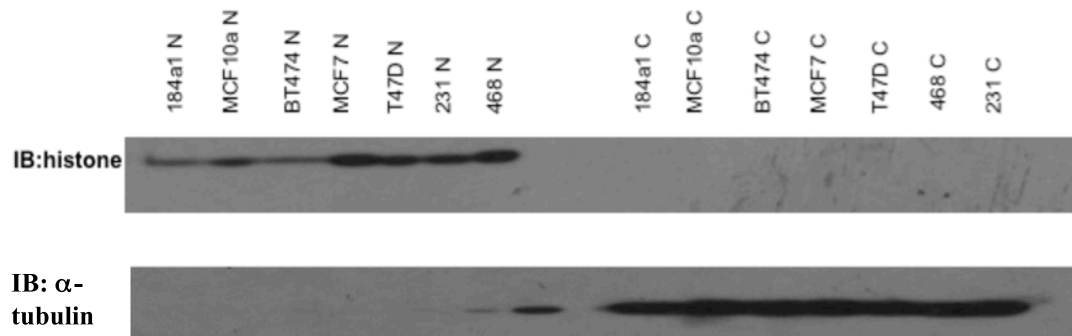

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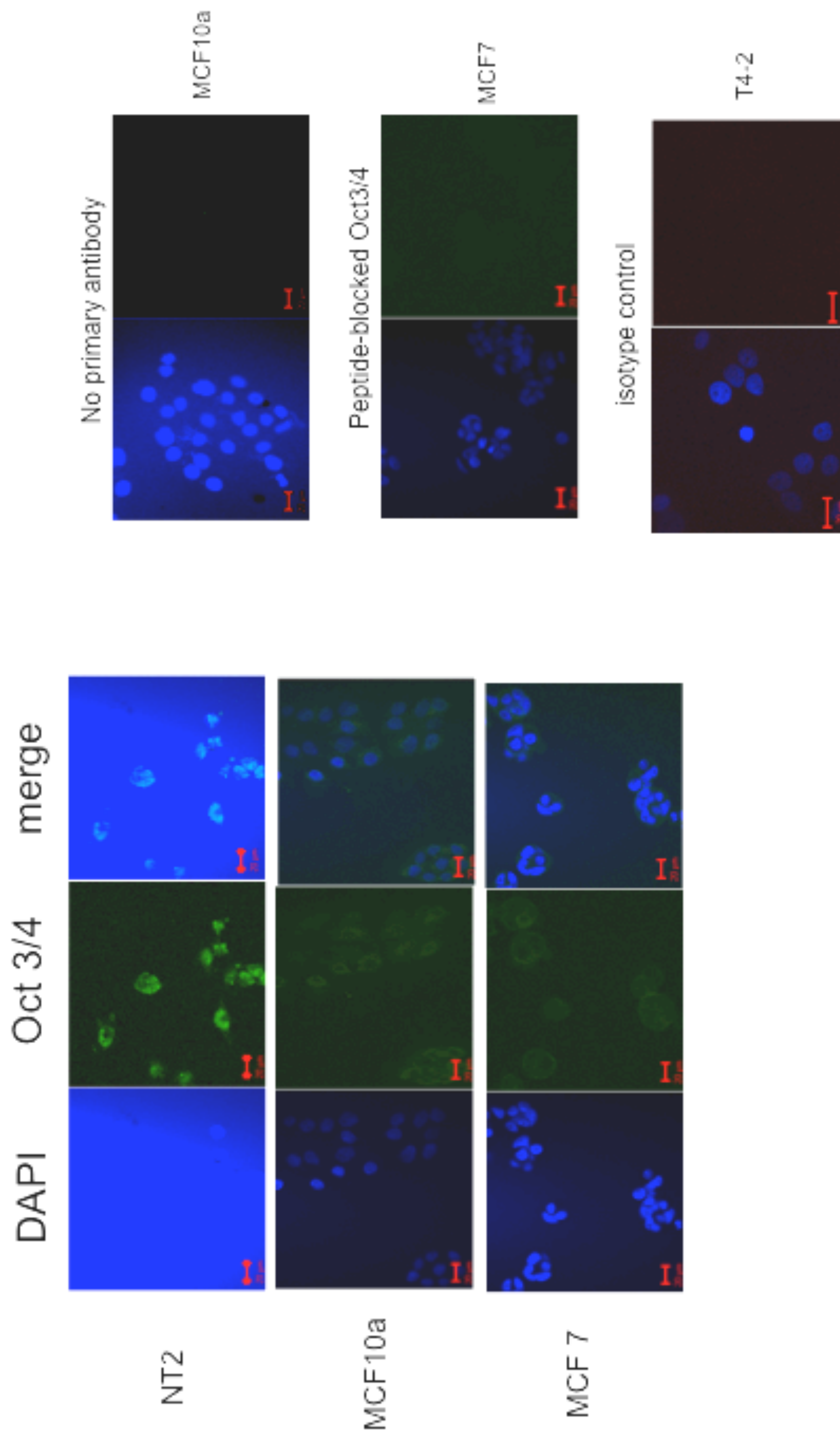

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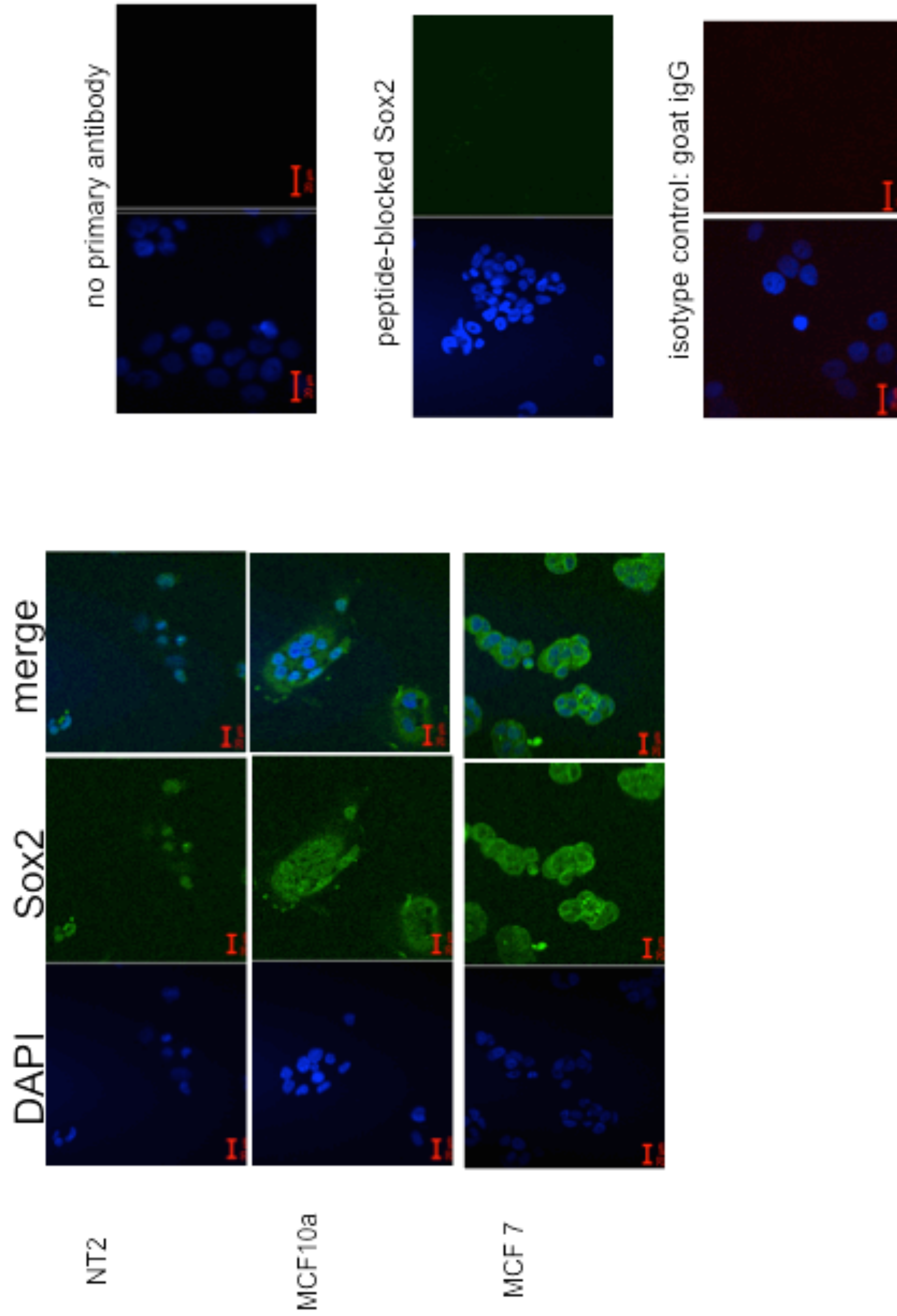
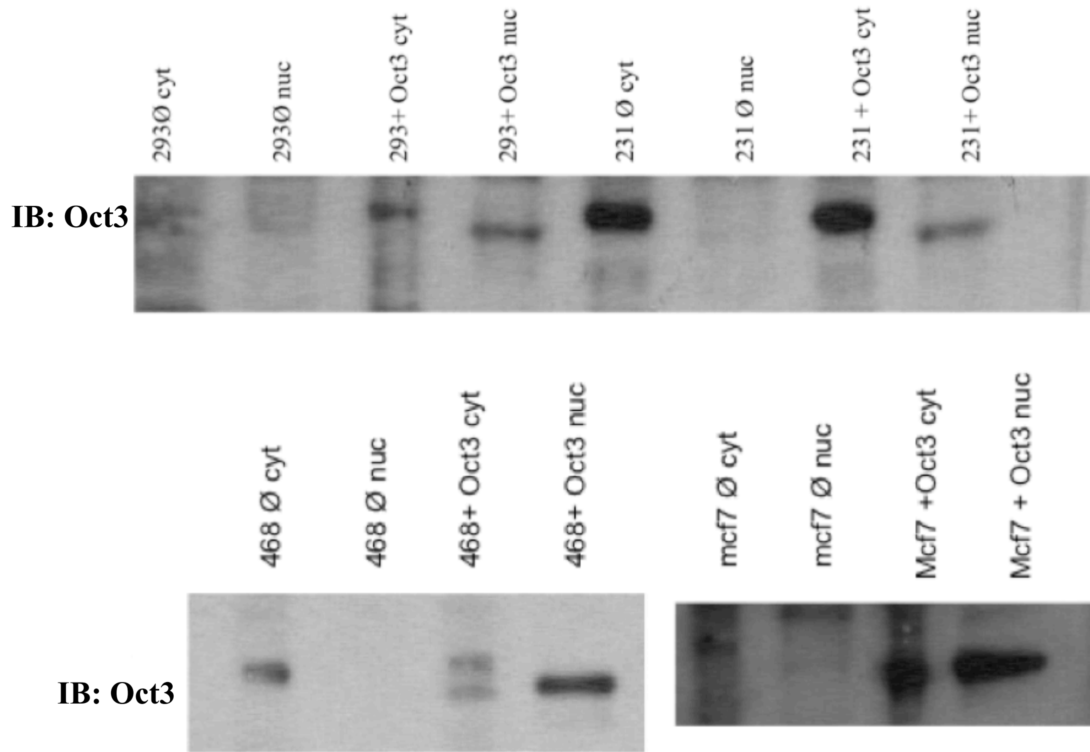
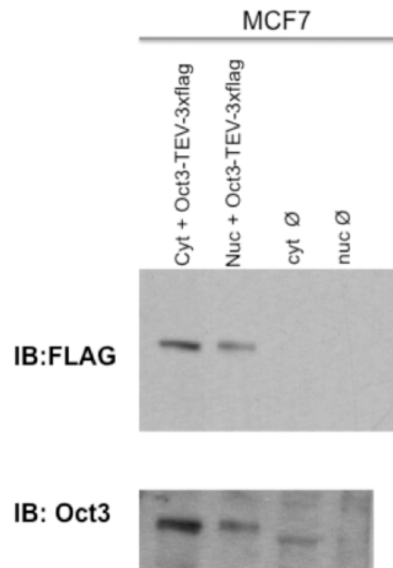


Figure 3-5





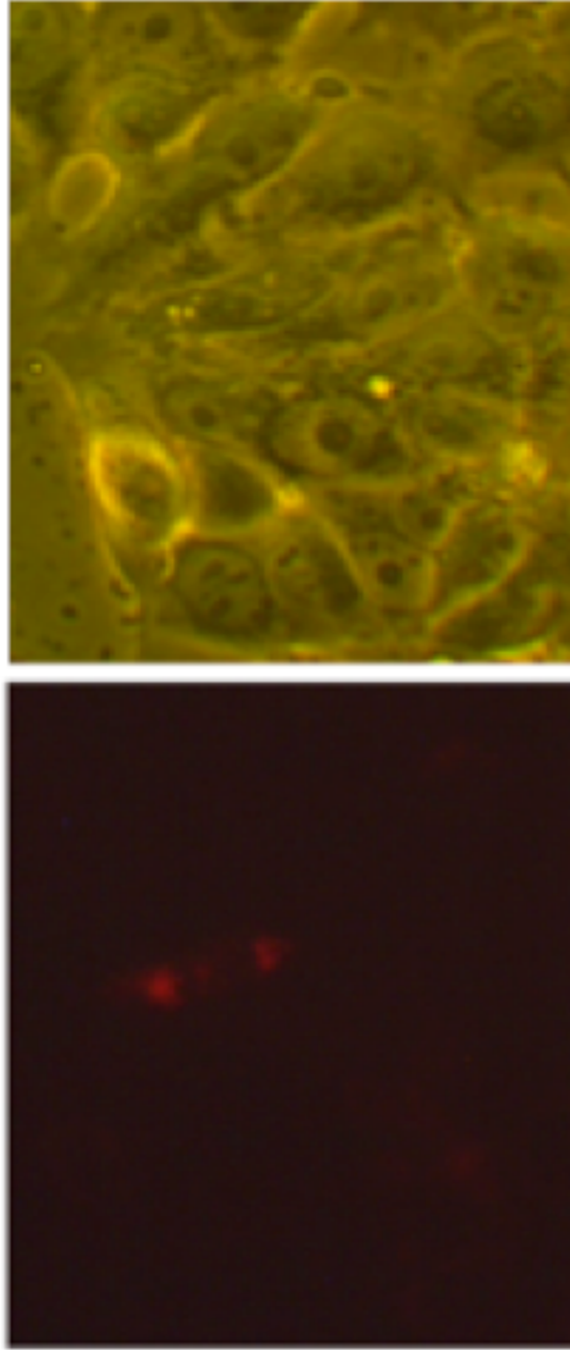
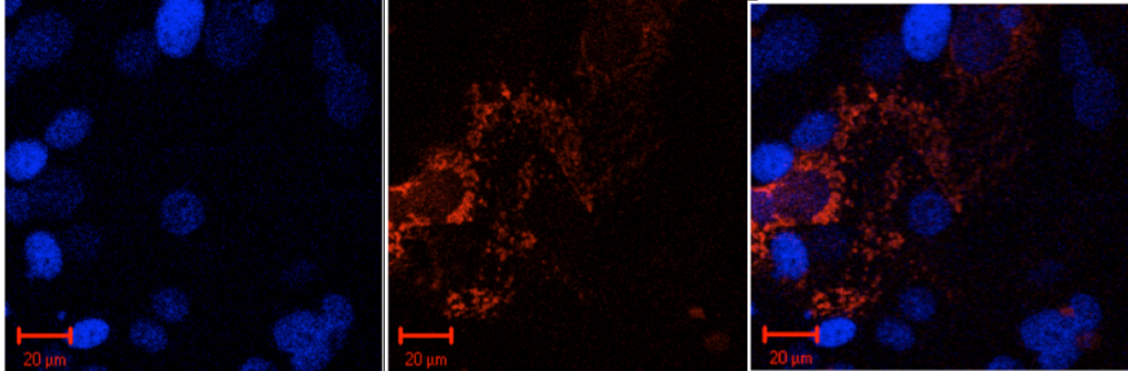
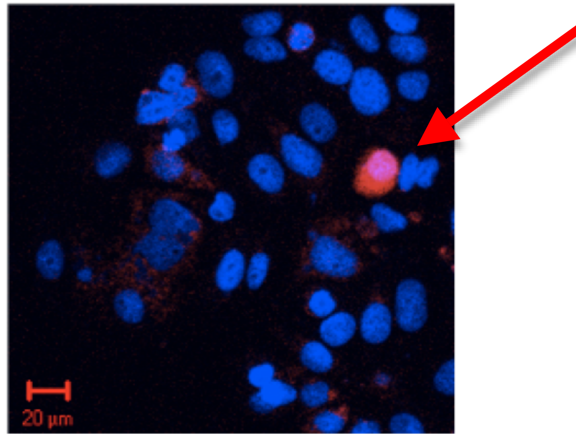


Figure 3-6
pmCherry-N1 Oct3 WT
MCF7 cells

Figure 3-7

a) pmCherry-N1 Oct3 WT





b) pmCherry-N1 Oct3ΔNTD

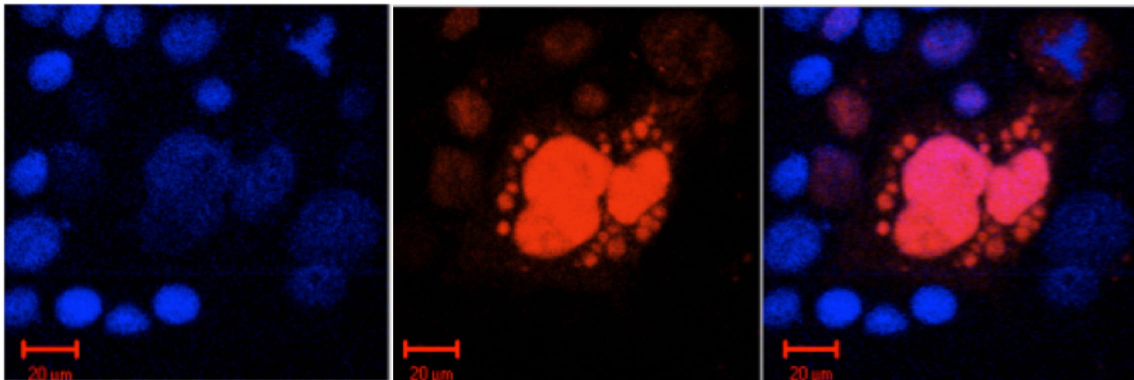
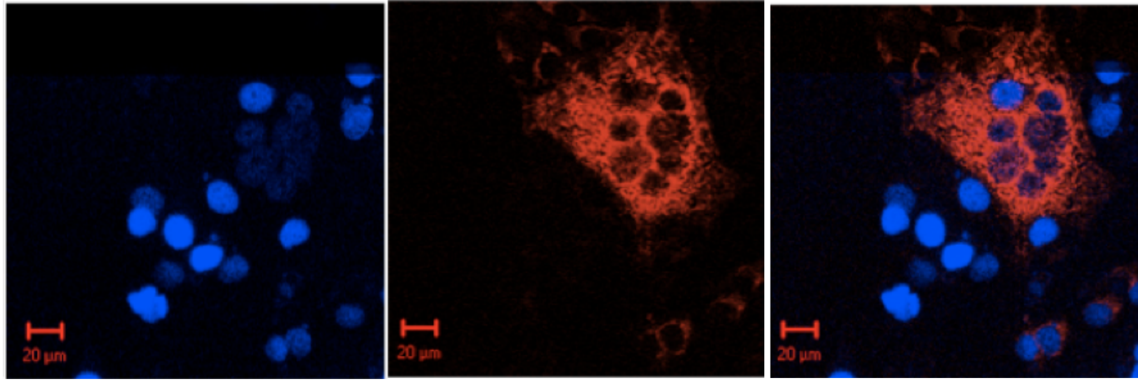


Figure 3-7 (cont.)

c)

pmCherry-N1 Oct3ΔPOU



d)

pmCherry-N1 Oct3ΔCTD

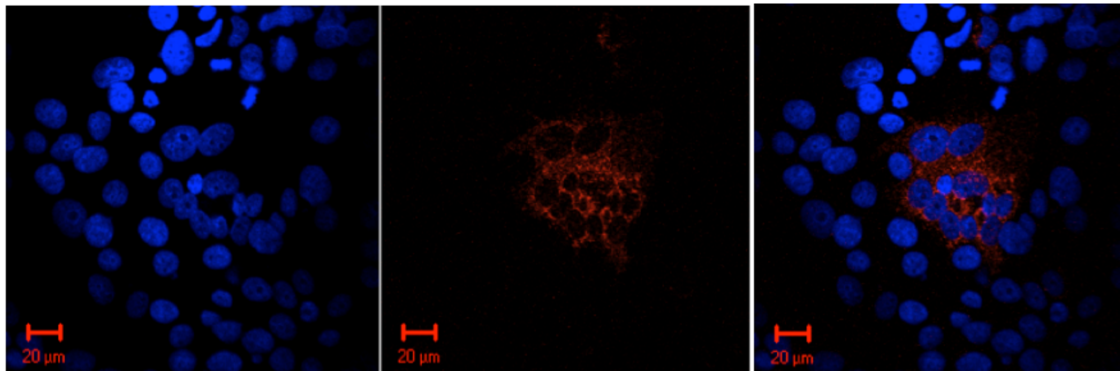
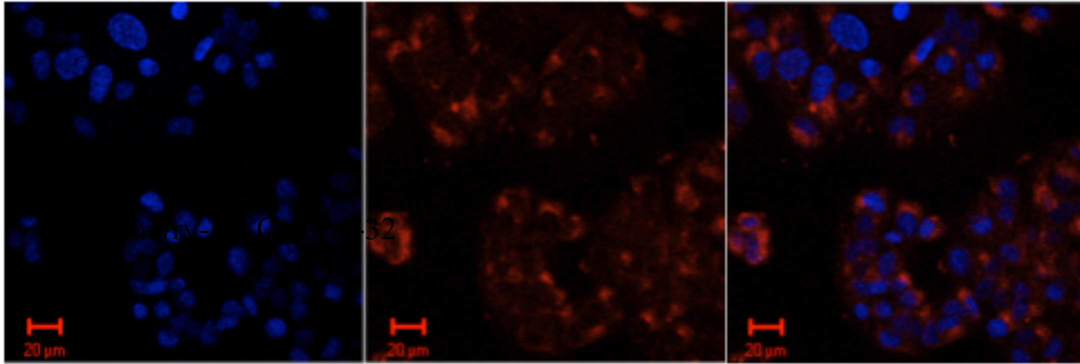
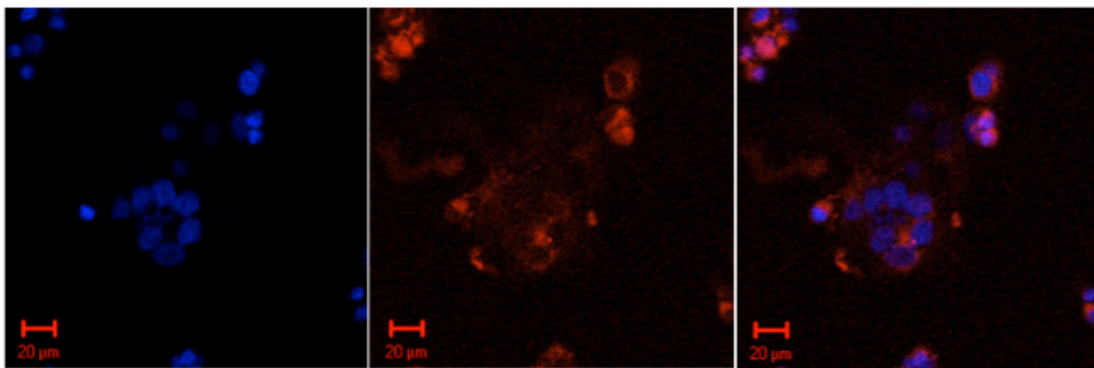


Figure 3-8

a) pmCherry-N1 Oct3Δ1-8



b) pmCherry-N1 Oct3Δ1-16



c) pmCherry-N1 Oct3Δ1-24

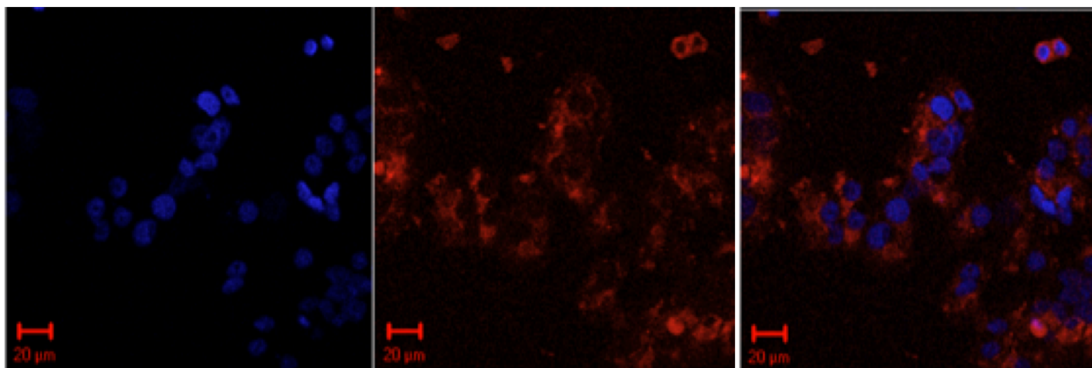
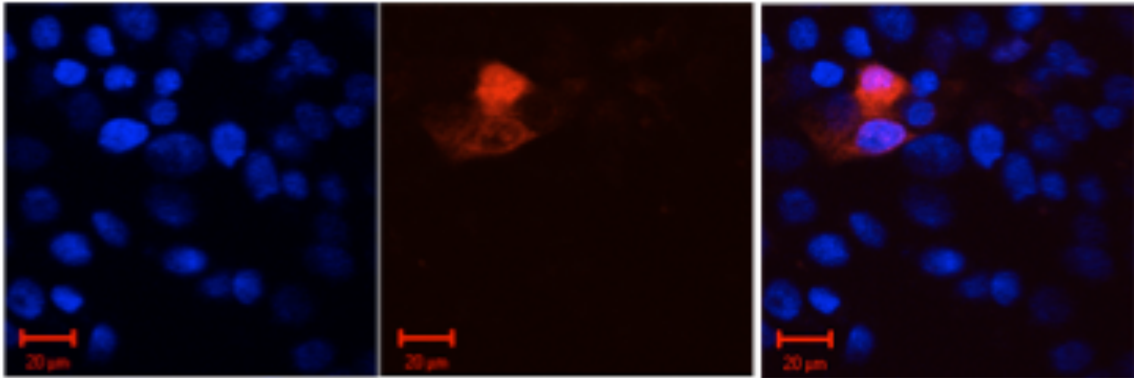


Figure 3-8 (cont.)

d) pmCherry-N1 Oct3Δ1-34



e) pmCherry-N1 Oct3Δ1-38

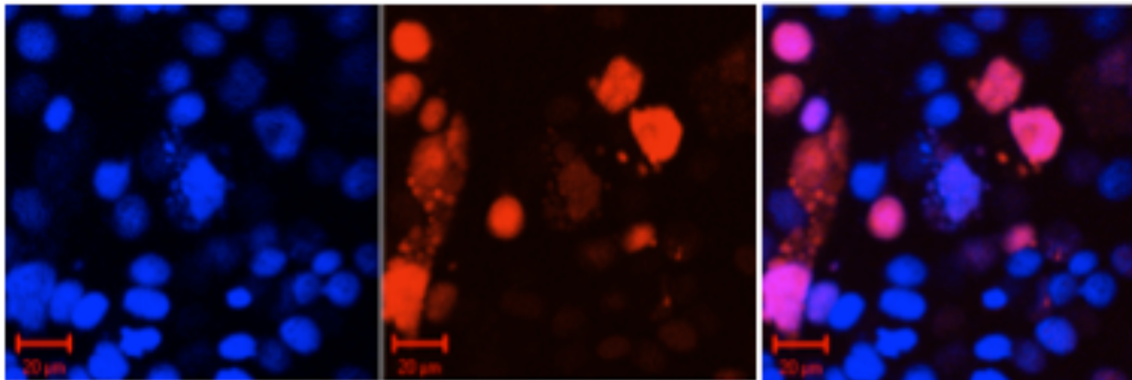
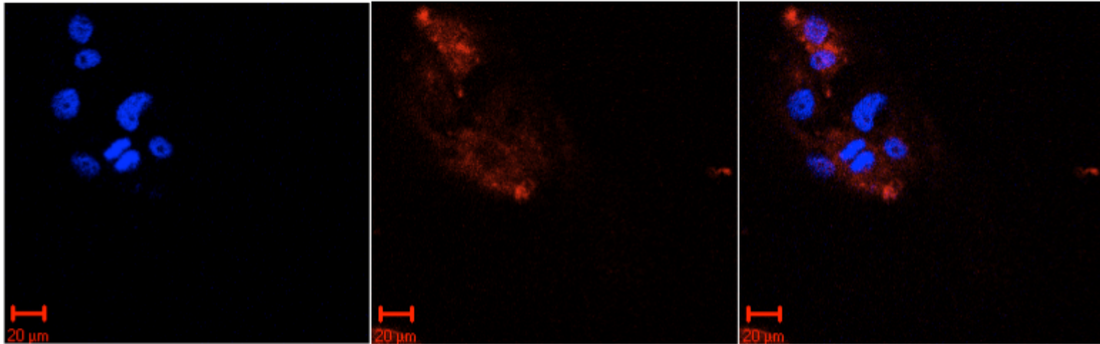
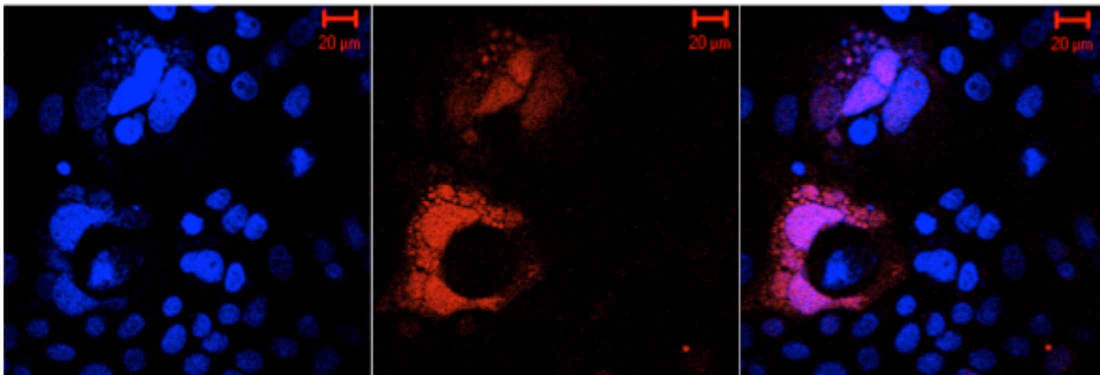


Figure 3-9

a) pmCherry-N1 Oct3 WT



b) pmCherry-N1 Oct3 ΔNTD



c) pmCherry-N1 Oct3 ΔNTD GSRVD

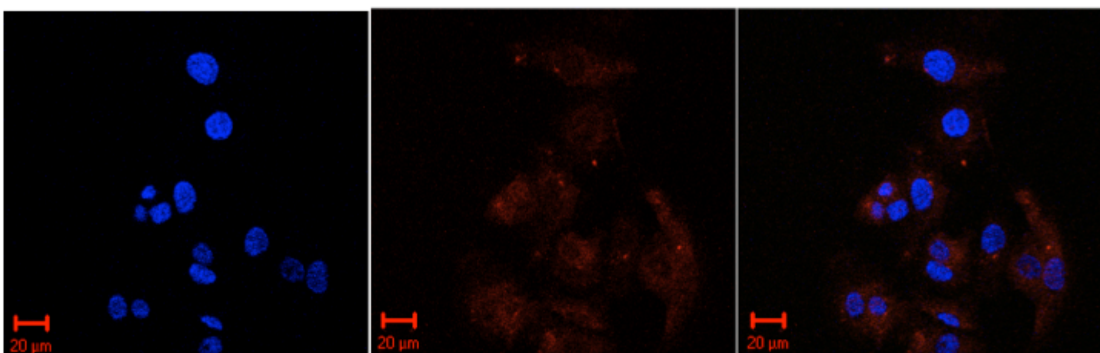
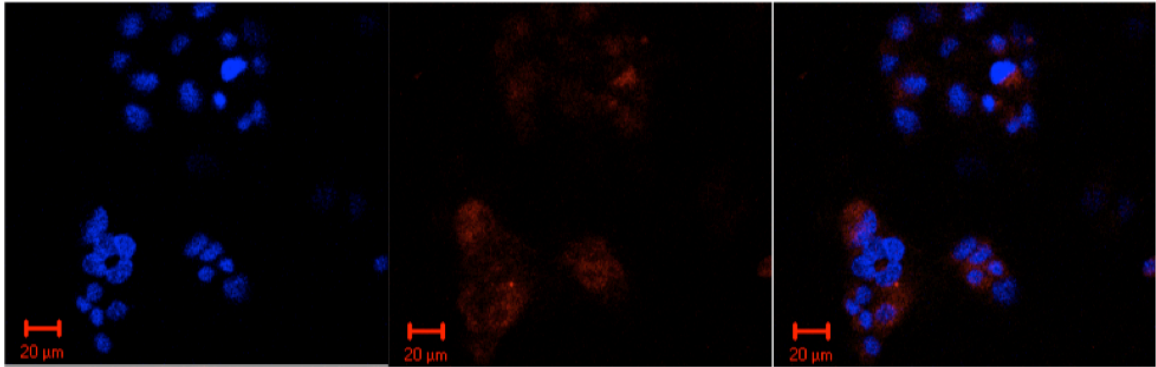


Figure 3-10

a)

pmCherry-N1 Oct3 ΔNTD S141A



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pmCherry-N1 Oct3 ΔNTD S141D

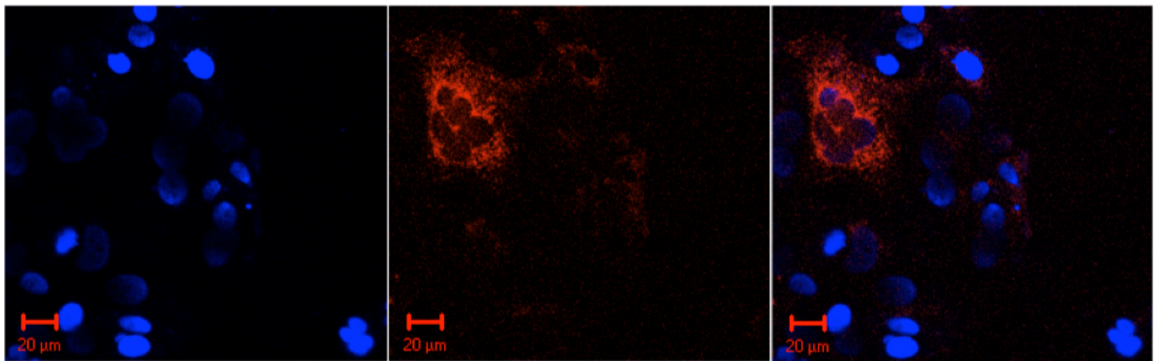

Figure 3-11

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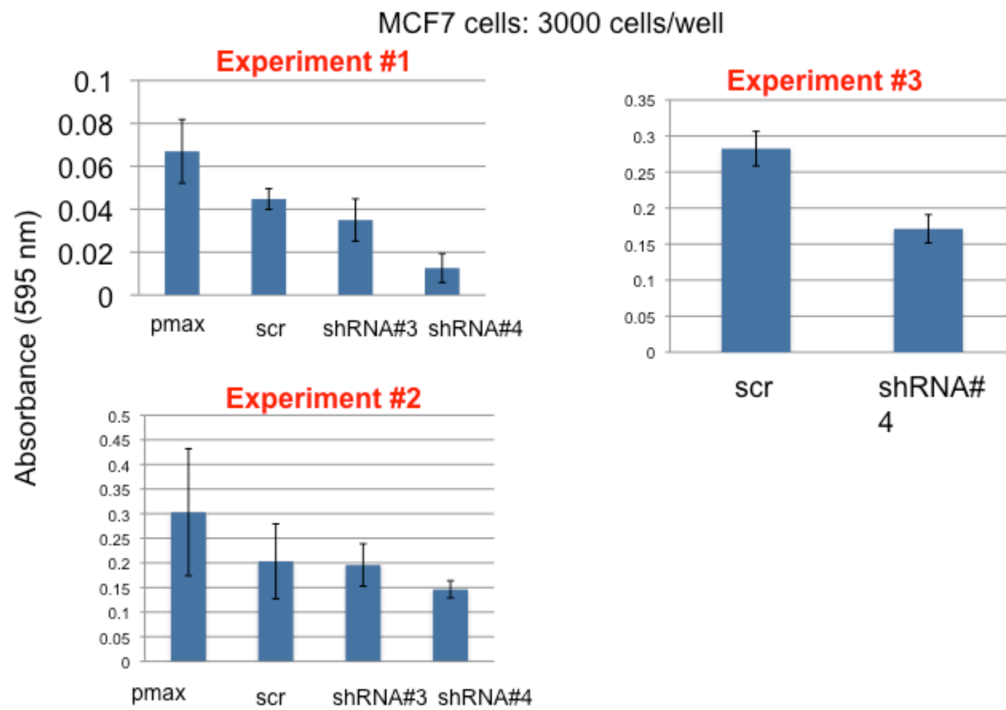

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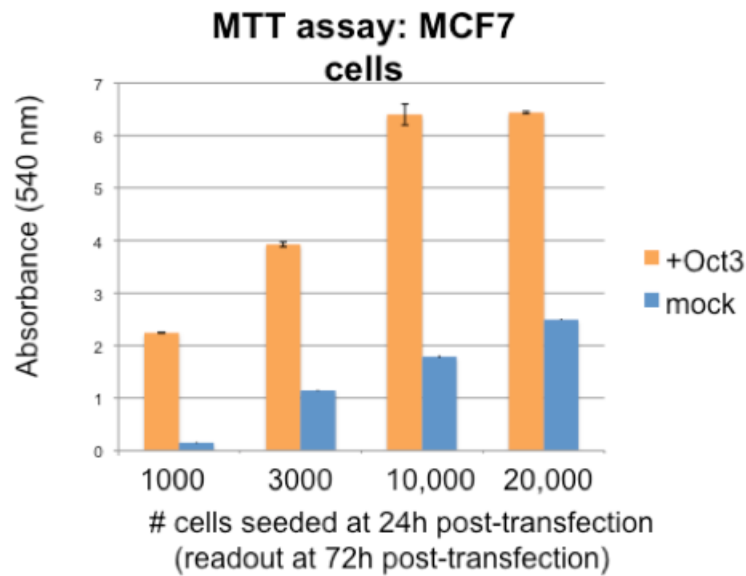
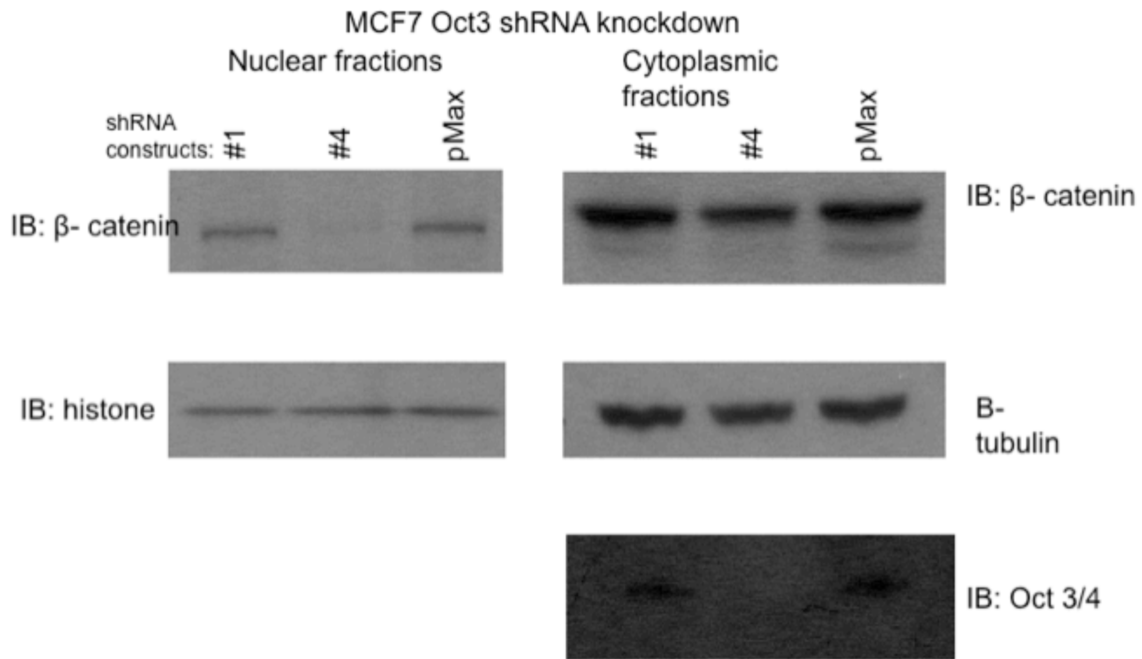


Figure 3-12

a)



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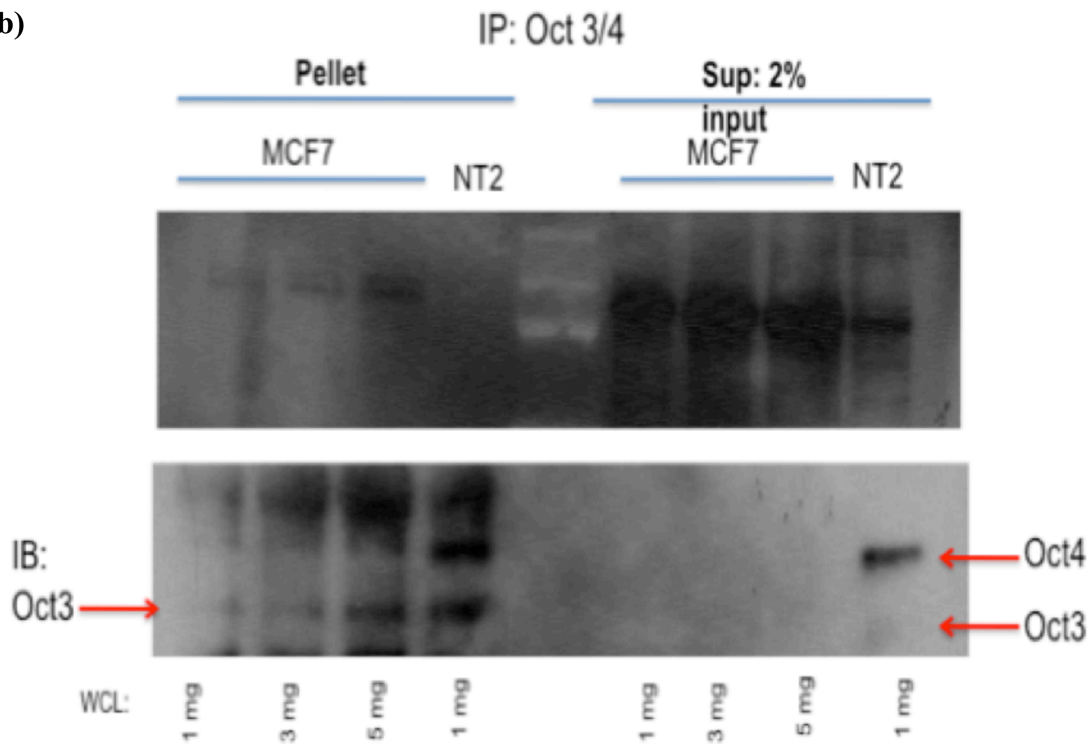
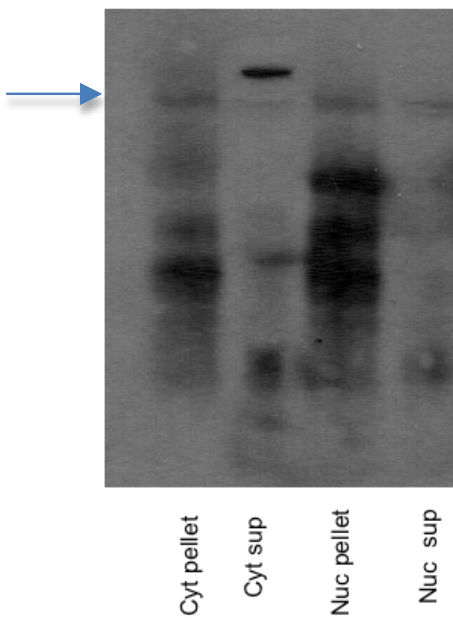


Figure 3-13

IP:Sox2

IB: phospho- β-catenin (S33/S37/T41)



IB: β-catenin

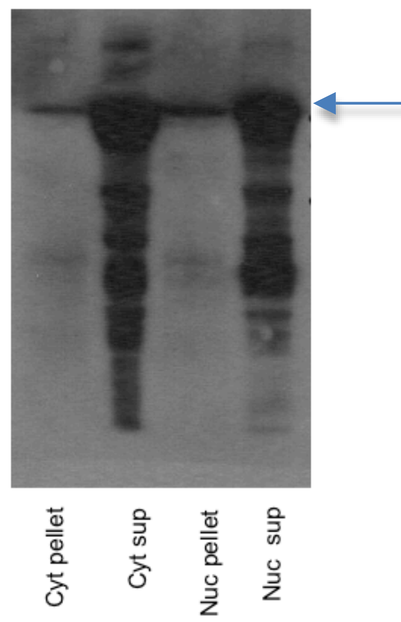
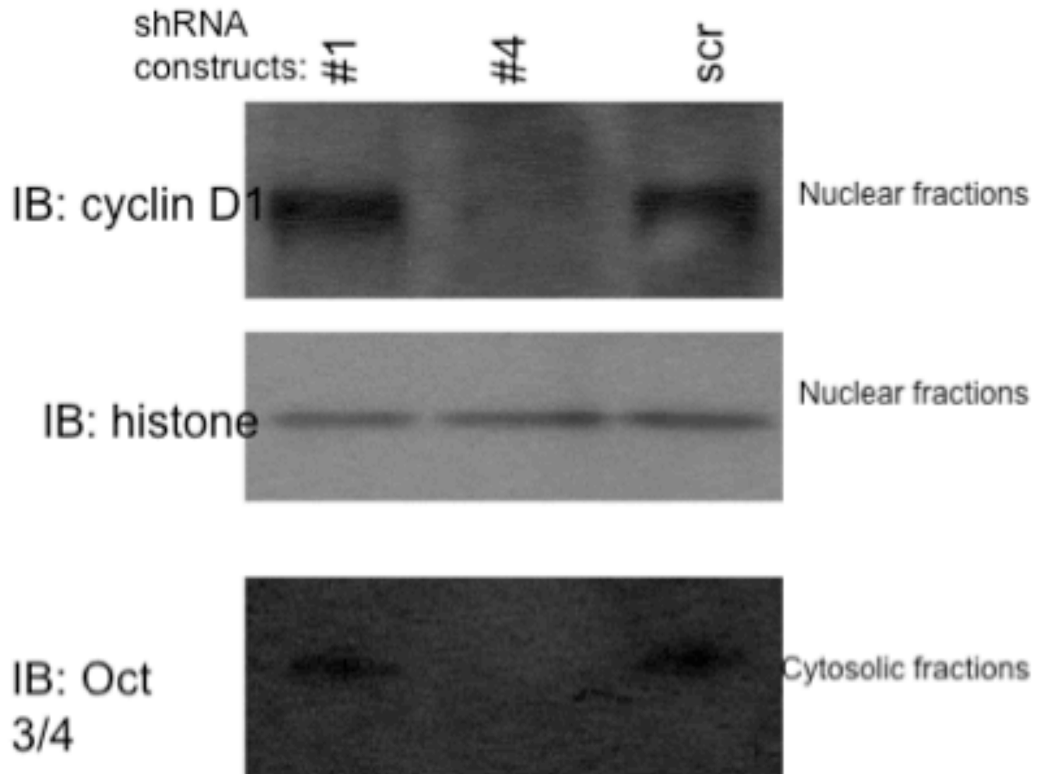


Figure 3-14

a)



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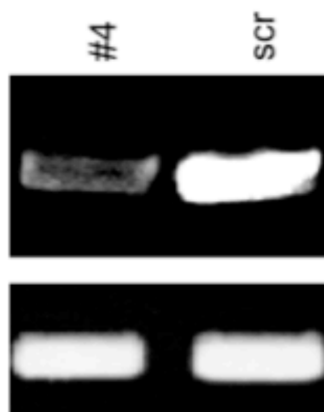

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c)

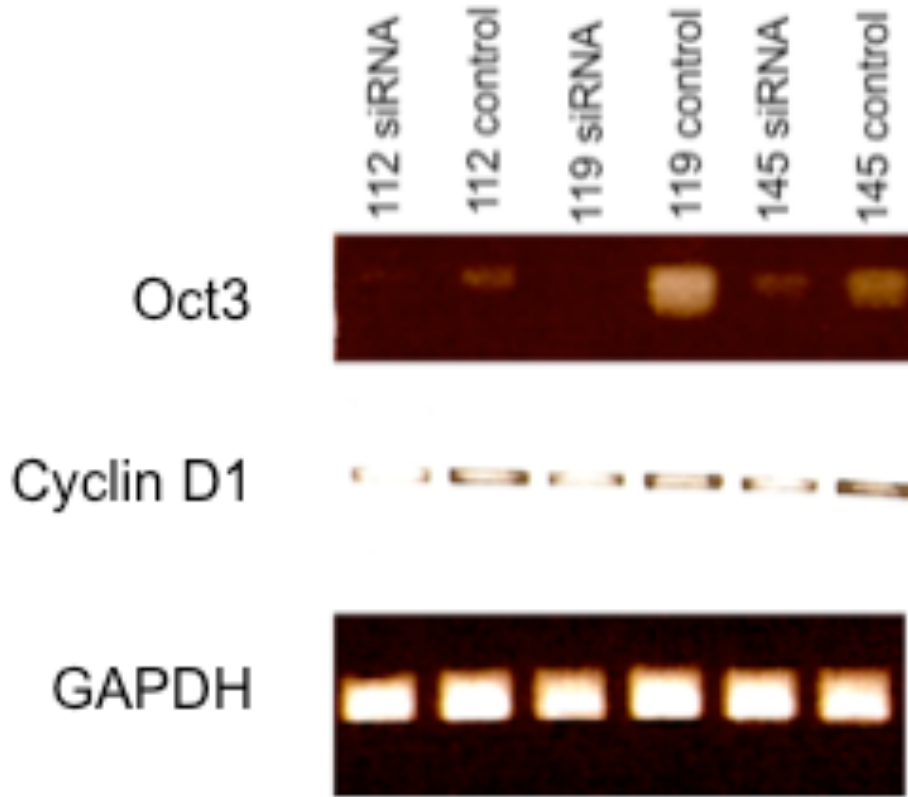


Figure 3-15

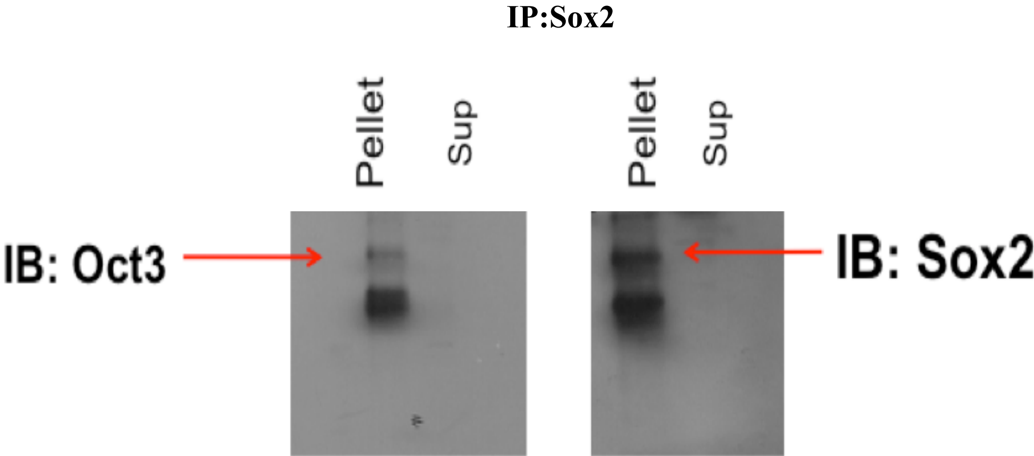
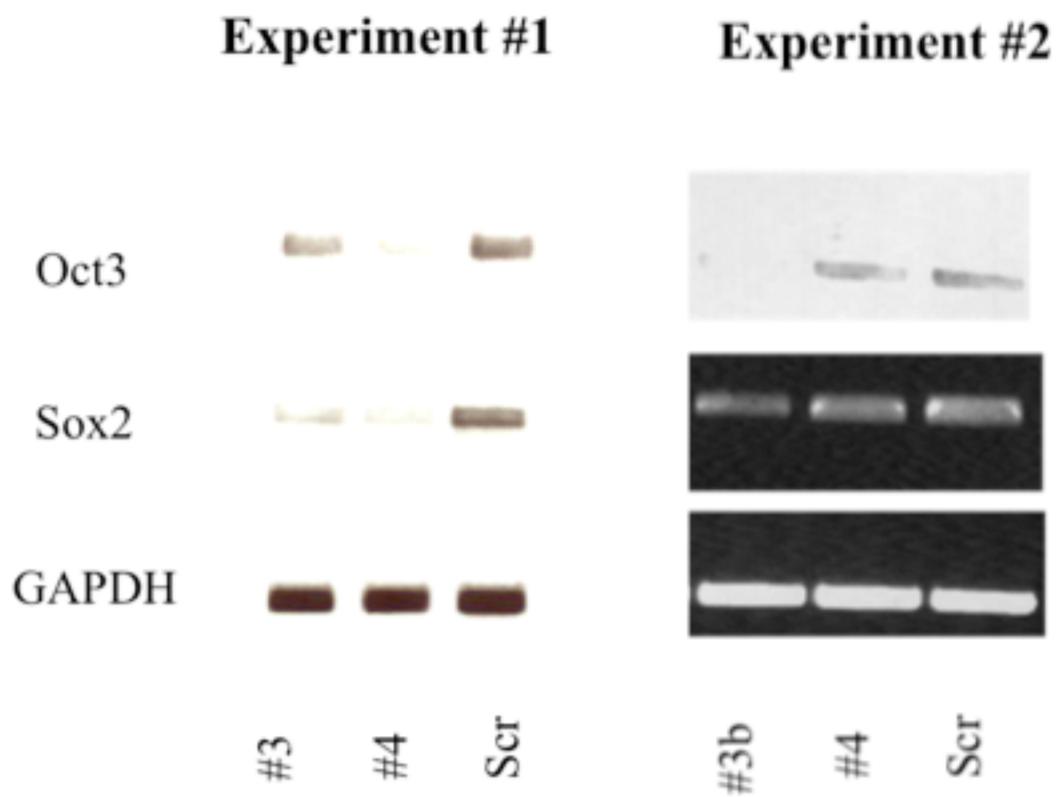


Figure 3-16



Chapter 4

Discussion

Tumor progression is a complex process that is mediated by an array of signaling networks. The phenotypic similarities of cancer cells and ESCs led us to question whether the regulatory mechanisms that govern pluripotency in ESCs might be similar to those that promote tumor progression. The central regulatory circuitry of ESC pluripotency has been described by a number of previous reports to consist of the transcription factors Nanog, Sox2, and Oct3/4, which act both independently and in concert with each other to elicit numerous downstream effects. Although it was previously thought that these pluripotency factors were rapidly downregulated upon differentiation and that these proteins are not expressed in adult tissues, it has recently been reported that Nanog, Sox2, and Oct3/4 are expressed in a number of tumor types. However, little work has been done to characterize these factors in the context of cancer cells, nor has much work been done to elucidate the precise mechanisms by which they might play cancer-promoting roles. Thus, I set out to better understand how pluripotency factors might be involved in the process of tumor cell maintenance and progression. I hypothesized that the ectopic expression of transcription factors that maintain pluripotency in ESCs might contribute to the continued proliferation and block in differentiation observed in breast cancer. The goal of this study was to examine the expression of embryonic pluripotency factors in breast cancer cells and to determine whether Nanog, Sox2, and/or Oct3/4 play a role in the cancer phenotype.

Initial screening of a panel of mammary carcinoma cell lines by immunoblot and quantitative RT-PCR revealed that Nanog and the Oct4 isoform are not expressed in breast cancer cells, but that Sox2 and the Oct3 isoform are expressed in all cell lines tested. Additionally, Sox2 and Oct3 are overexpressed in a number of breast cancer cell lines when compared to non-tumorigenic mammary cells. These results highlight the confusion that may arise from the confounding factors of pseudogenes and the presence of highly homologous alternatively spliced transcripts. Previous studies may have erroneously reported expression of Nanog and Oct4 in cancer cells because they did not take into consideration the existence of Nanog and Oct4 pseudogenes in designing PCR primers. Most publications have not distinguished between the Oct3 and Oct4 alternatively spliced isoforms, suggesting that experimental results indicating "Oct4" or "Oct3/4" expression in breast cancer may have actually been showing Oct3 isoform expression. A more recent study that does specify between the splice variants in prostate cancer cells corroborates our results, finding Oct3 expression but no Oct4 expression in their model system (Monsef et al., 2009). Our expression studies are also bolstered by findings in embryonic stem cells demonstrating that Oct3 does not promote pluripotency whereas Oct4 is a key regulator of pluripotency (Lee et al., 2006).

Cellular localization is a critical factor in protein function, and in order for transcription factors to bind to DNA, they must localize to the nucleus. Our results support a role for Sox2 as a transcription factor in breast cancer cells that shuttles between the cytoplasm and the nucleus. I found that Sox2 is localized to the cytoplasm in breast cancer cells, but significant nuclear expression was also found in several cell lines. In contrast, endogenous Oct3 expression was only seen in the cytoplasm, with the notable, albeit preliminary observation that during cell division, it may be able to bind to DNA, suggesting that Oct3 localization regulation is at the level of the nuclear membrane.

Overexpression studies using several different Oct3 expression plasmids with and without epitope tags allowed us to determine that wild type Oct3 can localize to the

nucleus upon forced overexpression. While the majority of overexpressed Oct3 remains cytoplasmic, a small minority of cells exhibit strong nuclear expression of the overexpressed protein. These experiments also suggested that Oct3 might be post-translationally modified in the cytoplasm, and that this putative modification may be involved in retaining Oct3 in the cytoplasm. I was unable to identify the modification; however, previous studies and sequence analysis of Oct3 indicate that there are several potential sites present in the Oct3 NTD that are candidates for phosphorylation. Taken together, the data from the overexpression experiments suggest that a cellular mechanism for retention of Oct3 in the cytoplasm might be overwhelmed upon expression of high levels of the protein, leading to nuclear import.

Given the above results, I decided to investigate further the regulatory mechanism(s) of Oct3 localization by making deletion mutants of the three major Oct3 domains by PCR-based site-directed mutagenesis. I individually deleted the 40 amino acid (aa) N-terminal domain (NTD), the 154 aa POU domain, or the 71 aa C-terminal domain (CTD) of Oct3, and cloned these mutants into the pmCherry-N1 expression plasmid for visualization by fluorescence microscopy. The use of the mCherry tagged vector allowed us to visualize the overexpressed protein without immunostaining, eliminating the possibility of background staining artifacts.

Upon deletion of the Oct3 NTD, which is the unique domain of the isoform, the protein localized almost entirely to the nucleus upon overexpression in MCF7 cells. This result was in contrast to wild-type Oct3, the POU deletion mutant, and the CTD deletion mutant, all of which exhibited predominantly cytoplasmic localization. These results indicate that the Oct3 NTD contains an autoinhibitory region that blocks nuclear transport, and that the CTD and POU domain are required for nuclear localization.

Examination of the role of the RKRKR NLS in regulating Oct3 localization allowed us to determine that a functional NLS is critical for Oct3 nuclear localization and that the NTD of Oct3 blocks the activity of the NLS sequence. I generated double mutations in the Oct3 sequence, deleting the NTD and substituting the NLS sequence with the non-functional GSRVD sequence. One previous published report generated Oct4 mutants with this same GSRVD substitution in an otherwise intact Oct4 sequence, resulting in nuclear export of Oct4. Since endogenous Oct4 is normally retained in the nucleus and acts as a transcription factor, the RKRKR NLS was therefore shown to be critical for nuclear localization of Oct4, and the NTD of Oct4, unlike that of Oct3, does not inhibit nuclear localization. In our experiments, the GSRVD substitution resulted in a "rescue" phenotype of the NTD deletion mutant, with the Oct3 double mutant localizing to the cytoplasm. Therefore, lack of an NTD and the presence of a functional NLS are both required for Oct3 nuclear localization.

The critical Oct3 NTD sequence responsible for retaining the protein in the cytoplasm despite the presence of its known POU domain nuclear localization signal (NLS) was also clarified by this study. Additional mutagenesis experiments further characterized the NTD of Oct3. I performed serial truncations of the 40-amino acid Oct3 NTD, allowing us to pinpoint the critical localization sequence. Much of the NTD, from amino acids #1-34, does not appear to impact on Oct3 localization, since upon deletion there was no change compared to wild type and Oct3 remained primarily in the cytoplasm. However, upon deletion of amino acids #1-38, a dramatic change in localization occurred, and the overexpressed Oct3 mutant was found to be almost entirely in the nucleus. Therefore, the sequence between amino acids #35-38 is critical for

maintaining Oct3 in the cytoplasm. This could be due to blocking of the NLS directly by this sequence. Alternatively, the critical regulatory sequence could mediate a phosphorylation or other modification event that blocks the NLS, or it may bind to another protein that in turn blocks the NLS. However, it is notable that the regulatory amino acids identified here are not candidates for tyrosine or serine/threonine kinase phosphorylation, as the putative amino acid sequence (from aa #35-#39) is: leu-pro-phe-lys-ile.

A likely candidate for phosphorylation in the Oct3 sequence is serine 141, located within the POU domain. Saxe et al. (2009) showed that the homologous murine residue of Oct4, serine 229, is post-translationally modified by phosphorylation, and that this phosphorylation partially controls Oct4 transactivation activity. However, localization of Oct4 is not affected by the phosphorylation state of serine 229, although it is adjacent to the RKRKR NLS and phosphorylation at this position could potentially block NLS function. To determine whether phosphorylation of serine 141 of human Oct3 plays a role in its function and/or localization, I generated Oct3 double mutants with NTD deletions plus either a non-phosphorylatable S→A mutation or an S→D mutation that has been shown to be a phosphorylation mimetic for the Oct4 isoform (Saxe et al., 2009). Both of these double mutants were cloned into pmCherry-N1 and expressed in MCF7 cells, and both exhibited predominantly cytoplasmic localization. Therefore, a substitution of the serine at position 141 "rescues" the nuclear localization seen when the NTD is deleted. It is clear that the presence of serine 141 is required for nuclear localization, but we have no evidence that the phosphorylation state has an effect on localization of the protein.

While I primarily focused on regulation of Oct3 localization and downstream effects of Oct3 and Sox2 expression in cancer cells, I was also interested in how Oct3 and Sox2 expression might be regulated in these cells. Src family members, in particular c-Yes, regulate expression of Oct3/4 in ESCs, and Oct3 regulates Sox2. Thus, I asked whether Src family kinases might regulate Oct3 and Sox2 expression in breast cancer cells. Using a pharmacological inhibition approach, I found that repression of Src activity resulted in a decrease in Oct3 and Sox2 expression levels without changes in localization patterns. Notably, Src is a key modulator of cell proliferation, and our results suggest that Oct3 and Sox2 could be downstream effectors in Src-regulated pathways that impact cancer cell proliferation.

The function of Oct3 in normal and cancer cells remains mostly unknown, although results presented here point to a potential role in regulating the cell cycle and thus regulating cell proliferation. Upon overexpression of Oct3, an MTT assay revealed a significant increase in MCF7 cell proliferation, and when Oct3 is repressed, proliferation is decreased. In a single experiment, Oct3 knockdown by RNAi resulted in complete abrogation of cyclin D1 expression, although further experiments did not produce consistent results due to the difficulty in reproducing effective Oct3 knockdown. In experiments where a significant knockdown of Oct3 was achieved, a concurrent reduction in cyclin D1 mRNA expression was also observed. This result was seen using two different RNAi systems, an shRNA system and an siRNA system.

β-catenin shuttles between the cytoplasm and the nucleus, and binds in complex with Sox2 to the cyclin D1 promoter to regulate the cell cycle. To ascertain whether Oct3 might also be involved in this regulatory pathway, I performed Oct3 RNAi using shRNAs against the Oct3 transcript and showed in an immunoblot that the nuclear pool of β-

catenin was completely abolished upon effective Oct3 knockdown. The cytoplasmic pool of β-catenin was also slightly reduced when Oct3 was repressed. These results were seen in only a single experiment, since I encountered difficulties with achieving consistent oct3 knockdown in further experiments. However, to strengthen the hypothesis that Oct3 may be involved in a β-catenin-Sox2 regulatory pathway, I also performed co-immunoprecipitation experiments in which Oct3 interaction with β-catenin was observed. Moreover, the predominant form of β-catenin that was observed to co-immunoprecipitate with Oct3 was the phosphorylated form. Phosphorylated β-catenin is normally rapidly degraded in the cytoplasm, so our results suggest that the interaction with Oct3 may be serving a protective function to stabilize phospho-β-catenin. This stabilization of phospho-β-catenin may be an upstream mechanism that impacts cyclin D1 expression, ultimately impacting the cell cycle and cellular proliferation. These results are preliminary and further experiments with additional controls would be required to make definitive conclusions.

It is likely that Oct3 is not acting as a transcription factor, as it cannot bind DNA in non-dividing cells due to its cytoplasmic localization. It is possible that small amounts of Oct3, undetectable in the assays used here, translocates to the nucleus. However, even if it does localize to the nucleus, the sequences in the NTD that inhibit DNA binding make it unlikely that it can bind to a downstream gene promoter (Lee et al., 2006). Another possible scenario in which Oct3 might regulate expression of downstream genes is by interacting with Sox2 in the cytoplasm. Oct3 may act in concert with Sox2, which shuttles between the cytoplasm and the nucleus and is known to bind the cyclin D1 promoter in complex with β-catenin. The idea that Oct3 may bind Sox2 in the cytoplasm to elicit downstream effects is supported by the fact that Oct4 binds the Sox2 homeodomain via its POU domain. This POU domain sequence is identical in Oct3 and Oct4 except for the absence of two aa's at the 3' end of the Oct3 POU domain. Therefore, it is likely that the Oct3 POU domain can bind the Sox2 homeodomain in breast cancer cells. Using a co-immunoprecipitation technique, I determined that Sox2 is likely to interact with Oct3, although further controls would be necessary to confirm these results.

Regulation of Sox2 by Oct3/4 in ESCs has been reported by numerous groups, so I endeavored to determine if the Oct3 isoform regulates Sox2 in breast cancer cells. Experiments were not definitive since Oct3 knockdown did not consistently result in a reduction in Sox2 mRNA levels. Further experiments are needed to clarify these results. Since Oct3 does not act as a transcription factor, it cannot regulate Sox2 expression at the level of transcription, but it may act upstream by interactions with by other factors, perhaps with β-catenin or Sox2 itself, to affect the amount of Sox2 expressed. Oct3 interaction with Sox2 might be particularly relevant here, since Sox2 is known to bind to its own promoter in a loop regulating its expression levels.

The idea that cancer cells resemble ESCs is not new, but investigations into parallel mechanisms that might account for these similarities are ongoing. The complexities of pluripotency regulation seem to be controlled by a relatively small core network of factors, including Sox2, Oct3/4 and Nanog. Although I focused on the Sox/Oct/Nanog circuitry in these studies, other regulatory genes are clearly involved in maintaining pluripotency. These include Klf4, Lin28 and c-Myc, which were used along with Sox2 and Oct3/4 and Nanog to create induced pluipotent stem cells (iPS cells) from both mouse and human fibroblasts. Retroviral-mediated expression of a combination of

Sox2, Oct3/4, Klf4, and c-Myc genes was sufficient to induce pluripotency in adult human cells in one study (, while forced expression of Sox2, Oct3/4, Nanog, and Lin28 was sufficient to produce the pluripotent phenotype from adult human cells in a separate study using a lentiviral delivery system. The ability of these small networks of genes to effect such profound phenotypic changes, essentially "de-differentiating" adult cells to a stem cell state, highlights the complexity of the downstream regulation that must occur. As with Sox2 and Oct3, Klf4 and Lin28 overexpression are also associated with the cancer phenotype (Pandya et al., 2004; Viswanathan et al., 2009), and c-Myc is a well-known proto-oncogene (Vennstrom et al., 1982; Hoffman and Lieberman, 1998; Askew et al., 1991; Evan and Littlewood, 1993). Therefore, I must point out that the observed "stem-like" phenotype of cancer cells may be controlled by genes beyond those investigated in the present study, as was shown in a recent publication identifying a Myc-controlled network that can account for the pluripotency phenotype of cancer cells (Kim et al., 2010). This Myc network was shown to be functionally separable from the Sox/Oct/Nanog core network and the authors assert that the pluripotency transcription program in cancer cells is composed of distinct, independent modules. Thus, the data presented here should be placed in the context of a wider array of pluripotency regulatory networks that seem to be active in cancer cells.

Taken together, the data presented here build upon previous work that examined the roles of Oct3/4 and Sox2 in cancer progression. This study further clarifies that Oct3 is the only isoform of the two splice variants that is expressed in breast cancer cells, and that, as previous work describing Oct3 and Oct4 functions in ESCs has shown, Oct3 is not acting as a pluripotency factor in cancer cells. This is the first study to report that the Oct3 isoform is specifically expressed in the cytoplasm of breast cancer cells. Preliminary results presented here also suggest that Oct3 may be involved in regulation of the cell cycle in breast cancer. Our studies contradict previous reports that have claimed Nanog and Oct4 expression in cancer cells, and I note that our observations from the expression studies are strengthened by our use of extensive controls, an awareness of pseudogenes, and consideration of the homology of the Oct3 and Oct4 transcripts.

Chapter 5

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