CYB5D2 PRODUCES TUMOR SUPPRESSING EFFECTS IN BREAST CANCER

CYB5D2 ATTENUATES BREAST CANCER AND CELL SURVIVAL

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ABSTRACT

Breast Cancer (BC) is the leading cause of cancer related deaths among women worldwide. Its etiology includes inactivation of tumor suppressors. In line with this notion, our laboratory has recently reported that CYB5D2 possesses tumor suppressing activities in cervical cancer; evidence also suggests CYB5D2 as a novel tumor suppressor of BC. I thus hypothesize that CYB5D2 mitigates cell propagation *in vitro*. To examine this possibility, I transiently expressed CYB5D2 in two typical BC subtype cell lines, HCC 1954 (HER2+) and MCF7 (Luminal A). Remarkably, cell population was diminished over a period of at least five days post-transfection when compared to empty vector (EV). To characterize CYB5D2-derived inhibition of BC cell proliferation, I generated a Tet-On inducible system in both cell lines in which CYB5D2 expression is induced upon addition of doxycycline. As expected, induction of CYB5D2 led to a decline of cell propagation and colony formation based on cell proliferation and colony formation assays respectively. Moreover, knockdown of CYB5D2 via lentivirus-based shCYB5D2 transfection in HCC 1954 and MCF7 cells resulted in an incline of cell propagation and colony formation when compared to the shCTRL (control) line. To examine possible apoptotic mechanism of tumor suppressor, we performed TUNEL assay analysis in cell lines expressing CYB5D2. Both HCC 1954 and MCF7 obtained similar results exhibiting evidence of apoptotic cells when compared to their respective controls. Interestingly, upon CYB5D2 expression HCC 1954 also displayed evidence of halting G1 phase progression when performing cell cycle analysis, suggesting a promising alternate

tumor suppressing mechanism. Lastly, to corroborate with previous observations of an existing molecular interaction between CYB5D2 and tumor suppressor Phosphatase and tensin homolog (PTEN) we performed a Co-Immunoprecipitation (Co-Ip) assay. As expected, endogenous CYB5D2 and PTEN lysate from HCC 1954 and MCF7 were demonstrated to interact on a molecular level. These observations support the notion that CYB5D2 elicits tumor-suppressing activities in both Her2+ and Luminal A breast cancer cell lines. It is important to note that we detected a greater tumor suppressing impact of CYB5D2 in HCC 1954 when compared to MCF7 suggesting a potential mechanism to favour HER2+ status. Additional research in determining potential tumor-suppressing pathway of CYB5D2 in BC is encouraged as we established promising insight of novel tumor suppressor.

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These past two years of graduate studies have been a tremendous achievement. I experienced at first hand the challenges encountered with clinical research, which can sometimes be discouraging. However my passion for innovative clinical research inspired me to push through the difficult moments and helped me realize that being a part of individuals who are passionate towards working to find a cure or better treatment for a disease is highly rewarding. I look forward to continuing my career in clinical research where I can make a positive impact in the scientific community.

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AJCC	American Joint Committee on Cancer
AP1	Activator protein 1
ATCC	American Type Culture Collection
ATM	Ataxia- telangiectasia mutated
BC	Breast Cancer
BRCA 1	Breast cancer type 1
BSA	Bovine Serum Albumin
CDK	Cyclin D Kinase
CFA	Colony formation assay
CK-2	Casein kinase-2
Co-Ip	Co-Immunoprecipitate
CPA	Cell proliferation assay
CYB5D2	Cytochrome b5 domain containing 2
D	Aspartic acid
DAPI	4’,6- diamidino-2-phenylinodole
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor

ER	Estrogen receptor
ERD	Estrogen receptor downregulators
ETOP	Etoposide
EV	Empty Vector
FBS	Fetal bovine serum
FoxO	Forkhead box O
G_1	Gap 1phase
G_2	Gap 2 phase
GAP	GTPase activator protein
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GP	Gag-pol expressing vector
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IP	Immunoprecipitate
IRS-1	Insulin receptor substrate 1
LCIS	Lobular carcinoma in situ
LHRH	Luteinizing hormone-releasing hormone
LOH	Loss of heterozygosity

MAPR	Membrane associated progesterone receptor
NET	Progestin norethisterone
PAIRBP1	Plasminogen Activator Inhibitor 1 RNA-Binding
PGRMC	Progesterone receptor membrane component 1
P_{min} CMV	Minimal CMV promoter
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinases
PIK3	Phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PR	Progesterone receptor
PSB	Protein sample buffer
PTEN	Phosphatase and tensin homolog
pTet-ON	Plasmid tetracycline “on” inducible system
R	Argenine
RB1	Retinoblastoma 1
RHEB	Ras homolog enriched in brain
RTK	Receptor tyrosine kinase
S	Synthesis phase
SERM	Selective estrogen-receptor response modulator
rtTA	Reverse tetracycline transactivator
TBS-T	Tris buffer saline

TdT	Terminal deoxynucleotidyl transferase
Tet O	Tet operon sequence
TM	Transmembrane domain
TNBC	Triple negative breast cancer
TRE	Tetracycline response element
tTA	tetracycline transactivator
TSC1/2	Tuberous sclerosis proteins 1 and 2
TetR	Tet repressor protein
tetO	Tetracycline operator
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VSV-G	Vesicular stomatitis virus glycoprotein

DECLARATION OF ACADEMIC ACHIEVEMENT

While I was the primary contributor for the work presented in this thesis, several individuals collaborated to successfully fulfill this project.

Dr. Damu Tang and I designed a structural approach for this project consisting of experiments and theoretical background knowledge. Former members of Dr. Tang's lab provided the cloned vectors expressing CYB5D2 used for generating stable lines and transient transfections in HCC 1954 and MCF7. Dr. Xiaozeng Lin, Dr. Wenjuan Mei, Yan Gu and Colleen MacKenzie provided essential training in experimental techniques.

CHAPTER 1: INTRODUCTION

1.1 Overview of Breast Cancer

1.1.1Breast Cancer Epidemiology

Cancer is the result of genomic instability in a cell leading to uncontrollable and rapid cell division causing mass formations commonly known as malignant tumors (1). Breast cancer (BC) is the most commonly diagnosed cancer and cause of cancer related deaths among women worldwide (1, 2). It is estimated that in 2018 more than two million women were diagnosed and more than 600,000 deaths were accounted for BC (1, 3). While this remains the case for women worldwide, BC is the second cause of cancer related deaths in developed countries (4). It is believed BC survival in developed countries has constantly improved in comparison with developing countries as a result of the use of early detection mammography screening, advanced surgical procedures, selective therapy and overall awareness; options that are typically unattainable or unaffordable in low income countries (5). For instance, the 5-year survival rate in 2010-2014 in the United States and Australia was 90% while in Malaysia was only 65% (3). In Canada, it is estimated that 1 in 8 women will develop BC during her lifetime whereas 1 in 31 women will perish from it (2). In 2017 approximately 25% of all new cancer cases and 13% of all cancer mortality in Canadian women were attributable to BC (2, 5). While mortality rates for BC have significantly decreased over the past decades, this disease remains a life threatening risk for women's health (2). This is primarily because of our limited knowledge on the etiology and pathophysiology of BC. Nonetheless, extensive research suggests risk factors in the development of BC include early menarche, later onset menopause, nulliparous women and minimal to no breast-feeding (6, 7).

1.1.2 Classification and selective treatment of BC

Breast cancer typically originates as a carcinoma in the lining of lactiferous ducts or lobules of the gland known as invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) respectively (8, 9). BC is highly heterogeneous and can be separated into different subtypes varying in overexpression of estrogen receptors (ER+), progesterone receptors (PR+) and human epidermal growth factor receptor 2 (HER2+) (10, 11). ER and PR are typically located on the cell membrane surface or in cytosolic space; however in the absence of estrogen or progesterone these receptors remain mostly in cytosolic space (12, 13, 14). Binding of estrogen and progesterone to ER and PR respectively causes dimerization of receptors initiating migration to the nucleus activating nuclear cell response signalling pathways (15). Activation of ER promotes binding of DNA-binding domain zinc finger motifs to estrogen response elements (13). While regular cellular response from PR initiation remains unclear, ER activation promotes breast epithelial differentiation (14). Nonetheless, in the event of tumorigenesis, overexpression of ER activation is believed to stimulate BC cell proliferation. Over 70% of all BCs exhibit ER+ status while 65% of cases presents with PR+ (16). HER2 is a receptor tyrosine kinase (RTK) located on cell membrane surface (17, 18). It belongs to the epidermal growth factor receptor (EGFR) family (HER1, HER2, HER3, and HER4);

however, unlike all EGFRs, HER2 does not have a known ligand (18). Activation of HER2 is thought to commence via hetero-dimerization with HER3 and HER4 triggering phosphorylation of tyrosine residues residing in cytosolic space (18, 19). Phosphorylation subsequently initiates signal transduction pathways such as MAPK and PI3K/Akt, ultimately stimulating cell proliferation and survival (17, 19). It is important to note that over 90% of HER2+ cases display gene overexpression and protein receptor amplification (20).

Triple positive BC is classified as ER+, PR+ and HER2+ status (20, 21). On the other hand, BC not overexpressing aforementioned receptors is categorized as triple negative status resulting in a poorer prognosis due to its more aggressive and unrecognized tumorigenic activity (22, 23). Tumorigenesis in the luminal epithelium of lactiferous ducts or lobules can give rise to luminal A and B breast cancer (21, 22). Luminal A displays a status of ER+ and PR+ but not of HER2+. On the other hand, Luminal B displays ER+ and varies for PR+ and HER2+ status, causing luminal B to have a poorer prognosis in comparison with Luminal A status (21, 23). Moreover, HER2+ status accounts for 15-25% of diagnosed BC and exhibits no ER or PR amplification (24). Tumor cells originating in myoepithelial cells of lactiferous ducts are classified as basal-like BC (21, 24). This subtype displays a triple negative status representing approximately 15% of IDC cases having the poorest prognosis of mentioned subtypes. Basal-like BC is also typically associated with familial tumor suppressor breast cancer 1 (BRCA1) gene mutation (25).

Clinically, the American Joint Committee on Cancer (AJCC) classifies BC in a staging system referred as TNM, which incorporates tumor size, growth status in nearby tissues, presence of a mass in local lymph nodes and metastatic status (23, 26). Additional guidelines were implemented in 2018 that would further categorize the type of malignant mass in breast tissue. Tumor grade defines morphological disparities between tumor cells and regular epithelial cells (25, 26). While grade 1 suggests tumor cells are well differentiated and divide at a slower rate, tumor grade 3 BC cells appear distinctly abnormal and poorly differentiated, leading to rapid cell division and higher metastatic rate (26, 27). Oncotype DX score is a diagnostic and prognostic test comprised of a genomic-based risk assessment for BC recurrence in early stages of ER+ where no evidence of lymph node invasion is detected (27). The genomic test identifies 21 genes, generates a score (1-100) representing the likelihood of BC recurrence and evaluates the benefits from chemotherapy, radiation or highly targeted therapy (27, 28). Patients with a score above 31 are classified at a high risk of recurrence and chemotherapy is recommended (26, 29). Staging in BC identifies the size and location of the tumor. Stage zero represents a non-invasive benign mass originating in ducts or lobule glands of the breast classified as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) respectively (29). Stage one encompasses invasive cancer cells with tumor sizes up to 2cm in diameter (27, 30). Additionally, there is no evidence of tumor regional spread outside the breast including nearby lymph nodes (30). Stage two is recognized as a tumor mass between 2-5cm in diameter and evidence of regional spread in 1-3 axillary lymph nodes (30, 31). Invasive tumors larger than 5cm in size with a regional spread to nearby tissues including 4-9 axillary lymph nodes are classified as stage 3 BC. Lastly, metastatic breast cancer cells spreading and colonizing distant regions of the body represent a stage four BC (30).

Patients with BC exhibiting ER+ status are believed to benefit from targeted hormonal therapy (31, 32). Tamoxifen is a selective estrogen-receptor response modulator (SERM) operating as a receptor antagonist to ER (32). Aromatase inhibitors mitigate estrogen production in adrenal glands and adipose tissue (31, 32). Because it does not inhibit estrogen production in the ovaries, aromatase inhibitors are more suited for post-menopausal women (31). Pre-menopausal women with ER+ BC are believed to benefit from luteinizing hormone-releasing hormone agents (LHRH), which target and suppress estrogen production in the ovaries. An alternative therapy from SERM is estrogen receptor downregulators (ERDs) recommended for patients with highly aggressive BC. ERDs block ER, thus preventing ligand activation and reduce concentration of receptors in cell membrane space (31, 33). Moreover, BC patients with HER2+ status are recommended HER2 treatment-targeting proteins with a high specificity. Trastuzumab is a recombinant monoclonal antibody that selectively binds to domain four of HER2, thereby preventing down-stream activation (33). Pertuzumab is an alternative monoclonal antibody treatment often used in combination with Trastuzumab therapy in locally recurrent or metastatic HER2+ BC (33,34). Pertuzumab binds to domain two of HER2 and sterically prevents hetero-dimerization with HER3 (33). Trastuzumab also works as a conjugate complex with cytotoxic agent emtansine. This adjuvant therapy selectively binds to HER2 inducing cell internalization, allowing emtansine to stimulate cell cycle arrest and apoptosis (35, 36). Treatment with a reduced selective specificity often used in combination with hormonal therapy include surgical lumpectomy or mastectomy to remove localized mass, radiation to reduce localized tumor and chemotherapy to shrink or eliminate masses that have spread regionally or distantly (37).

1.1.3 Loss of heterozygosity in BC

Loss of heterozygosity (LOH) is a hallmark on the pathogenesis of BC. Allelic alterations of tumor suppressor genes have been identified in various cancers including but not exclusive to colorectal cancer, small cell lung cancer, ovarian cancer and breast cancer (38). Loss or alterations of chromosome 17p is commonly found in BC. Tumor suppressor TP53 locus residing in chromosome 17p12.1 is lost in 20-40% of BC cases and occurs at an early stage of tumorigenesis (39). Stress conditions including hypoxia, DNA damage and oncogenic activation in a cell induces TP53 activation; ultimately impeding cell cycle growth, cell survival and preserving genomic integrity (39). Mutations of TP53 are most prevalent within exons 5-8 accounting for over of 90% of mutations. Evidence suggests stroma-specific allelic imbalance correlates with promoting TP53 mutations in BC, allowing tumorigenesis to further develop in a rapid and aggressive manner (40). It is believed that basal tumor suppressor activity is essential to diminish BC survival; research suggests the efficacy of radiotherapy and some types of chemotherapy highly depend on the functionality of TP53 pathway (41, 42). It is important to emphasize that in comparison with a negative status, TP53 mutations significantly increase the relative risk of death within ten years in BC patients that have undergone surgery independent of tumor size, node status or ER/PR status (43, 44). Subsequent to LOH, hereditary mutation of tumor suppressor breast cancer type 1 (BRCA1) plays a critical role in developing BC. While it is estimated that 20-25% of BC patients contain a family history of BRCA1 mutation, 5-10% demonstrate an autosomal dominant inheritance increasing the risk of developing BC in a lifetime to 82% (45). Mutation and LOH of tumor suppressor retinoblastoma 1 (Rb1) located in chromosome 13q14.2 has been correlated with the progression of triple negative breast cancer (TNBC) (44). Evidence suggests Rb1 negative TNBC displays a significantly higher sensitivity to gamma-irradiation and a moderate greater sensitivity to chemotherapeutic agents doxorubicin and methotrexate when compared to Rb1 positive TNBC (44, 46). Moreover, substantial research has identified the LOH of tumor suppressor phosphatase and tensin homolog (PTEN) in some types of BC. PTEN is believed to inhibit PI3K/AKT pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) into its inactive form Phosphatidylinositol (4,5)-bisphosphate (PIP2). It is suspected that promoter methylation of PTEN gene may prevent transcription resulting in contribution to tumorigenesis and tumor progression in BC (47). It should be noted that loss of PTEN has a greater impact of correlation with disease-related deaths, predicted lymph node metastasis, and with loss of estrogen receptor signaling all resulting in poor prognosis (48, 49). Tumor suppressor activity plays a key role in preventing tumorigenesis. Consequently, substantial evidence emphasizes a strong association of LOH with the development of therapy-resistant BC and overall increased risk of disease-related death (49).

1.2 PI3K/AKT and MAPK molecular pathways

1.2.1 PI3K/AKT signaling pathway and its relevance in BC

The Phosphoinositide 3-kinases (PI3K)/AKT is an intracellular signaling pathway fundamentally essential for cell growth regulation by suppressing pro-apoptotic proteins and promoting cell proliferation and differentiation (50). Binding of extracellular growth factor (EGF) ligand to its conjugate receptor tyrosine kinases (RTKs) triggers homo-dimerization or hetero-dimerization of monomers resulting in heterologous auto-phosphorylation at intracellular tyrosine residues (50). Activation of PI3K by phosphorylation can occur in three different pathways; through direct binding of phosphorylated RTK tyrosine residues, indirect RTK binding via phosphorylated insulin receptor substrate 1 (IRS-1) or by binding to active GTP Ras from the membrane bound GTPase Ras complex independently of RTK activation (51). Active second messenger PI3K in turn migrates to cellular membrane-bound PIP2 and phosphorylates component into PIP3 allowing it to become an effective docking site for AKT activation (52, 53). Binding to PIP3 causes conformational changes in AKT exposing sites serine-473 and threonine-308 available for phosphorylation by kinases mTOR2 and PDK1 respectively; this in turn provides AKT its complete activation form (54, 55). Remarkably AKT serves as a complex master regulator capable of initiating activation of several pathways ultimately promoting cell survival (56). Substantial research has identified the binding of AKT to inactivate tumor suppressor BAX from inducing apoptosis via activation of the caspase cascade and consequently generating large pores in the outer mitochondrial membrane (57). Interestingly, AKT activity is also observed in activation of translation factor S6K serving as a translational regulator through the binding with 40S ribosomal protein and inducing translation of mostly angiogenic and cell growth factor proteins (56, 58). Activation of S6K is initiated upstream by molecular disassociation between Ras homolog enriched in brain (RHEB) and tumor suppressor tuberous sclerosis proteins 1 and 2 (TSC1/2). Inhibition of TSC1/2 occurs by AKT binding and phosphorylating TSC1/2 at the serine-939 and threonine-1462 sites generating aforementioned molecular dissociation; allowing RHEB to take its active form and in turn activate mTOR (59). Consequently mTOR phosphorylates and activates translation factor S6K. Moreover, extensive research has reported the reduction in tumor suppressor forkhead box O (FoxO) concentration by AKT involvement (59, 60). Phosphorylated FoxO via AKT binding is thought to serve as a substrate for the enzyme ubiquitin ligase. Consequently, transferring ubiquitin peptides to FoxO results in protein degradation via proteasome ubiquitination (60).

Overexpression of PI3K/AKT pathway has been identified in several cancers including BC, particularly in malignancies with resistance in endocrine therapy, Trastuzumab resistance in HER2 + patients and cytotoxic therapy (61). Phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene encodes for protein p110α, the catalytic subunit for PI3K (60, 61). While PI3K also contains an additional polypeptide corresponding to its regulatory subunit p85, somatic mutation on exon 20 of gene PIK3CA is more common in BC resulting in PI3K overexpression (61). Genes PIK3CA and PTEN are believed to be most common mutations in BC throughout cancer progression, accounting for 70% of cases. In BC patients, aberration of gene PIK3CA is present in 35% of hormone resistant, 20-25% in HER2+ and with a lower frequency in TNBC (61, 62). Preclinical trials have detected activated AKT independent of PI3K activation presenting a challenge for HER2+ BC patients; resulting in Trastuzumab resistance. Alternatively it has been reported that while Trastuzumab blocks signaling pathway upstream from PI3K, a downstream aberration like in the case of PTEN alteration can override PI3K/AKT pathway (62). In the interest of overcoming Traztuzumab resistance in HER2+ patients, studies have suggested therapy be supplemented with mTor inhibitors (62). Furthermore, AKT activation has been associated with advanced BC and resistance to endocrine therapy resulting in ER activation independent of estrogen concentration (61). Endocrine therapy in combination with AKT and mTor inhibitors has shown to overcome endocrine therapy resistance in ER+ and PR+ (60, 62). PI3K/AKT is an intricate pathway yet to be fully understood. Further knowledge on signaling cascades hold promising novel therapies for BC resistant to presently used therapies.

1.2.2 MAPK signaling pathway and its relevance in BC

Mitogen-activated protein kinase (MAP kinase) cascade is a complex signaling pathway that transmits and amplifies induction of cell proliferation. MAP kinase activation begins by the binding of epidermal growth factor (EGF) to its conjugate RTK causing dimerization and as a result heterologous auto-phosphorylation at intracellular tyrosine residues (58). Protein adaptor Growth factor receptor-bound protein 2 (GRB2) in turn binds to phosphorylated residues and serves as an active docking site for protein SOS. Consequently, activated Guanine nucleotide exchange factor protein SOS catalyzes the exchange of Guanosine diphosphate (GDP) from small G-protein GTPase Ras protein for Guanosine triphosphate (GTP) (62, 63). In its active Ras-GTP state, membrane bound protein activates effector protein B-Raf. Subsequently effector protein phosphorylates and activates signaling cascade initiated by mitogen-activated kinases MEK 1/2, which in turn allocates ERK 1/2 its activate form; ultimately promoting activation of transcription factors fos and jun by inducing hetero-dimerization (62). Hetero-dimer complex fos-jun then target promoters of the activator protein 1 (AP1) family in the nucleus by binding to the motif of DNA resulting in transcription of growth factor proteins such as cyclins and cytokines (63). Consistently with MAP kinase signaling cascade, transcription of growth factors primarily promote cell proliferation and differentiation. Cyclin D complexes with CDK4 and CDK6 (CDK 4,6), both phosphorylated by ERK1/2, regulate cell cycle growth by coordinating transition of G1 to S phase (63). Concentration of CDK 4,6 increases in late G1 phase, inhibiting tumor suppressor RB1 thereby allowing cell cycle growth to progress.

Hyperactivity of the MAP kinase-signaling cascade has been detected in several types of cancers including BC (64). A recent study identified approximately half of malignancies in breasts to express hyperactivity of MAP kinase compared to surrounding benign tumors (63, 64). Experimental research suggests induced stress in cancer cells by estrogen deprivation triggers an up-regulation of MAP Kinase signaling pathway observed in both tissue culture and xenografts. Moreover, in ER+ malignancies activated MAP Kinase signaling pathway is thought to activate ER independently of the presence of estrogen and promote cell cycle growth; thus causing greater challenges for the efficiency of endocrine therapy. On the other hand, further mutagenesis in BC is believed to promote activation of the MAP Kinase pathway via non-genomic estrogen binding to membrane-associated receptors such as G- protein coupled receptors (63). Estrogen is capable of stimulating cell proliferation independently of ER binding by increasing growth factor production via MAP Kinase activation. Triggering aforementioned pathway leads to a mechanism that evades ER activation while resuming cell proliferation stimulation, making tumor cells resistant to tamoxifen and other types of ER+ endocrine therapy. This is because blocking ER via drug intervention no longer inhibits rapid cell proliferation stimulated by estrogen; rather estrogen finds an alternate route to promote cell growth via promoting MAP Kinase signaling cascade activation (64). Remarkably, hyperactivity of MAP Kinase pathway may occur independently of estrogen through Ras mutations (63, 64). Mutation in the Ras-GTP complex produces conformational changes that prevent GTPase activator protein (GAP) from catalyzing the exchange of GTP into inactive form GDP resulting in permanent Ras protein activity. Nonetheless, studies suggest the majority of MAP Kinase hyperactivity in BC highly depends on the enhancement of growth factor production suggesting that oncogenic pathways play a key role in the development of resistant BC (64).

1.3 Membrane Associated Progesterone Receptors (MAPR) and their role in cancer

1.3.1 MAPR family

While activation of progesterone receptors (PRs) via progesterone binding exhibit genomic functions, progesterone may also stimulate non-genomic ones by binding and activating single transmembrane PRs belonging to the membrane associated progesterone receptors (MAPR) family (65). MAPR are proteins characterized by N-terminal single transmembrane domain (TM) and C-terminal cytochrome b5 domain (CYB5) providing a high affinity for heme binding (65). Heme-binding domain is essential for MAPR-progesterone interaction thus allocating cytochrome b5 a crucial role for conjugate progesterone interaction (66). The MAPR family consists of neuferrecin also referred as CYB5D2, neudesin, and progesterone receptor membrane component 1 and 2 (PGRMC 1,2) (67). CYB5D2 expression has been identified in the brain, liver and kidney and has recently been detected as a novel tumor suppressor in cervical cancer.

Neudesin is a multifunctional protein expressed in the brain, adipose, heart, lung and kidney tissue that is function-dependent on heme binding and thought to initiate activation by intracellular pathways mostly via G protein-coupled receptors (68). In line with this notion, neudesin expression has been identified in neural functions, energy metabolism and tumorigenesis. It was first detected as a neurotrophic protein; a study detected up-regulation of neudesin in neurons located in brain and spinal cord of embryonic mice suggesting expression of protein contributes to neuron differentiation and development via activation of MAP kinase and PI3K pathways (69). A study also observed neudesin-knockout mice to exhibit anxiety-like behaviour proposing neudesin to play a key role in the hippocampal anxiety circuitry (67, 69). Consistent with multifunctional capabilities, experimental data indicated up-regulation of neudesin to suppress adipogenesis in white adipose tissue by promoting MAP kinase activation in 3T3-L1 cells (69). Administration of neudesin in mice resulted in a decrease of food intake and body weight suggesting a physiological role in hypothalamic nuclei (69).

PGRMC 1 is the most extensively researched MAPR up to date and like all MAPR, it contains a cytochrome b5 domain facilitating heme-binding activity. PGRMC 1 has also been identified to bind to cytochrome P450 promoting cholesterol synthesis (70). While PGRMC 1,2 contain two conserved introns suggesting their initial formation developed from common ancestral genes, neudesin has non-conserved introns indicating no common ancestry to other members of the MAPR family (70). Nonetheless, experimental data suggest both neudesin and PGRMC 1 contain tumorigenic activity in BC.

1.3.2 Neudesin and PGRMC 1 in BC

In the interest of identifying tumorigenic activity in BC, substantial evidence suggests neudesin and PGRMC 1 promote tumorigenesis in breast malignancies, mostly in resistant and advanced tumors (70). It is important to note that while neudesin is primarily known as a neurotrophic protein expressed at the embryonic stage, it has also been recently discovered as a novel potential oncoprotein involved in promoting tumorigenesis in breast tissue among other malignancies. Neudesin gene is located in chromosome 1 p33 and its expression is exclusive to vertebrate species (71). Overexpression of this protein has been detected in malignancies of the breast, uterine, colon, lung and skin. Recent data indicate an up-regulation of neudesin mRNA in immortalized cells while proteomics analyses revealed increased abundance of neudesin protein in ER+ and PR+ malignancies (70, 71). Consistent with BC tumorigenesis, neudesin expression in MCF7 cells has been identified in promoting invasiveness in cell culturing *in vitro* and tumorigenicity *in vivo* (72). It is believed neudesin promotes tumorigenesis primarily through activation of MAP kinase and PIK3 pathways. To emphasize oncogenic activity, ectopically expressed neudesin in BC cell lines via retroviral transfection methodologies resulted in higher cell proliferation rate (73). Hyperactivity of Neudesin expression is thought to arise as a result of hypomethylated regions within the gene facilitating spatial access for continuous transcription (74). Experimental data suggests knockdown of neudesin via RNA interference supresses cell growth significantly in BC proposing an inhibition effect in AKT and MAP kinase activation (74, 75). Remarkably, neudesin is a novel oncogene and a promising therapeutic target.

PGRMC 1 is a protein co-localized with endoplasmic growth factor receptor (EGFR) in cytoplasmic sites. Interestingly, PGRMC 1 has also been identified at the plasma membrane and in the nucleus of some cell types (76). Extensive research has reported PGRMC 1 overexpression in BC, more specifically in malignancies with resistance to endocrine therapy and in TNBC (76, 77). While its exact mechanism of action is not well understood, it is believed PGRMC 1 may trigger estrogen dependent proliferation in BC cell lines. Previous findings have demonstrated expression of this oncoprotein to promote proliferation of progestogens resulting in contribution to tumorigenesis (76, 78). PGRMC 1 expression is also elevated in ER negative particularly in TNBC. Its hyperactivity is believed to be a requirement for PRs to reduce apoptotic effects of topoisomerase II inhibitor chemotherapeutic agent doxorubicin *in vitro* and to facilitate tumor formation and growth in TNBC *in vivo* (79). Consistently with tumorigenic activity, studies have detected interaction and complex formation of PGRMC 1 and Plasminogen Activator Inhibitor 1 RNA-Binding (PAIRBP1) to serve as a quasi-PR allowing to mediate anti-apoptotic activity in spontaneously immortalized granulosa cells (78, 80). Known for its role in regulating mRNA stability, PAIRBP1 is believed to bind to the C-terminus between amino acid 70-130 of PGRMC 1 (80). Overexpression and hyper-activation of PGRMC 1 may also indicate an increased risk for developing BC in post-menopausal women undergoing hormone therapy where both estrogens and synthetic progestins are administered (81). While the effect of progestins in normal breast tissue remains unclear, it is known that progestins substantially increase cell proliferation in BC particularly in MCF7 overexpressing PGRMC 1; possibly by transducing membrane-initiated progestin signals (79, 81). Progestin norethisterone (NET) is a drug used as a treatment for post-menopausal women containing synthetic progestins (80). It is used in combination with estrogen to prevent endometrial hyperplasia and therefore formation of malignancies caused by the presence of estrogen (82). These progestins are believed to induce phosphorylation of PGRMC 1 at the casein kinase-2 (CK2) phosphorylation site Ser181 in BC cell lines MCF7 and T47D thereby initiating signalling cascade that ultimately results in cell survival and propagation (81, 82). Consequently women receiving both progestins and estrogens as hormonal therapy have a higher risk of developing BC than women receiving solely estrogen.

1.4 Protein CYB5D2

1.4.1 Multifunctional properties of CYB5D2

Cytochrome b5 domain containing 2 (CYB5D2) is a member of the MAPR family and while highly homologous to PGRMC 1,2 (20) CYB5D2 is a secretory protein similarly to neudesin (83). Human CYB5D2 consists of a 264 amino acid protein with a TM domain near n-terminus (residues 1-29) and cytochrome b5 domain near c-terminus (residues 35-134) (83). CYB5D2 was initially identified as neuferricin expressing mostly in the brain of embryonic mice. Postnatal stage revealed further expression in brain, heart, kidney and adrenal glands (83). CYB5D2 was first recognized in attenuating growth and development of Neuro2a cells (83, 84). Evidence suggests down-regulation of CYB5D2 RNAi correlates with an increase in cell survival and cell proliferation in Neuro2a cells followed by increased expression of apoptotic suppressor Bcl-2; however no difference of expression was detected in P53 or Bax (84). Moreover, experimental data indicated that ectopic expression of CYB5D2-RNAi resulted in an increase of cell propagation confirming CYB5D2 stimulates proliferation in Neuro2a cells (84). On the other hand, expression of CYB5D2 promotes neurite differentiation of Neuro2a cells during brain development in embryonic mice (84, 85). *In vitro* studies stimulated neurite differentiation by introducing neural precursor cells with 10% FBS and detected a decreased nestin gene expression, a marker for promoting neural proliferation; and a gradual increased expression of neuron and astrocyte differentiating genes MAP2 and GFAP respectively along with CYB5D2 (85). Consistent with this notion, evidence proposes expression of CYB5D2 in neurons stimulates MAP2 and has no effect on GFAP indicating that CYB5D2 selectively promotes neural differentiation but not of astrocytes (85). Alterations in the heme-binding domain do not seem to stimulate MAP2 expression; thereby suggesting CYB5D2 neurotrophic role requires heme binding (85).

CYB5D2 has also been reported to protect Hela cells from etoposide-induced cytotoxicity by substantially increasing cell survival. In agreement with this, both TM and CYB5 domains are required for Hela cell protection (86). CYB5D2 mechanism of action preventing etoposide-induced poisoning in Hela cells is not well understood; however when analysing DNA damage response (DDR) pathways, CYB5D2 did not seem to have any effect in activating ATM pathway proposing a mechanism of action independently of DDR (85, 86). Further experimental data also indicated that ectopic expression of CYB5D2 in Hela cells does not protect cells from induced apoptosis via UV or TNFa exposure.

Moreover, CYB5D2 has been linked with tumor suppressor capabilities in cervical cancer (87). Consistently with the well-recognized cell growth attenuation effect progesterone produces in Hela cells, experimental data suggests ectopic expression of CYB5D2 together with progesterone administration in Hela cells has no impact on inhibiting cell proliferation (87). On the other hand knocking down CYB5D2 via ShRNA in Hela cells enhances cell invasion. In line with this notion, CYB5D2 expressed in Hela xenograft tumors cells substantially reduces metastatic masses in the lungs in comparison with empty vector tumor cells (87). Arginine (R) in residue 7 and Aspartic acid (D) in residue

86 are essential for TM and heme-binding affinity respectively (87). Alterations of CYB5D2 in the 7th residue (R7P and R7G) prevented any effect in attenuating Hela cell invasion but continued to have high affinity to heme binding thereby suggesting TM domain plays a crucial role in tumor-suppressing effects. On the contrary, ectopic expression of CYB5D2-(D86G) was unable to bind to heme but sustained Hela cell invasion properties (87).

1.4.2 CYB5D2, a novel tumor suppressor in BC

Extensive research has identified deletion of 17p 13.1 P53 allele in most types of BC (85). CYB5D2 is located in chromosome 17p 13.2, a region commonly lost or altered in various cancer types including BC. Evidence suggests down-regulation of CYB5D2 plasy a role in tamoxifen resistant MCF7 cells (83). Interestingly, expression of CYB5D2 is significantly reduced in primary BC tumors compared to healthy breast tissue and in Her2+ or TNBC compared to ER+ (83, 85). Consistent with this notion, down-regulation of CYB5D2 is in relation with mutations in PIK3CA, GATA3, MAP3K1, CDH1, TP53 and RB1 (85). Down-regulation of CYB5D2 significantly correlates with a reduction of overall survival in patients with BC (85). This study focuses on investigating potential tumor suppressor properties of CYB5D2 in BC. Ectopic expression of CYB5D2 in HCC 1954 (HER2+) and MCF7 (luminal A) breast cancer cell lines was established to measure and analyse attenuation of cell proliferation and apoptotic effects. It should be noted that tumor suppressor PTEN is downregulated in most BCs. PTEN acts as a phosphatase protein in the PI3K/AKT pathway by accomplishing dephosphorylation of PIP_3 to PIP_2.

Previous research suggests evidence of CYB5D2-PTEN interaction potentially providing promising knowledge for CYB5D2 possible tumor-suppressing pathways.

1.5 Tet-On Inducible system

Previous efforts in establishing MCF7 stable line ectopically expressing CYB5D2 have been unsuccessful. We suspect tumor suppressor effects of CYB5D2 overexpression diminishes cell count significantly preventing cell colonies to survive. Alternatively, we have established a Tet-On inducible line expressing CYB5D2 in HCC 1954 and MCF7 cell lines. *Escherichia Coli* expresses tet repressor protein (TetR), acting as an inhibitor of the tetracycline-resistance gene in the absence of tetracycline by blocking the tet operon sequence (tetO) (88). In the interest of establishing the inducible system, transcriptional repressor is reversed into a transcriptional activator. This is accomplished by fusing tetR with the herpes simplex virus VP16 activation domain forming the tetracycline-controlled transactivator (tTA) able to activate transcription in the absence of tetracycline (88, 89). In the Tet-On system, tetR is altered thereby reversing its effect (rtetR). rtetR combined with VP16 forms the rtTA (reverse tetracycline transactivator) domain, activating gene of interest in the presence of tetracycline (90). Tet-On inducible system is composed of two vectors: first vector consists of the rtTA domain activated by a CMV promoter; second vector contains the gene of interest under the control of tetracycline response element (TRE) comprised of a tetO (90, 91). The tetO is located upstream of minimal CMV promoter (P_{min}CMV) lacking strong enhancers to avoid gene expression without the presence of rtTA (Fig. 1). Together, these two vectors maintain an effective and tightly regulated gene expression allowing us to evaluate the tumor suppressor role of CYB5D2 in breast cancer cell lines Hcc 1954 and MCF7. Since doxycycline is 100-fold more potent than tetracycline (92, 93), we used doxycycline for all experiments related to tet-On inducible lines.

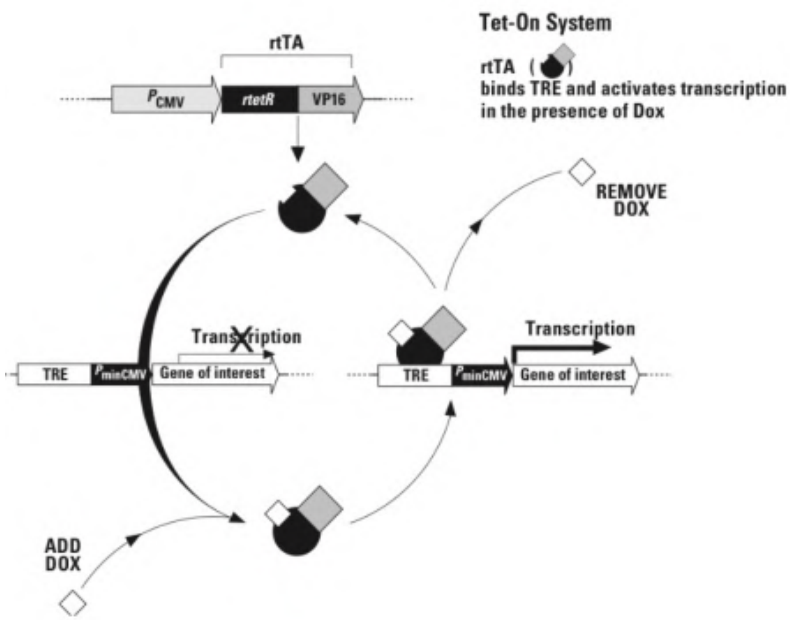

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Figure 1. Representation of Tet-On inducible system construct.

First vector pTet-On consists of rtTA domain from rtetR-VP16 complex. Second vector pTRE contains gene of interest (CYB5D2). Both vectors are integrated with CMV promoters downstream of transcriptional DNA. Doxycycline exposure to Tet-On system co-transfected stable line initiates induction of gene expression by binding and activating rtTA. Activated rtTA then binds to pTRE promoter thereby initiating transcription of the gene of interest. The removal of doxycycline deactivates rtTA and gene transcription stops.

1.6 Primary hypothesis, specific hypothesis and main objectives

1.6.1 Thesis question

Does CYB5D2 protein assist in attenuating tumor progression in BC cell lines HCC 1954 and MCF7?

1.6.2 Central Hypothesis

Recent research has reported substantial evidence of CYB5D2 stimulating tumor suppressor properties in cervical cancer. We are now shifting our interest to BC and suspect CYB5D2 elicits tumor suppressor activities by halting cell growth or inducing apoptosis.

1.6.3 Objectives

Aim #1

- Establish tumor-suppressing effect of ectopically expressed CYB5D2 in HCC 1954 and MCF7 cell lines via transient transfection methods.

Aim #2

- Construct Tet-On inducible system expressing CYB5D2 in BC cell lines to quantify mitigated cell propagation effect upon doxycycline induction in HCC 1954 and MCF7 cell lines.

Aim #3

- Identify potential apoptotic effect of ectopically expressed CYB5D2 in HCC 1954 and MCF7 cell lines via transient transfection and gene regulation. Evidence of cell death may suggest possible pathway of potential tumor suppressor CYB5D2.

.**Aim #4**

- Perform cell cycle analysis in HCC 1954 and MCF7 ectopically expressing CYB5D2 to identify variations in cell cycle.

Aim #5

- Construct HCC 1954 and MCF7 stable lines silencing CYB5D2 expression.

Aim #6

- Determine whether molecular protein-to-protein interaction exists between CYB5D2 and PTEN.

CHAPTER 2: MATERIALS AND METHODS

2.1 In vitro cell manipulation

2.1.1 Cell culture

HCC 1954, MCF7 and 293T cells were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (HCC 1954) and DMEM (MCF7 and 293T) media supplemented with 10% FBS (Sigma Aldrich) and 1% Penicillin-Streptomycin (Thermo Scientific Fisher).

2.1.2 CYB5D2 transient expression

Hcc 1954 and MCF7 cells were transiently transfected with retrovirus-based PLHCX vector subcloned with GFP-CYB5D2 or PLHCX GFP vector. Polyjet 3000 In Vitro DNA Transfection Reagent (SignaGen Laboratories) kit was used for viral infection according to the manufacturer's instructions.

2.1.3 CYB5D2 – Tet-On inducible stable line construct

Production of Tet-On inducible lines expressing CYB5D2 in HCC 1954 and MCF7 consisted of two consecutive retroviral transfections. First stable line was initiated via transfection of vector pRev Tet-On or empty vector pRev using a calcium phosphate transfection protocol in 293T cells. Transfection cocktail consisted of 10µg of Vesicular stomatitis virus glycoprotein (VSV-G) and 10µg of gag-pol (GP) viral packaging proteins, 10µg of desired DNA plasmid, 0.22- µm filtered 50µl of 250Mm $CaCl_2$ totalling solution to 500µl with autoclaved ddH_2O and complementing it to 500µl of 2x HePES buffer. Transfection cocktail was then introduced to 293T cells at approximately 30% confluency cultured in 100mm tissue culture plate. Viral load was harvested 48 hours post-transfection by collecting supernatant from 293T and aliquot viral concentration in HCC 1954 and MCF7 cells for viral infection. pRev Tet-On plasmid contains the Neomycin resistant gene thus selection was accomplished with neomycin (800µg/ml for MCF7 and 600µg/ml for HCC 1954). Following a two-week period of antibiotic selection, second stable transfection followed the same aforementioned retroviral method introducing the pRev TRE vector encoding for CYB5D2, green fluorescent protein (GFP) and Hygromycin resistant gene or the pRev TRE empty vector. Hygromycin B was used for selection (200µg/ml for both cell lines). Additionally, single cell clones from stable lines were each seeded in 96-well tissue culture plate. Emerging colonies were then trypsinized and seeded in 24-well tissue culture plate to stimulate further proliferation. At approximately 50% confluency, cells were induced with 2µg/ml of doxycycline to measure and compare CYB5D2 expression via GFP intensity. Single clones were also compared on basal CYB5D2 expression in the absence of doxycycline. It should be noted that stable cell lines exhibiting highest GFP intensity upon doxycycline induction and least GFP intensity in the absence of doxycycline were selected to measure CYB5D2 tumor suppression effects in HCC 1954 and MCF7.

2.1.4 Knockdown of CYB5D2

Hcc 1954 and MCF7 cells were transfected with lentivirus-based shCTRL (control) or shCYB5D2 (a pool of three individual knockdown constructs; Santa Cruz). Aforementioned stable-line transfection protocol was performed utilizing 293T cells to assemble viral load using VSV-G, GP and REV packaging proteins in pMD2 vector. Transfected lines were selected with puromycin 48 hours post-transfection for 7 days.

2.1.5 Colony Formation Assay

CYB5D2 knockdown and Tet-On inducible cell lines were seeded in 6-well tissue culture plate at $1X10^3$/well. Cells were incubated for two weeks at 37°C and 5% CO_2 changing fresh media every 2-3 days. Cells were fixed in a solution containing 2% formaldehyde, 0.2% glutaraldehyde for 20 minutes. Subsequently, crystal violet solution (0.5% crystal violet, 20% methanol, 150mM NaCl) was added for 30 minutes. Wells were then rinsed with water before quantifying and taking images of formed colonies.

2.1.6 Cell Proliferation Assay

Cells were seeded at $5X10^4$/well in 6-well tissue culture plate followed by 18 days of incubation (37°C and 5% CO_2) changing media every 2-3 days. For cell proliferation and colony formation assays, Tet-On – CYB5D2 inducible cell lines were exposed with 2μg/ml of doxycycline every 3 days followed by cell count using the hemocytometer technique.

2.1.7 Cell death Assay

HCC 1954 and MCF7 cells were seeded in chamber slides and transiently transfected with PLHCX – GFP-CYB5D2 or PLHCX – GFP for 48 hours. TUNEL assay was then performed using the In Situ Red TUNEL Kit (Abcam) according to the manufacturer’s protocol. Enzyme terminal deoxynucleotidyl transferase (TdT) was exposed to add tagged BrdUTP (5-Bromo-2´-Deoxyuridine 5´-Triphosphate) at end of fragmented DNA strands. Anti-Brdu-Red antibody was then introduced to detect tagged dUTP-DNA complex followed by counterstaining with DAPI. Detection of apoptotic cells was accomplished via fluorescence microscopy. Detection of apoptosis was further investigated in HCC 1954 and MCF7 Tet-On – CYB5D2 – GFP inducible lines expressing novel tumor suppressor.

2.1.8 Cell Cycle Analysis

HCC 1954 ectopically expressing CYB5D2 via doxycycline induction was washed using 1x PBS and disassociated from 100mm tissue culture plate using 10% trypsin solution. Fresh RPMI 1640 media was then added. Separation of cells was accomplished by Pipetting up and down repeatedly followed by collecting cells into 15ml tube and isolating cells by centrifugation at 2000 rpm for 5 minutes at 4°C. Supernatant was discarded and 1xPBS, 2%FBS was introduced to re-suspend wash and isolate cells via centrifugation; repeating washing step a total of three times. Subsequently cells were resuspended with prepared Propidium Iodide (PI) solution containing 10µl RNase A per 1ml of solution and incubated at room temperature for 20 minutes in the dark. Final prepared solution was used for cell cycle analysis via flow cytometry according to the manufacturer's protocol. Raw output data was analysed using FloJo software quantifying separated peak densities in cell growth cycle.

2.1.9 Western blot analysis

Cells were concentrated and lysed in solution consisting of 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin (Sigma Aldrich, Oakville, ON). 50μg of protein from total cell lysate was separated on an SDS-PAGE and transferred onto Amersham hybond ECL nitrocellulose membranes (Amersham, Baie d'Urfe, QC). Membranes were blocked with 5% skim milk for one hour at room temperature followed by incubation with anti-CYB5D2 (Abcam, 1:1000) or anti-actin (Santa Cruz, 1:1000) overnight at 4°C. Signal was then developed by incubating membrane with corresponding horseradish peroxidase-conjugated secondary antibody and an ECL Western Blotting Kit (Amersham, Baie d'Urfe, QC). Protein band intensity was quantified via ImageJ software (National Institutes of Health).

The primary and secondary antibodies and the concentrations used were:

anti-CYB5D2 (Abcam, 1:1000)

β- actin (1:1000; Santa Cruz)

α- tubulin (1:1000; Santa Cruz)

M2- flag (1:1000; Sigma Aldrich)

anti- mouse IgG (1:3000; Bio Rad)

anti-goat IgG (1: 3000; Bio Rad)

2.2.1 Co-immunoprecipitation (Co-Ip) of CYB5D2 and PTEN

rProtein G Agarose beads (Invitrogen) were prepared for Co-Ip by precipitating beads at 2000 rpm and 4°C for 5 minutes followed by re-suspending beads in Co-Ip buffer consisting of 50mM Tris-Cl pH 7.5, 150 mM NaCl, 1mM EDTA and 0.1% Triton x-100. Co-Ip cocktail (Co-Ip buffer, rProtein G agarose beads, 2mg of protein from total cell lysate, and either 1µg of antibody of interest or mouse IgG as control) was incubated overnight at 4°C. Beads were washed by centrifugation and re-suspension in Co-Ip buffer a total of 8 times followed by re-suspending final pellet in 2X protein sample buffer (PSB) and heating it at 100°C for 3. Detection of CYB5D2 or PTEN was accomplished using via blot analysis.

2.2.2 Statistical Analysis

The two-tailed Student's t-test was used for statistical analysis. A value of $p<0.05$ is considered statistically significant. Data are presented as mean ± SEM.

CHAPTER 3: RESULTS

3.1 Transient transfection of CYB5D2 supresses cell proliferation in HCC 1954 and MCF7

Preliminary research strongly supports CYB5D2 as a tumor suppressor in cervical cancer. CYB5D2 resides in chromosome 17p13.2, a region commonly lost in BC (85). Previous analysis has revealed downregulation of mRNA expression in BC compared to normal breast tissues exposing CYB5D2 as a tumor suppressor candidate for BC. HCC 1954 and MCF7 cells transiently transfected with CYB5D2 displayed attenuated cell propagation. Images through microscopy were captured two days post-transfection showing approximately 30% confluency in HCC 1954 – GFP and 15% in HCC 1954 – GFP-CYB5D2. HCC 1954 expressing ectopic GFP reached 100% confluency by day five; whereas HCC 1954 transfected with CYB5D2 was only 40% confluent (Fig. 2A). Both MCF7 – GFP and MCF7 – GFP-CYB5D2 displayed approximately 40% confluency two days post-transfection. However, by day five MCF7 – GFP was 90% confluent while MCF7 – GFP-CYB5D2 was only 65% (Fig. 2B). Interestingly, cells continued to exhibit mitigated cell propagation post-transient expression suggesting CYB5D2 may act as a secretory protein to supress cell proliferation.

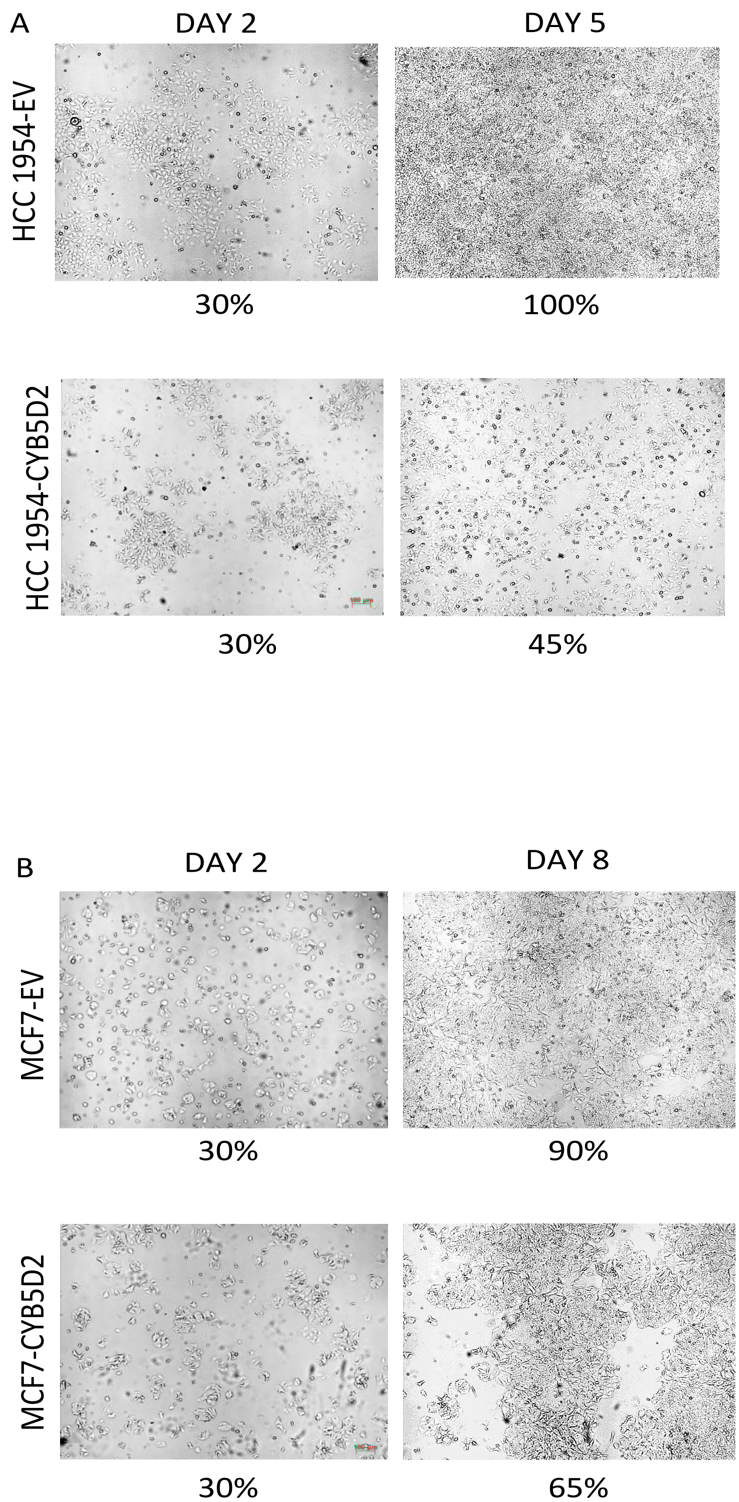
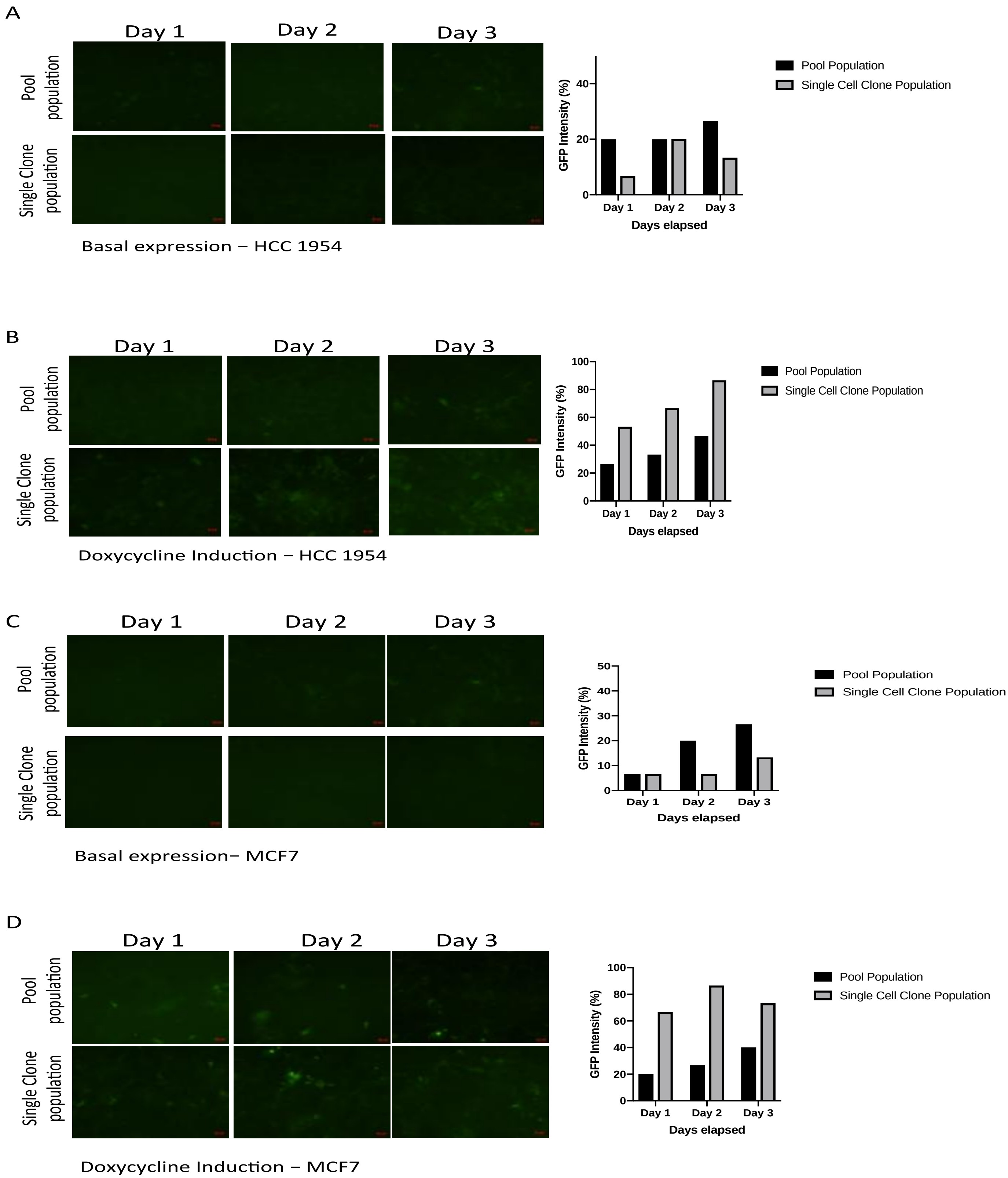

A
DAY 2
DAY 5
HCC 1954-EV
30%
100%
HCC 1954-CYB5D2
30%
45%
B
DAY 2
DAY 8
MCF7-EV
30%
90%
MCF7-CYB5D2
30%
65%

Figure 2. Ectopic CYB5D2 expression attenuated cell growth in HCC 1954 and MCF7 BC cell lines.

At approximately 30% confluency, cells were transiently transfected with GFP-CYB5D2 or GFP using the Polyjet 3000 In Vitro DNA Transfection Reagent (SignaGen Laboratories). Transfection was confirmed via fluorescence microscopy analyzing GFP intensity (data not shown). Cell propagation was monitored each day post transfection. (A) HCC 1954 – EV proliferated to full confluency by day 5. HCC 1954 – CYB5D2 reached 40% confluency by day 5. (B) MCF7 – EV cells reached approximately 90% confluency where as MCF7 – CYB5D2 merely reached 65% by day 8.

3.2 Tet-On – CYB5D2 construct

Recent attempts of generating stable expression of CYB5D2 in MCF7 cells have been unsuccessful on account of suspected CYB5D2 tumor suppressor effects. Alternatively we have decided to generate a Tet-On inducible system activating CYB5D2 expression in the presence of doxycycline. Because the Tet-On system requires consecutive transfections of two vectors (pTet-On and pTRE), there have been reports of weak gene expression and basal expression in the presence and absence of doxycycline respectively. In pursuance of producing a higher efficacy and accuracy for the Tet-On system, single cell clones of Tet-On inducible lines were seeded in 96-well tissue culture plate. 12 and 7 wells formed colonies in the HCC 1954 and MCF7 inducible lines respectively (data not shown). Formed colonies were then transferred into 24-well tissue culture plate where proliferation continued. Eight colonies survived in HCC 1954 single clone population allowing colonies to be induced with doxycycline at approximately 40% confluency (data not shown). Five colonies of MCF7 single cell clones were selected for measuring induction and basal expression (data not shown). Single cell clone population producing highest inducibility rate while maintaining minimal basal expression were selected and compared to pool population (Fig. 3A, B, C, D). Comparison of single cell clone to pool population revealed noticeable distinctions yielding higher inducibility and lower basal expression in single clone population. Identifying CYB5D2 in selected Single clone population was then detected via western blot analysis for optimal concentration of doxycycline yielding highest inducibility rate while showing no sign of cytotoxicity. Lysate was collected two days post-induction. Whereas HCC 1954–Tet-On-CYB5D2 displayed highest CYB5D2 expression at 2μg/ml of doxycycline (Fig. 3E), MCF7 Tet-On-CYB5D2 showed highest CYBD2 expression to be at 6μg/ml with no noticeable evidence of cytotoxicity (Fig. 3G). It is important to note doxycycline concentration 10μg/ml did not have a significant effect on CYB5D2 expression in HCC 1954 compared to 2μg/ml; it did however have a negative effect in MCF7 reducing expression. While this report used 2μg/ml as the standard concentration for doxycycline induction in Tet-On lines, concentrations of 6μg/ml to induce CYB5D2 was further investigated in MCF7 inducible line and compared for potential variable growth supressing effects. Moreover Tet-On lines were induced once followed by collection of each lysate per day to detect CYB5D2 for a total of five days. It is paramount to note CYB5D2 continued to be actively present five days post-doxycycline induction in both HCC 1954 and MCF7 cell lines (Fig. 3F,H).


A
Day 1
Day 2
Day 3
Pool population
Single Clone population
GFP Intensity (%)
Days elapsed
Pool Population
Single Cell Clone Population
Basal expression – HCC 1954
B
Day 1
Day 2
Day 3
Pool population
Single Clone population
GFP Intensity (%)
Days elapsed
Pool Population
Single Cell Clone Population
Doxycycline Induction – HCC 1954
C
Day 1
Day 2
Day 3
Pool population
Single Clone population
GFP Intensity (%)
Days elapsed
Pool Population
Single Cell Clone Population
Basal expression– MCF7
D
Day 1
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Single Cell Clone Population
Doxycycline Induction – MCF7

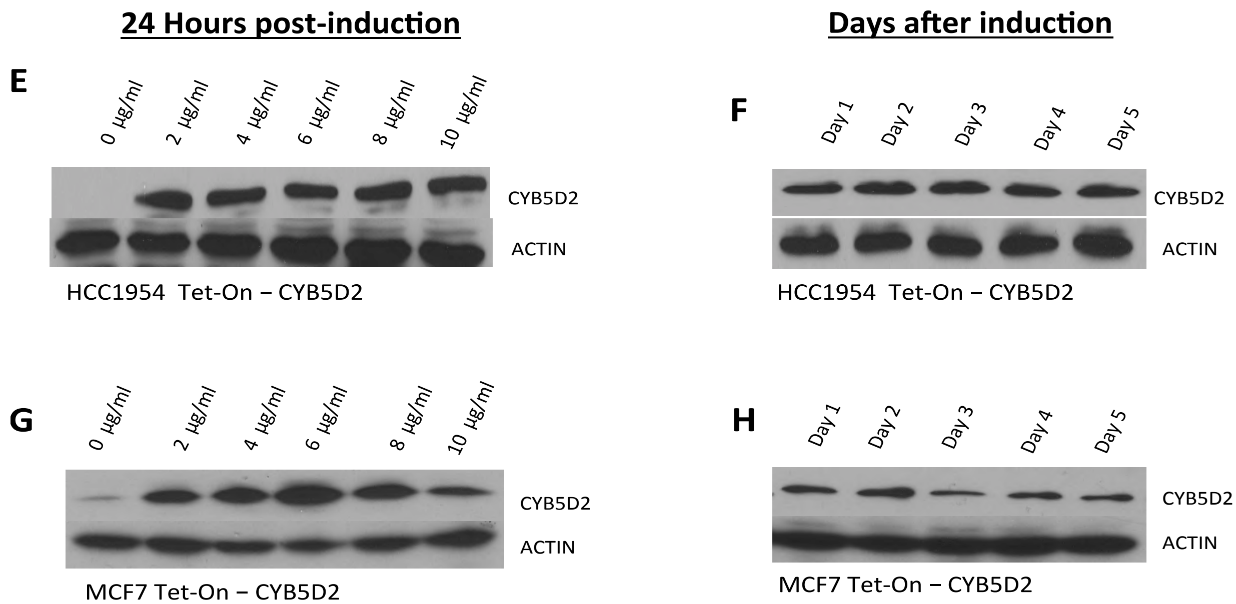
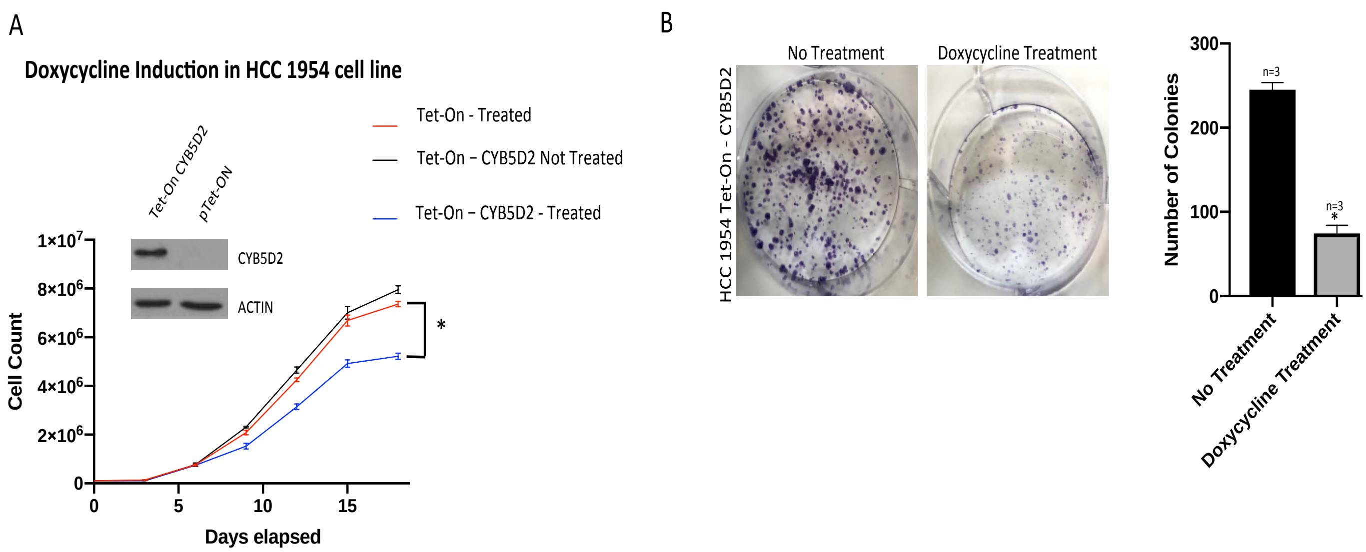


Figure 3. Single cell clones of Tet-On – GFP-CYB5D2 construct elicits higher inducibility rate while maintaining lower basal expression compared to pool population.

Various concentration of doxycycline in Tet-On constructs revealed optimal dose. Cells with Tet-On constructs were only induced with doxycycline 24 hours after being seeded in 6-well tissue culture plates. CYB5D2 continued to be active after five days post-induction. Single cell clones of the Tet-On construct were seeded in 96-well tissue culture plate. Emerging single cell cloned colonies were then transferred onto 24-well tissue culture plate and induced with 2µg/ml of doxycycline. Basal expression of GFP-CYB5D2 was also observed and compared to pool population (control) for 3 days. Inducibility was confirmed and analyzed using fluorescence microscopy for GFP detection. (A) HCC 1954 single cell clone Tet-On displays higher inducibility and (B) lower basal expression than pool population. The same is true for MCF7 single clone Tet-On (C) inducibility and (D) basal expression. Tet-On construct cells were exposed with different concentrations of doxycycline varying from 0μg/ml to 10μg/ml. CYB5D2 expression was then measured with western blot analysis 48 hours after induction. Presence of CYB5D2 after 5 days of 2μg/ml of Doxycycline was observed via western blot analysis. (E) HCC 1954 Tet-On construct exhibited similar intensity of CYB5D2 expression when induced with doxycycline ranging from 2μg/ml to 10μg/ml. (F) Presence of CYB5D2 continued after 5 days of induction. (G) MCF7 Tet-On construct revealed a peak of CYB5D2 expression when induced with 6μg/ml of Doxycycline. Cells induced with 10μg/ml of Doxycycline reveal minimal expression. (H) MCF7 reveals presence of CYB5D2 5 days post-induction.

3.3 Characterization of CYB5D2-derived inhibition of cell proliferation in HCC 1954 and MCF7

With the availability of tet-inducible HCC1954 tet-CYB5D2 and MCF7 tet-CYB5D2 lines, I have characterized the inhibitory effects of CYB5D2 on the proliferation of HCC1954 and MCF7 cells. HCC 1954 tet-CYB5D2 cells were induced for CYB5D2 expression by doxycycline (DOX, 2 µg/ml) (Fig 4A, inset). Induction of CYB5D2 significantly reduced HCC1954 tet- CYB5D2 cell proliferation in comparison to the cell proliferation profile without CYB5D2 induction (Fig 4A). The reduction in cell number was not caused by DOX, as HCC1954 tet-on (negative control without ectopic expression of CYB5D2) cells displayed an identical proliferation profile in the presence of DOX (2 µg/ml) as HCC1954 tet-CYB5D2 cells without CYB5D2 induction (Fig 4A). To further examine CYB5D2-derived inhibition of HCC1954 cell proliferation, I have performed colony formation assay. HCC1954 tet-CYB5D2 cells were seeded at a low density and cultured for 15 days in the presence and absence of DOX. The induction of CYB5D2 with DOX significantly reduced cell colony numbers (Fig 4B). Similar results were also obtained in MCF7 tet-CYB5D2 lines. In comparison to MCF7 tet-on and MCF7 tet-CYB5D2 lines, induction of CYB5D2 expression significantly inhibited MCF7 cell proliferation (Fig 4C) and colony formation (Fig 4D). Collectively, these results demonstrate that CYB5D2 inhibits the proliferation of HCC1954 HER2+ and MCF7 ER+ BC cells. Since DOX at 6 µg/ml induces peak induction of CYB5D2 in MCF7 tet-CYB5D2 cells (Fig 3G), I have determined the CYB5D2 inhibitory effects under this condition. As expected, DOX at 6 µg/ml substantially reduced the number of colonies formed in MCF7 tet-CYB5D2 cells and addition of DOX at this dose to MCF7 tet-on cells produced comparable number of colonies as MCF7 tet-CYB5D2 cells in the absence of DOX (Fig 4E), suggesting that DOX at 6 μg/ml impacts MCF7 cell proliferation through induction of CYB5D2 but not its potential cytotoxic effects. Additionally, in comparison to the inhibitory effects of MCF7 tet-CYB5D2 cells treated with DOX at 2 μg/ml (Fig 4D), DOX at 6 μg/ml does not appear to significantly elevate the inhibition of colony formation of MCF7 tet-CYB5D2 cells (Fig 4E). In this regard, I have chosen to use DOX at 2 μg/ml to investigate CYB5D2's effects on in vitro oncogenic events of MCF7 cells.



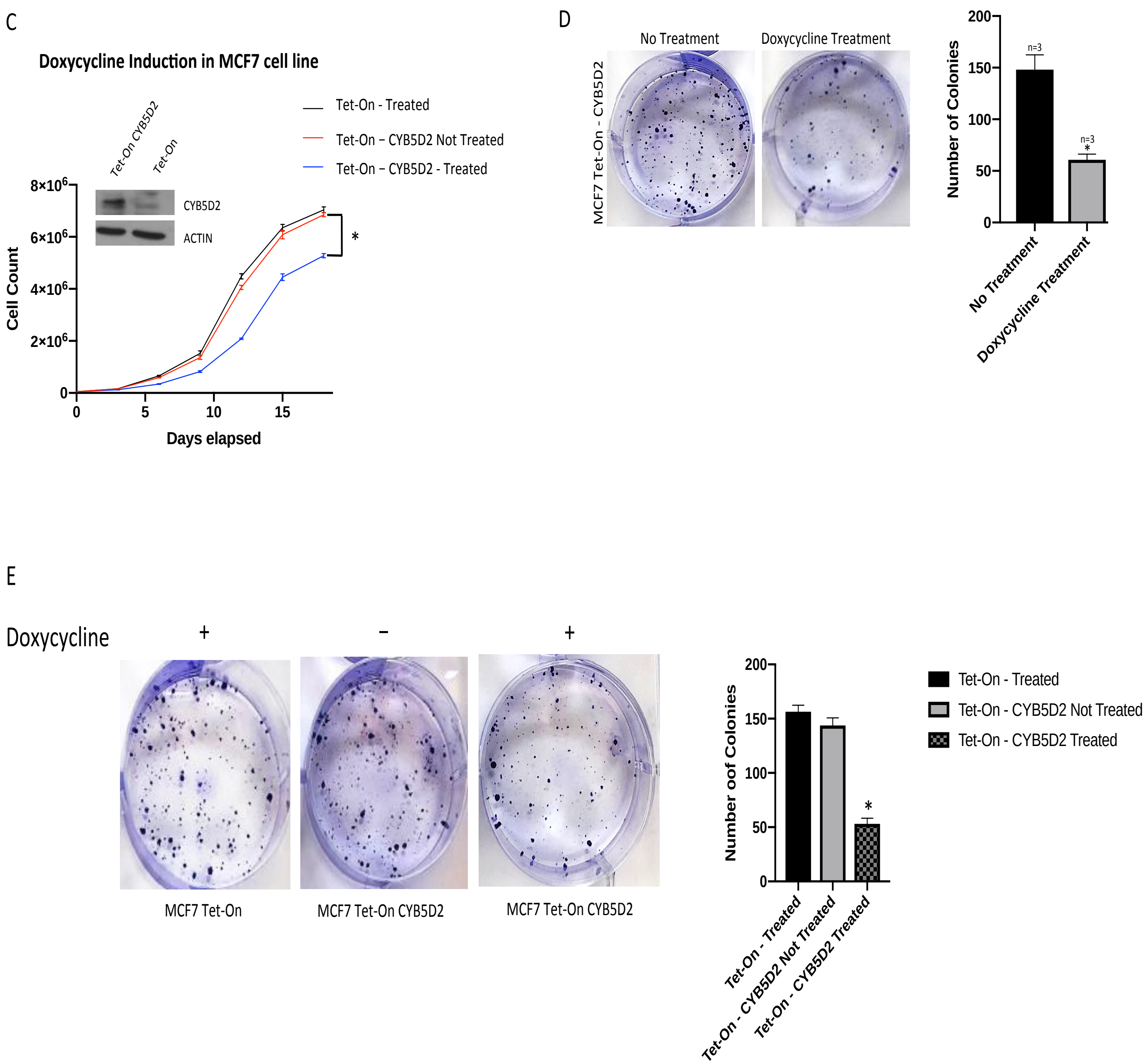
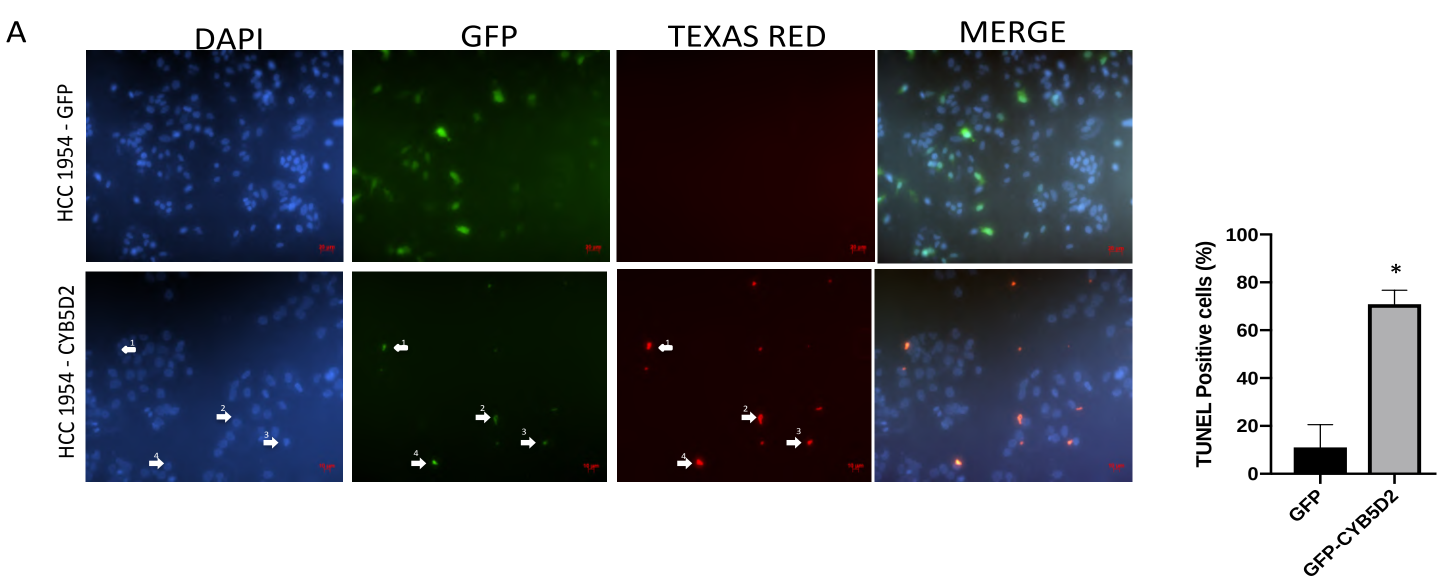


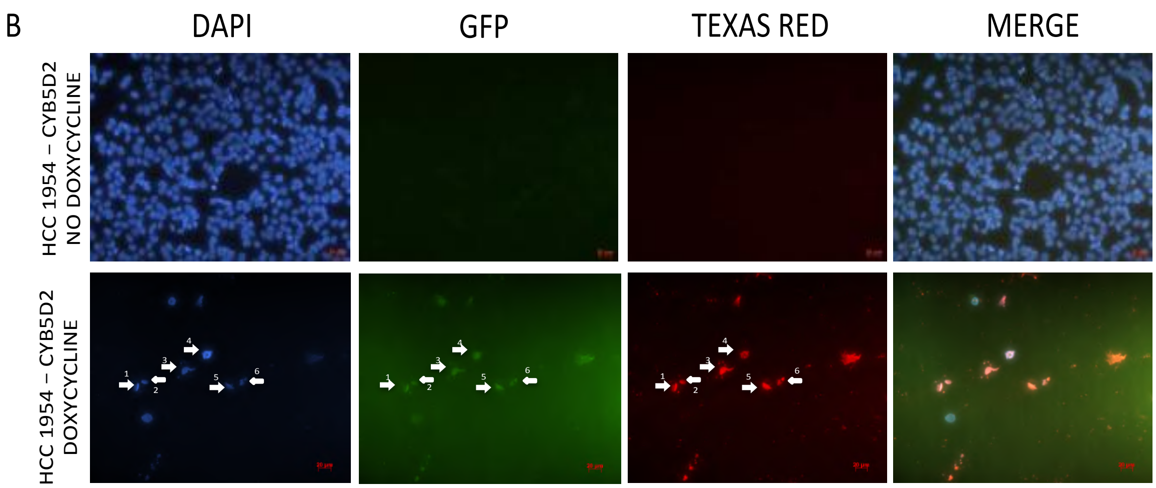
Figure 4. Induction of CYB5D2 expression inhibits the proliferation of HCC 1954 tet-CYB5D2 and MCF7 tet-CYB5D2 cells.

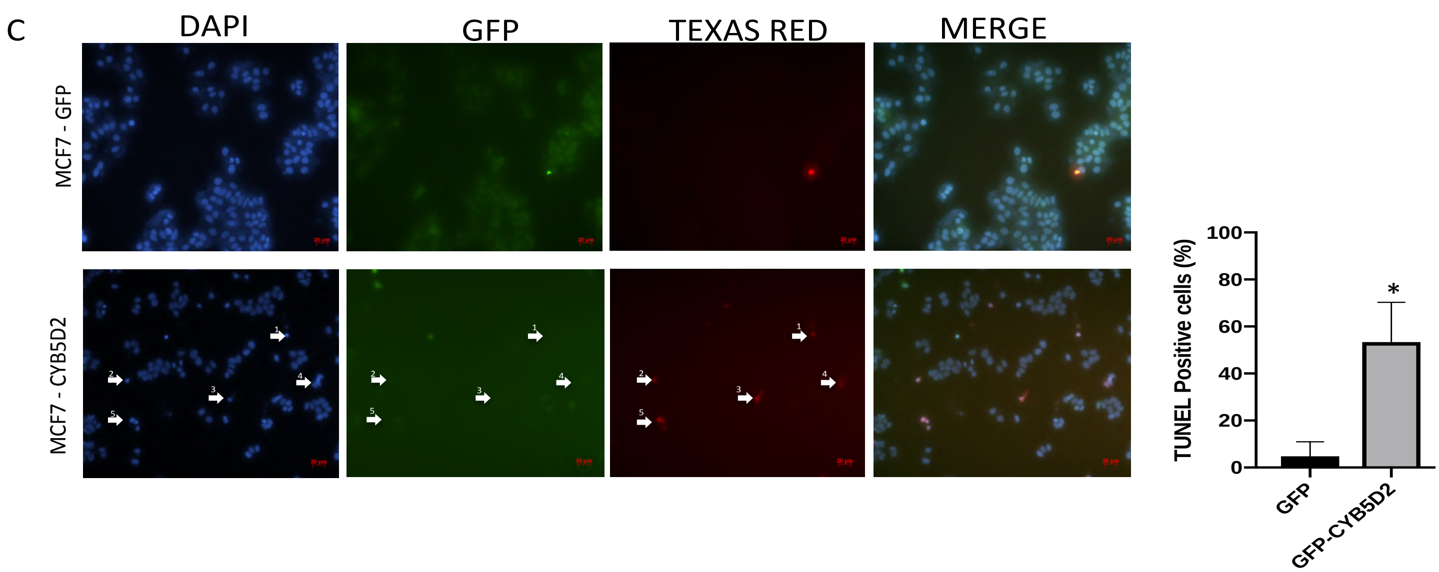
(A) HCC1954 tet-on and HCC1954 tet-CYB5D2 cells were seeded in 6-well tissue culture plate at $5x10^4$ /well and cultured in the presence or absence of DOX (2µg/ml) as indicated. CYB5D2 induction was confirmed by Western blot (inset). Cells were cultured for 18 days with cell numbers counted every 3 days. Experiments were repeated 3 times; means ± SD are graphed. Statistical analyses were performed using 2-way ANOVA for the curves of HCC1954 tet- CYB5D2 cells with and without DOX. *p<0.05. (B) HCC1954 tet-CYB5D2 cells were seeded in 6-well tissue culture plate at 10^3 /well and cultured for two weeks with and without DOX (2μg/ml). Colonies were stained by crystal violet; the numbers of colonies were recorded and presented in Appendix 1. Experiments were repeated 3 times; typical image from the same repeated were shown; means ± SD (-158±8.165) are graphed. Symbol * indicates p<0.05 when compared to control (two-tailed Student's t-test). (C) Growth curves of MCF7 tet-on, MCF7 tet-CYB5D2 cells were generated at the same conditions as the counterparts of HCC1954 cells. (-96 ± 3.27) (D) Colony formation of MCF7 tet-CYB5D2 cells following the same condition as described in panel B. Colony numbers are presented in Appendix 1. (E) MCF7 tet-on and MCF7 tet-CYB5D2 cells were seeded at 10^3 /well in 6-well tissue culture plates and cultured for two weeks with and without DOX (6μg/ml) as indicated. Experiments were repeated 3 times; typical image from the same repeated were shown; means ± SD are graphed. Symbol * indicates p<0.05 when compared to control (two-tailed Student's t-test).

3.4 CYB5D2 induces apoptosis in HCC 1954 and MCF7 cells

A potential mechanism for CYB5D2 to inhibit HCC1954 cell proliferation is through induction of apoptosis. To examine this possibility, HCC1954 cells were transiently transfected with either GFP or CYB5D2-IRES-GFP plasmids (illustrated in Appendix 4); TUNEL-positive cells were significantly increased in cells transfected with the CYB5D2 plasmid for 24 hours (Fig 5A). Additionally, induction of CYB5D2 in HCC1954 tet-CYB5D2 cells with DOX (2 µg/ml) also increased cells positive for TUNEL (Fig 5B), supporting the possibility of CYB5D2-mediated apoptosis in HCC1954 cells. While evidence also suggests apoptosis upon transient expression of CYB5D2 in MCF7 cells (5C) or induction of CYB5D2 in MCF7 tet-CYB5D2 cells (5D), the process was not as robust as in HCC1954 cells as MCFC7 cells exhibited less evident signs of apoptosis. Nonetheless, evidence supports that CYB5D2 is capable of inducing apoptosis in BC cells.




B
DAPI
GFP
TEXAS RED
MERGE
HCC 1954 – CYB5D2
NO DOXYCYCLINE
HCC 1954 – CYB5D2
DOXYCYCLINE


C
DAPI
GFP
TEXAS RED
MERGE
MCF7 - GFP
MCF7 - CYB5D2
TUNEL Positive cells (%)
100
80
60
40
20
0
*
GFP
GFP-CYB5D2

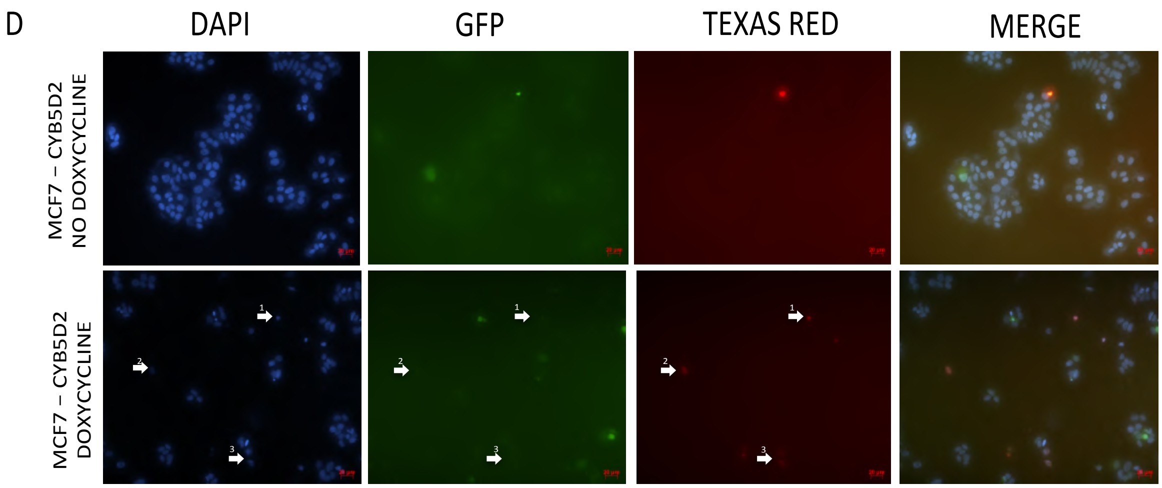


Figure 5. CYB5D2 induces apoptosis in HCC 1954 cells

(A) HCC1954 and (C) MCF7 cells were transiently transfected with either GFP or GFP-CYB5D2; TUNEL assay was performed 24 hours post transfection with nuclei being counter-stained with DAPI. Cells positive for GFP and TUNEL (TEXAS RED) were imaged, counted, and analyzed. Experiments were repeated 3 times; typical image from the same repeated were shown; means ± SD are graphed. Symbol * indicates $p<0.05$ when compared to control (two-tailed Student's t- test illustrated in Appendix 2). Arrows indicate apoptotic cells. (B) HCC1954 tet-CYB5D2 and (D) MCF7 tet-CYB5D2 cells were induced for CYB5D2 expression for 24 hours, followed by TUNEL analysis. Nuclei were counter-stained with DAPI. Successful induction of CYB5D2 is indicated by expression of GFP (GFP is translated via IRES in the configuration of CYB5D2-IRES-GFP illustrated in Appendix 5).

3.5 CYB5D2 induces cell cycle arrest in HCC1954 cells

Another mechanism contributing to CYB5D2-induced inhibition of HCC1954 cell proliferation is via delay cell cycle progression. To elucidate on this notion, HCC1954 tet-CYB5D2 cells were induced for CYB5D2 expression with DOX (2 μg/ml) for 24 hours; cell cycle distribution was then determined using flow cytometry. In comparison to non-stimulated cells, induction of CYB5D2 slightly but significantly increased cells in G1 phase with a concurrent reduction of cells in G2/M phase (Fig 6), indicating that CYB5D2 attenuates G1 phase progression in HCC1954 cells.

A

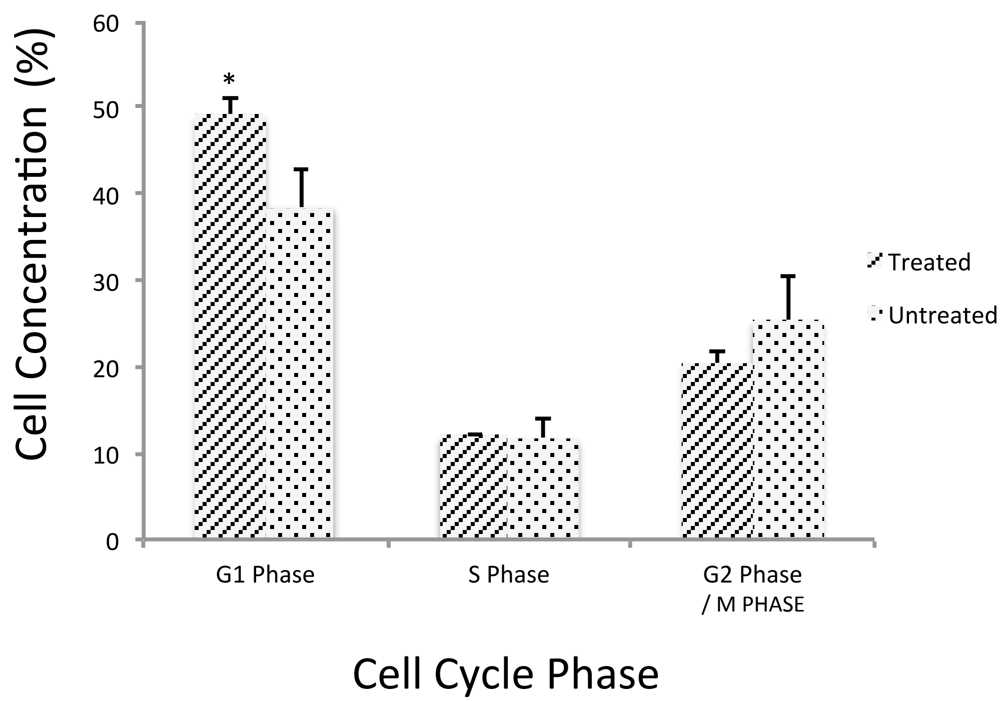


Figure 6. CYB5D2 expression decreases progression of cell cycle growth in HCC 1954 BC cell line.

CYB5D2 induces G1 arrest in HCC 1954 cells HCC 1954Tet-CYB5D2 cells were introduced with 2μg/ml of doxycycline. At 24 hours of post- doxycycline exposure, cells were collected, washed, and stained with PI solution containing 10μl of RNase A per 1ml of solution. Cell cycle distributions were determined by flowcytometry and analyzed with FlowJo software analysis. Experiments were repeated 3 times; means ± SD for the indicated cell phases are graphed. Symbol * indicates $p<0.05$ in comparison to un-stimulated cells by two-tailed Student's test demonstrated in Appendix 3.

3.6 Knockdown of CYB5D2 increases cell proliferation in HCC 1954 and MCF7

It becomes clear that ectopic CYB5D2 expression promotes inhibition of tumor cell propagation via possible combination of apoptotic pathways and cell growth mitigation. To further sustain existing evidence, we then analysed effects of endogenous CYB5D2 expression in HCC 1954 and MCF7. HCC1954 cells stably expressing shCYB5D2 or shCtrl (control) were established; knockdown of CYB5D2 in HCC1954 shCYB5D2 line was confirmed by western blot analysis (Fig 7A, inset). In comparison to HCC1954 shCtrl cells, HCC1954 shCYB5D2 cells exhibit elevations in cell proliferation (Fig 7A) and colony formation capacity (Fig 7B). We also generated stable MCF7 shCtrl and MCF7 shCYB5D2 lines with the expected knockdown confirmed (Fig 7C, inset). Knockdown of CYB5B2 also enhanced MCF7 cell proliferation and the cell ability of forming colonies (Fig 7D). In comparison to HCC1954 cells, the effects of CYB5D2 knockdown on colony formation in MCF7 cells were not dramatic (see Chapter 4 Discussion for details). Collectively, evidence supports that the endogenous CYB5D2 possesses inhibitory effects on HCC1954 and MCF7 cell proliferation.

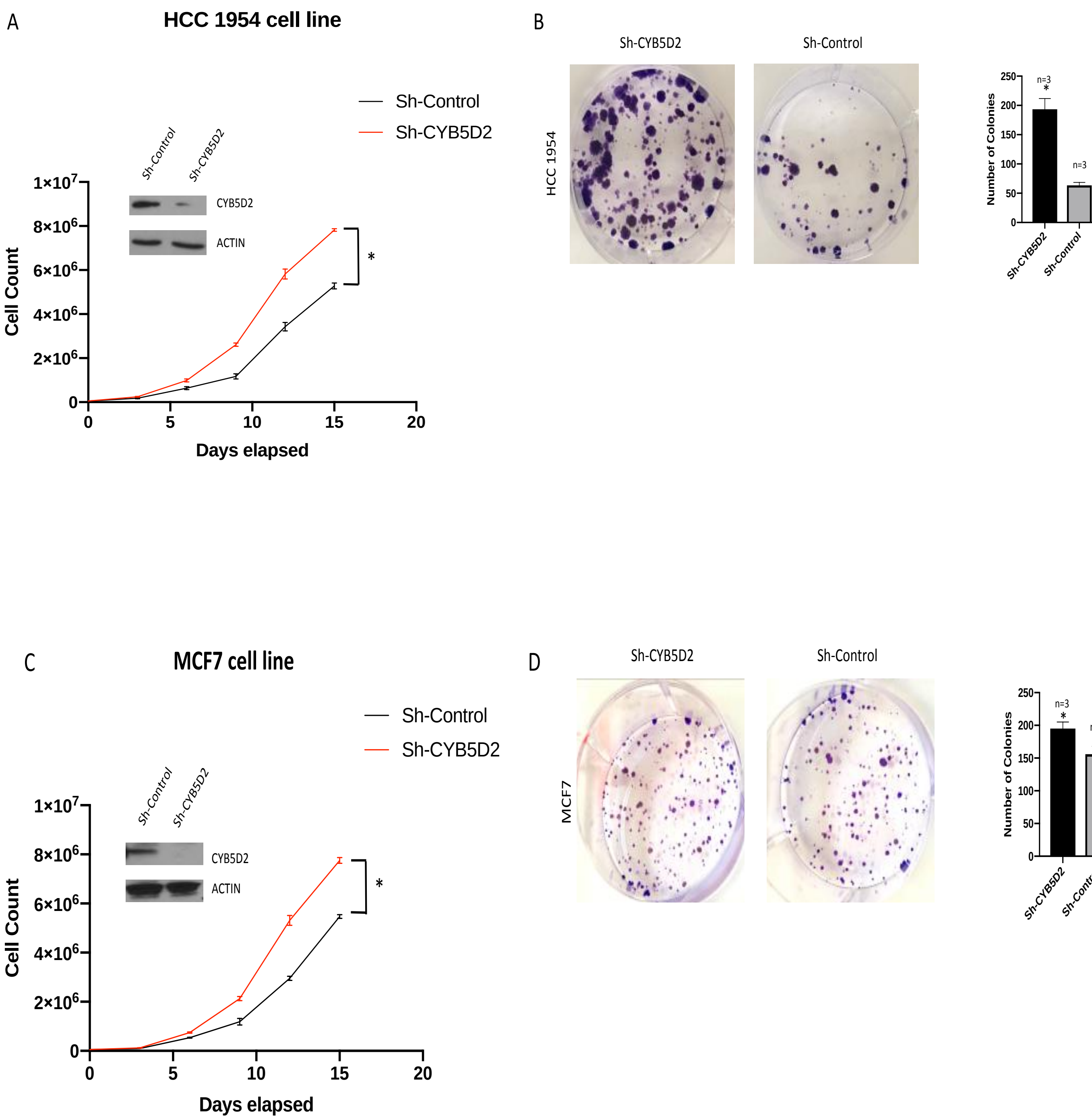

A
HCC 1954 cell line
Sh-Control
Sh-CYB5D2
Sh-Control
Sh-CYB5D2
CYB5D2
ACTIN
*
Cell Count
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
0
5
10
15
20
Days elapsed
B
Sh-CYB5D2
Sh-Control
HCC 1954
Number of Colonies
250
200
150
100
50
0
n=3
*
n=3
Sh-CYB5D2
Sh-Control
C
MCF7 cell line
Sh-Control
Sh-CYB5D2
Sh-Control
Sh-CYB5D2
CYB5D2
ACTIN
*
Cell Count
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
0
5
10
15
20
Days elapsed
D
Sh-CYB5D2
Sh-Control
MCF7
Number of Colonies
250
200
150
100
50
0
n=3
*
n=3
Sh-CYB5D2
Sh-Control

Figure 7. Endogenous CYB5D2 expression is associated with cell growth mitigation in HCC 1954 and MCF7 cells.

Stable knockdown of CYB5D2 was accomplished via lentivirus-based sh-CYB5D2 (a pool of three individual knockdown constructs; Santa Cruz) with selection using 1μg/ml of puromycin. The knockdown was confirmed in both HCC1954 shCYB5D2 and MCF7 shCYB5D2 lines (Fig 7A and 7C, insets). (A, C) The indicated cells were seeded at $5x10^4$ in 6-well tissue culture plate; cell numbers were counted every 3 days. Experiments were repeated 3 times; means ± SD are graphed. Statistical analyses were performed using 2-way ANOVA; *p<0.05. (B, D) The indicated cells were seeded at $1X10^3$ in 6-well tissue for colony formation analysis. Experiments were repeated 3 times; typical images from a single repeat are shown; means ± SD are graphed. Symbol * indicates p<0.05 in comparison to the respective shCtrl line by two-tailed Student's test. Colony numbers for these analyses are included in Appendix 4.

3.7 CYB5D2 interacts with PTEN

Previous research performed by our lab members using the transient overexpression system of 293T cells indicated an interaction between CYB5D2 and PTEN (Lin and Tang, unpublished observations). PTEN is a well-established tumor suppressor in all cancer types including BC; the association with PTEN suggests a potential mechanism by which CYB5D2 suppresses BC. To investigate this possibility, we examined the interaction using endogenous CYB5D2 and PTEN in HCC1954 and MCF7 cells. I was able to show that immunoprecipitation (IP) of PTEN precipitated PTEN from both MCF7 and HCC1954 cell lysates (Fig 8A, left panel) with concurrent precipitation of the endogenous CYB5D2 (Fig 8A, right panel). In the reverse manner, IP of the endogenous CYB5D2 (Fig 8B, left panel) let to co-IP of the endogenous PTEN in both HCC1954 and MCF7 cells (Fig 8B, right panel). Collectively, evidence supports an interaction between CYB5D2 and PTEN (see Chapter 4 Discussion for details).

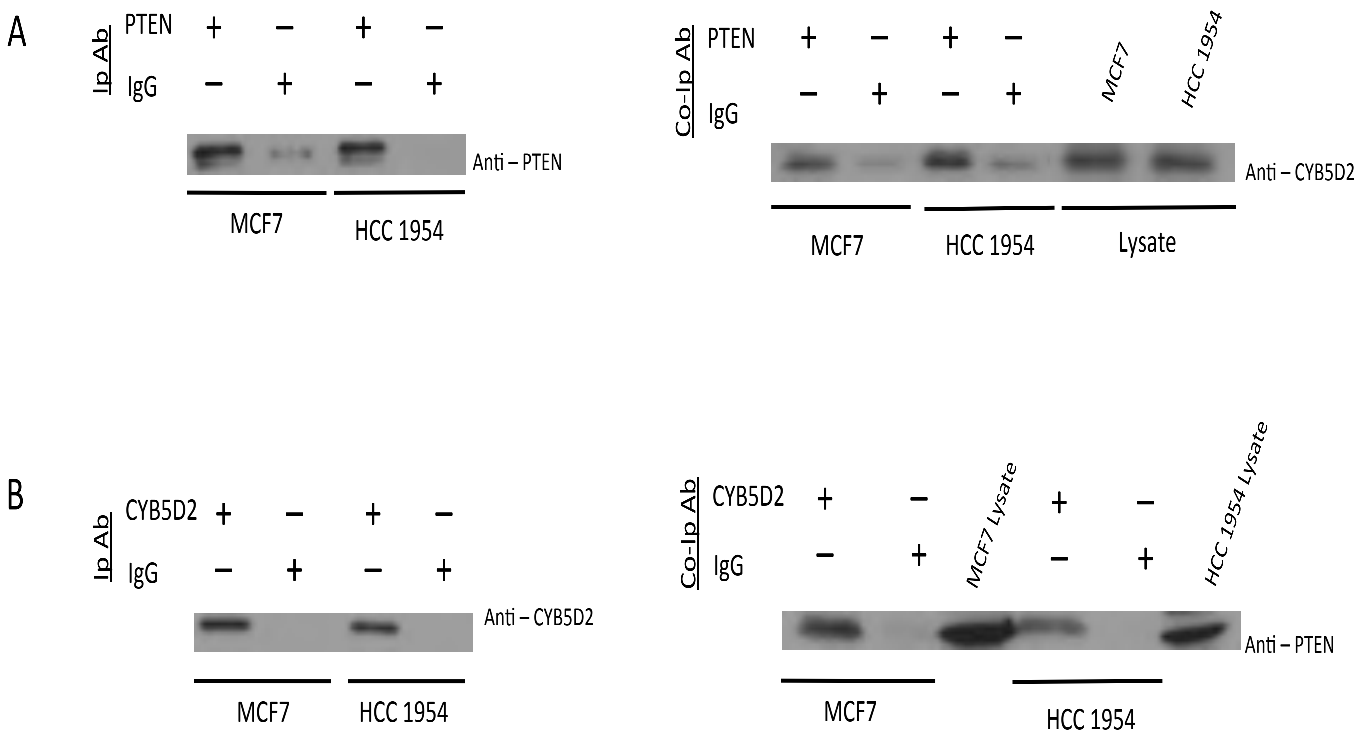


Figure 8. Co-Immunoprecipitation confirms endogenous CYB5D2-PTEN protein-to-protein interaction in HCC 1954 and MCF7 cells.

Cell lysates were prepared from HCC1954 and MCF7 cells, followed by IP of PTEN or CYB5D2 and western blot examination for the presence of CYB5D2 and PTEN. (A) Endogenous PTEN in HCC1954 and MCF7 lysates were immunoprecipitated with an anti-PTEN antibody or control IgG. Western blot analysis was then carried out for PTEN (left panel) or CYB5D2 (right panel); 10% of cell lysates used for IP were also analyzed for CYB5D2 expression. (B) The indicated cell lysates were IP for CYB5D2 and examined for the presence of CYB5D2 (left panel) and PTEN (right panel). Experiments were repeated 3 time; results from a typical repeat are included.

CHAPTER 4: DISCUSSION

BC remains to be the type of malignancy with highest incidence and the major cause of cancer mortality among women worldwide. While intensive research has advanced our understanding of the disease, the complex mechanisms underlying BC evolution and acquisition of therapy resistance remain incompletely understood. Nonetheless, cumulative evidence clearly reveals that cancer develops from genome instability, which results in numerous DNA mutations. These genomic alterations lead to amplification of oncogenes and deletions or silencing of tumor suppressors. The common tumor suppressors in BC include Tp53, BRCA1, BRCA2 and PTEN (39,47,49). However, it is also clear that additional oncogenic factors (both oncogenes and tumor suppressors) have yet to be added to list of BC factors. My research contributes to the establishment of CYB5D2 as a novel tumor suppressor in BC.

4.1 CYB5D2 attenuates BC cell propagation in vitro

We were the pioneers in providing evidence for CYB5D2 as a candidate of novel tumor suppressor by demonstrating its tumor suppression activities in cervical cancer in vitro and in vivo (xenograft tumor) as well as its downregulation in primary cervical cancer (21). This research led us to reason CYB5D2 as a novel tumor suppressor of BC; this possibility is supported by the genetic evidence of the CYB5D2 gene residing in the locus of chromosome 17p13.2, a segment of the genome commonly lost in BC. Furthermore, we have recently observed a common reduction of CYB5D2 expression in BC in comparison to normal breast tissue and in ER- BCs vs ER+ BCs (83, 85); importantly, downregulation of CYB5D2 significantly associates with poor prognosis in BC (85); in line with these observations, downregulation of CYB5D2 is correlated with typical BC mutations including PIK3CA, GATA3, MAP3K1 and TP53 (85). Collectively, research from our lab has provided a comprehensive set of translational evidence for an association of CYB5D2 downregulation with BC tumorigenesis, progression, and poor prognosis.

My research adds functional evidence supporting CYB5D2 as a novel tumor suppressor in BC. Through transient transfection, CYB5D2 surprisingly attenuates the proliferation of both HCC1954 and MCF7 cells (Fig 2A, B). The unexpected finding was attributed to transient expression system, which is commonly associated with a low percentage of transfection and thereby a low transient status of expression. While the detailed mechanisms underlying this overall and persistent impact on cell proliferation need further investigation, it is tempting to propose the secretory potential of CYB5D2 (83) contributing to tumor-suppressing effects. The inhibition on the proliferation of HCC1954 and MCF7 cells has also been demonstrated using tet-inducible HCC1954 tet-CYB5D2 and MCF7 tet-CYB5D2 lines that I have generated. Furthermore, CYB5D2-medaited inhibition of BC cell proliferation was further consolidated by knockdown of endogenous CYB5D2 in both HCC1954 and MCF7 cells, which led to enhancement of cell proliferation. HCC1954 and MCF7 cells are HER2+ and ER+ respectively, suggesting that CYB5D2 thus broadly inhibits BC cell proliferation. However, it appears that CYB5D2 inhibits more effectively HCC1954 cell proliferation compared to MCF7 cells; as this concept was based on studies involved two individual cells lines, the notion certainly needs to be confirmed in future investigations. Collectively, my study contributes a comprehensive set of in vitro evidence supporting CYB5D2 as a novel tumor suppressor of BC.

4.2 CYB5D2 suppresses BC cell proliferation in vitro affects both cell survival and cell proliferation

Tumor suppressors diminish tumorigenesis and cancer progression through a variety of mechanisms that impact cancer cells, stroma, and the communications between tumor cells and the stroma. For CYB5D2, my research demonstrates an inhibitory proliferation effect in BC cells, both HER2+ HCC1954 and ER+ MCF7 cells, via induction of apoptosis and attenuation of cell cycle progression.

For induction of apoptosis, we noticed that transient transfection of CYB5D2 in HCC1954 and MCF7 cells is more effective compared to DOX-mediated induction of CYB5D2 expression in HCC1954 tet-CYB5D2 and MCF7 tet-CYB5D2 cells. Multiple factors can contribute to this observation. For instance, the transient system may have a higher level of ectopic CYB5D2 expression than the tet-inducible lines; the integration of two plasmids, pTet-On and pTRE- CYB5D2, into the genome of HCC1954 tet-CYB5D2 and MCF7 tet-CYB5D2 cells may affect the apoptotic response of both lines induced by CYB5D2 expression. While my research indicates a potential of CYB5D2 in causing apoptosis in BC cells, the notion clearly needs substantial future investigations. These studies should focus on illustrating the mechanisms underlying CYB5D2-induced apoptosis; for example, is the extrinsic or intrinsic apoptotic pathway the major pathway activated by CYB5D2?

My research also suggests that CYB5D2 arrests cell cycle progression at G1 phase at least in HCC1954 cells. For cell proliferation, G1 phase is unique by response to external cues to coordinate cell cycle progression. For example, growth factor receptor signals stimulate cell proliferation at G1 phase. Tumor suppressors can halt G1 phase progression, like the Rb tumor suppressor. In this regard, the possibility of CYB5D2 in inhibition of G1 phase progression provides additional evidence for it being a novel tumor suppressor of BC.

With the possibility of CYB5D2 suppressing BC via induction of apoptosis and G1 phase arrest, it is an intriguing issue to determine which is the major pathway contributing to CYB5D2- mediated suppression of BC. While this issue will be addressed by future studies, it can be envisaged situations favouring apoptosis, such as when CYB5D2 is expressed at high levels with relative low oncogene activities allowing CYB5D2 signalling to eliminate cancerous cells via apoptosis; under other conditions where CYB5D2 is expressed at lower levels with concurrent elevations of oncogenic activity, the conditions may not support CYB5D2 to initiate cell death but instead initiate cell cycle arrest. We like to emphasize that induction of apoptosis and causing cell cycle arrest are not mutually exclusive. Other factors dictating which pathways to take may include factors that interact with CYB5D2. In view of the critical roles of Rb in inhibiting

G1 phase progression, it is intriguing to examine a potential functional connection between CYB5D2 and Rb.

4.3 CYB5D2 protein interacts with tumor suppressor

While whether CYB5D2 interacts with Rb remains unknown, my study reveals a binding between CYB5D2 and PTEN. Mirroring the common and major oncogenic functions of the PI3K-AKT pathway, as the central inhibitor of this pathway, inactivation of PTEN commonly occurs in essentially all cancer types including BC. Down-regulation of PTEN function is commonly found in BC. PTEN behaves as a phosphatase enzyme responsible for dephosphorylating PIP3 to PIP2 and thereby inactivating the second messenger in PI3K/AKT pathway. Up-regulation of HER2+ and down-regulation of PTEN in BC typically indicates a high level of aggressive pathogenesis with a varying resistance to tamoxifen and a poor prognosis (26, 27). With our current knowledge, we now have evidence of molecular interaction between CYB5D2 and PTEN via Co-Ip analysis. Remarkably, consistent evidence suggests CYB5D2 provokes higher tumor suppressing effects in HCC 1954 when compared to MCF7 BC cell lines proposing a possible CYB5D2-PTEN interaction affecting HER2+ pathway. While evidence supports protein-to-protein interaction external to their native cell environment, this does not confirm active basal interaction in tumor cells. In the interest of identifying protein-to-protein interaction within BC cell lines, further research such as co-immunofluorescence analysis is required to identify active location of proteins within the cell.

4.4 Future directions and Clinical implications

Tumor suppressors are essential in preventing tumorigenesis and cancer progression. Expanding our knowledge on tumor suppressors is vital not only for the understanding of cancer but also for the control of tumorigenesis. In this respect, our pioneer work in identification of CYB5D2 as a novel tumor suppressor is an important development in cancer research. My study yields the first functional evidence supporting CYB5D2 as a novel tumor suppressor of BC in vitro. The priority of future research should consolidate this functionality. Two domains of research are proposed. 1) CYB5D2 consists of multiple domains, including a transmembrane domain, a heme-binding domain functioning in heme association, and the critical residue D86 (Aspartic acid – 86) required for heme association. Whether these motifs are involved in CYB5D2-mediated suppression of BC should be investigated; these investigations will shed light on whether the clinical function of heme association is required for CYB5D2 to suppress BC. Additionally, this structural knowledge can be used to explore the possibility to restore CYB5D2 function as a therapeutic manipulation. For example, will individual motif bind PTEN or other critical factors required for CYB5D2 function? If so, will restoration of PTEN function or other factors sufficient to initiate CYB5D2 functions? 2) The CYB5D2-derived tumor suppression activities need to be studied in vivo. This can be achieved by implanting HCC1954 tet-CYB5D2 or MCF7 tet-CYB5D2 cells into immunocompromised mice to generate xenograft tumors, followed by induction of CYB5D2 expression by feeding mice with water supplemented with DOX. The goal is to see whether induction of CYB5D2 lessens tumor growth or induce tumor shrinking.

Following these studies, mice with breast specific knockout CYB5D2 can be generated and examined on potential development of breast cancer.

With the availability of the xenograft or tumor tissues generated from breast-specific CYB5D2 knockout mice, a systemic searching mechanisms underlying CYB5D2-derived tumor suppression in BC can be attempted. This can be done by profile gene expression using either microarray or RNA sequencing. Differentially expressed genes (DEGs) relative to CYB5D2 alteration can be defined; the pathway affected these DEGs can be analyzed; whether these DEGs have diagnostic and therapeutic values can also be researched.

While therapeutic development is promising requiring future research, current collective knowledge of CYB5D2 as a tumor suppressor may serve as a prognostic and diagnostic marker for BC. As previously discussed, CYB5D2 allelic location is commonly deleted or silenced in some types of BC. Down-regulation of CYB5D2 has been identified predominantly in HER2+, tamoxifen resistant MCF7 cells and TNBC all depicted as highly aggressive tumors with poor prognostics. It is important to emphasize expression of CYB5D2 is significantly reduced in primary BC tumors when compared to regular breast tissue. Interestingly, down-regulation of CYB5D2 may potentially be used as a biomarker for BC as it has been identified in relation with PIK3CA, GATA3, MAP3K1, CDH1, TP53 and RB1mutations. To address the relevancy, down- regulation of CYB5D2 correlates with overall survival in BC patients. Using current knowledge of tumor suppressor CYB5D2 expression in BC holds promising insight on BC diagnosis and prognosis.

CHAPTER 5: CONCLUSIONS

Our published research (83, 85, 86, 87) and on-going research support CYB5D2 as an important novel tumor suppressor. My research contributes to this development and thus cancer research in large in three major aspects.

- CYB5D2 suppresses the proliferation of HCC1954 HER2+ and MCF7 ER+ BC cells in vitro.
- The inhibition is in part mediated by induction of apoptosis and G1 phase arrest. The association with the well-established tumor suppressor PTEN likely contributes to CYB5D2's tumor suppression activities.
- Generation of unique materials: HCC1954 tet-CYB5D2 and MCF7 tet-CYB5D2 cell lines. Unlike oncogenes promoting tumorigenesis, studies of tumor suppressors need to consider whether constitutively expressing a tumor suppressor will lead to alterations that counter the tumor suppressor's tumor suppressing activity. The ideal way to ectopically express a tumor suppressor is thus via controllable expression, such as tet-inducible expression. However, generation of these inducible lines is time-consuming and sometime challenging. In this regard, the materials produced in my research will be very useful in research CYB5D2-derived tumor suppression in BC.

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APENDIX I

Table 1. Cell Proliferation Assay in HCC 1954 Tet-On – CYB5D2

Days elapsed	HCC 1954 pTet-On 2µg/ml Doxycycline			HCC 1954 pTet-On + pTRE-CYB5D2			HCC 1954 pTet-On + pTRE-CYB5D2 2µg/ml Doxycycline		
	Cell Count-1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3
0	50,000	50,000	50,000	50,000	50,000	50,000	50,000	50,000	50,000
3	110,500	102,400	169,900	121,350	138,600	159,601	117,350	98,400	103,700
6	757,700	660,900	890300	690,300	794,100	810,500	695,350	774,000	760,100
9	2,351,000	2,335,000	2,240,700	2,003,400	2,270,000	1,978,300	1,550,100	1,725,000	1,303,000
12	4,586,500	4,897,500	4,480,000	4,235,000	4,387,500	4,135,300	3,163,900	3,337,500	2,938,00
15	7,395,100	7,098,700	6,507,000	6,965,200	6,866,200	6,239,100	5,016,200	5,122,500	4,630,700
18	8,210,700	8,003,100	7,653,300	7,561,500	7,351,300	7,183,000	5,461,700	5,200,900	5,010,800

Table 2. Two-way ANOVA of Cell Proliferation Assay in HCC 1954 Tet-On CYB5D2

2-WAY ANOVA table	Sum of Squares	DF	Mean Square	F	P value
Interaction	$1.2X10^{13}$	12	$1.0X10^{11}$	34.23	P<0.0001
Time	$4.4X10^{14}$	6	$7.4X10^{12}$	2433	P<0.0001
Colum Factor	$1.2X10^{13}$	2	$8.3X10^{10}$	73.42	P<0.0001
Subject	$5.0X10^{11}$	6	$8.3X10^{8}$	2.739	P<0.0001

Table 3. Tukey's multiple comparisons test for HCC 1954 Tet-On CYB5D2

Days elapsed	HCC 1954	Mean Difference	Std Error	Sig
9	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	782,867	127,158	0.0333
12	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	1,508,200	170,479	0.0021
12	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	1,106,133	136,945	0.0053
15	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	2,077,133	300,782	0.0106
15	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	1,767,033	272,035	0.0100
18	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	2,731,233	208,649	0.0006
18	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	2,140,800	170,495	0.0006

***** The mean difference is significant at the 0.05 level

Table 4. Colony Formation Assay in HCC 1954 Tet-On CYB5D2 and t-test analysis

Repeats	HCC 1954 Tet-On - CYB5D2 NO DOX	HCC 1954 Tet-On - CYB5D2 DOX
Trial 1	255	88
Trial 2	233	77
Trial 3	232	81

Two-Tailed t-test	
D.F	4
C.V	2.45
α	0.05
t-Value	19.35
t-Test	<0.0001

Table 5. Cell Proliferation Assay in MCF7 Tet-On – CYB5D2

Days elapsed	MCF7 pTet-On 2µg/ml Doxycycline			MCF7 pTet-On + pTRE-CYB5D2			MCF7 pTet-On + pTRE-CYB5D2 2µg/ml Doxycycline		
	Cell Count-1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3
0	50,000	50,000	50,000	50,000	50,000	50,000	50,000	50,000	50,000
3	168,500	156,000	175,200	161,500	167,900	149,000	121,500	134,000	115,400
6	622,000	650,400	690,400	580,000	611,500	579,900	341,000	369,000	325,000
9	1,523,000	1,694,900	1,359,500	1,349,OOO	1,502,000	1,240,000	875,000	756,000	829,500
12	4,650,000	4,304,000	4,500,500	3,950,500	4,214,000	4,005,500	2,025,000	2,130,000	2,100,500
15	6,965,200	6,866,200	6,239,100	5,790,000	6,345,000	6,120,500	4,500,000	4,200,500	4,640,000
18	7,561,500	7,351,300	7,183,000	6,800,500	7,003,500	6,750,000	5,300,500	5,400,500	5,120,500

Table 6. Two-way ANOVA of Cell Proliferation Assay in MCF7 Tet-On CYB5D2

2-WAY ANOVA table	Sum of Squares	DF	Mean Square	F	P value
Interaction	$1.2X10^{13}$	12	$9.8X10^{11}$	52.71	P<0.0001
Time	$4.0X10^{14}$	6	$6.7X10^{13}$	3604	P<0.0001
Colum Factor	$1.4X10^{13}$	2	$7.1X10^{12}$	168.3	P<0.0001
Subject	$2.5X10^{11}$	6	$4.2X10^{10}$	2.290	P<0.0500

Table 7. Tukey's multiple comparisons test for MCF7 Tet-On CYB5D2

Days elapsed	MCF7	Mean Difference	Std Error	Sig
3	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	42,933	7,850	0.0119
3	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	35,833	7,795	0.0218
6	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	309,267	23,642	0.0010
6	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	245,467	16,611	0.0004
9	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	705,633	102,851	0.0214
9	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	543,500	83,522	0.0182
12	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	2,399,667	104,953	0.0013
12	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	1,971,500	86,128	0.0009
15	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	2,243,333	261,697	0.0056
15	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	1,638,333	106,843	0.0036
18	Tet-On – DOX vs. Tet-On CYB5D2 NO DOX	513,933	134,121	0.0471
18	Tet-On – DOX vs. Tet-On CYB5D2 – DOX	2,091,433	136,742	0.0004
18	Tet-On CYB5D2 DOX vs. Tet-On CYB5D2 NO DOX	1,277,500	112,749	0.003

***** The mean difference is significant at the 0.05 level

Table 8. Colony Formation Assay in MCF7 Tet-On CYB5D2 and t-test analysis

Repeats	MCF7 Tet-On - CYB5D2 NO DOX	MCF7 Tet-On - CYB5D2 DOX
Trial 1	152	55
Trial 2	145	47
Trial 3	147	54

Two-Tailed t-test	
D.F	4
C.V	2.45
α	0.05
t-Value	29.39
t-Test	<0.0001

Table 9. Colony Formation Assay of MCF7 Tet-On CYB5D2 in DOX (6μg/ml) and statistical analysis

2-WAY ANOVA table	Sum of Squares	DF	Mean Square	F	P value
Treatment (between columns)	19626	2	9813	121.6	P<0.0001

Tukey's multiple comparisons test

MCF7	Mean Difference	Std Error	Sig
Tet-On – DOX vs. Tet-On CYB5D2 – NO DOX	4.00	0.8525	0.8525
Tet-On – DOX vs. Tet-On CYB5D2 – DOX	101.00	170,479	<0.0001
Tet-On CYB5D2 NO DOX vs. Tet-On CYB5D2 DOX	97.00	136,945	<0.0001

* The mean difference is significant at the 0.05 level

APENDIX II

Table 1. Descriptive frequencies of apoptotic activity in HCC 1954 – CYB5D2

	TEST #1		TEST #2		TEST #3	
	GFP	CYB5D2	GFP	CYB5D2	GFP	CYB5D2
	0.071	0.800	0.071	0.875	0.176	0.467
	0.059	0.917	0	0.455	0	0.857
	0	0.750	0.111	0.600	0	0.471
	0.097	0.692	0.272	0.636	0.222	0.750
	0.105	0.500	0.0172	0.857	0.294	1.000
MEAN	0.066	0.732	0.126	0.685	0.139	0.709

Table 2. Independent t-test of apoptotic activity in HCC 1954 – CYB5D2

	GFP	CYB5D2
Mean	0.110	0.708
St.Dev	0.038	0.024
Variance	0.0010	0.0006
n	3	3
D.F		4
C.V		2.45
α		0.05
t-Value		22.98
t-Test		$2.12X10^{-5}$

Table 3. Descriptive frequencies of apoptotic activity in MCF7 – CYB5D2

	TEST #1		TEST #2		TEST #3	
	GFP	CYB5D2	GFP	CYB5D2	GFP	CYB5D2
	0.024	0.750	0	0.333	0.063	0.700
	0.043	0.500	0	0.714	0.037	0.727
	0	0.308	0	0.500	0.200	0.462
	0.130	0.875	0	0.211	0	0.600
	0.053	0.150	0.111	0.625	0.059	0.556
MEAN	0.050	0.517	0.022	0.477	0.072	0.609

Table 4. Independent t-test of apoptotic activity in MCF7 – CYB5D2

	GFP	CYB5D2
Mean	0.048	0.534
St.Dev	0.025	0.068
Variance	0.0006	0.0046
n	3	3
D.F	4	
C.V	2.45	
α	0.05	
t-Value	11.66	
t-Test	0.00031	

APENDIX III

Table 1. Descriptive statistics and independent t-tests for cell cycle analysis in HCC 1954 Tet-On – CYB5D2

	Untreated G1	Treated G1	Untreated S	Treated S	Untreated G2	Treated G2
Trial 1	43.40	51.10	9.96	12.10	20.20	19.20
Trial 2	34.50	47.50	13.89	11.70	30.62	22.02
Trial 3	37.50	48.50	11.50	12.00	25.10	20.10
Mean	38.467	48.433	11.883	11.930	25.307	20.440
St.Dev	4.528	2.701	1.980	0.208	5.213	1.440
Variance	20.503	7.293	3.920	1.468	27.176	2.075
n	3	3	3	3	3	3
D.F		4		4		4
C.V		2.45		2.45		2.45
α		0.05		0.05		0.05
t-Value		3.274		1.276		-1.559
t-Test		0.0307		0.271		0.194

APENDIX IV

Table 1. Cell Proliferation Assay in HCC 1954 – sh-CYB5D2

Days elapsed	HCC 1954 Sh-CYB5D2			HCC 1954 Sh-Control		
	Cell Count-1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3
0	50,000	50,000	50,000	50,000	50,000	50,000
3	278,400	260,000	210,500	228,800	136,000	180,900
6	1,005,500	1,100,300	858,000	624,000	525,000	759,000
9	2,485,000	2,750,000	2,590,300	1,350,000	950,000	1,200,400
12	5,370,000	6,100,500	6,000,500	3,140,500	3,800,500	3,350,000
15	7,850,000	7,900,000	7,700,600	5,200,000	5,100,500	5,540,500

Table 2. Two-way ANOVA of Cell Proliferation Assay in HCC 1954 Sh-CYB5D2

2-WAY ANOVA table	Sum of Squares	DF	Mean Square	F	P value
Interaction	$1.0X10^{13}$	5	$2.0X10^{12}$	56.18	P<0.0001
Time	$2.1X10^{14}$	5	$4.3X10^{13}$	1,185	P<0.0001
Colum Factor	$1.2X10^{13}$	1	$1.2X10^{10}$	336.4	P<0.0001
Subject	$1.4X10^{11}$	4	$3.4X10^{8}$	0.9607	P=0.4505

Table 3. Colony Formation Assay in HCC 1954 and t-test analysis

Repeats	HCC 1954 Sh-CYB5D2	HCC 1954 Sh-Ctrl
Trial 1	195	51
Trial 2	180	59
Trial 3	189	46

Two-Tailed t-test	
D.F	4
C.V	2.45
α	0.05
t-Value	23.56
t-Test	<0.0001

Table 4. Colony Formation Assay in MCF7 – sh-CYB5D2

Days elapsed	MCF7 Sh-CYB5D2			MCF7 Sh-Control		
	Cell Count-1	Cell Count -2	Cell Count -3	Cell Count -1	Cell Count -2	Cell Count -3
0	50,000	50,000	50,000	50,000	50,000	50,000
3	125,500	110,500	134,700	98,000	87,500	101,500
6	782,500	702,400	752,500	570,000	510,500	526,000
9	2,112,000	1,990,000	2,280,500	1,284,000	1,356,900	910,400
12	5,640,000	4,950,000	5,350,000	3,130,000	2,840,500	2,900,500
15	7,562,500	7,740,750	7,960,000	5,613,750	5,440,000	5,350,000

Table 5. Two-way ANOVA of Cell Proliferation Assay in MCF7 Sh-CYB5D2

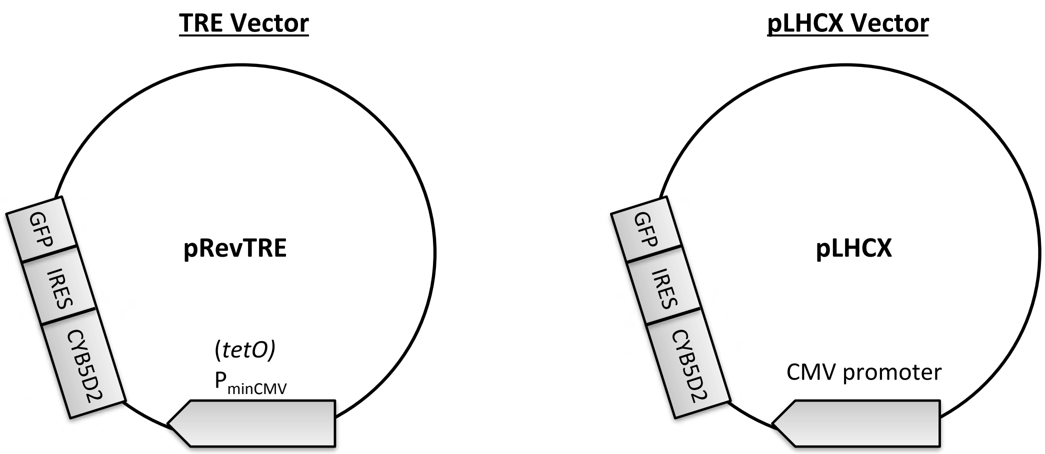
2-WAY ANOVA table	Sum of Squares	DF	Mean Square	F	P value
Interaction	$9.1X10^{12}$	5	$1.8X10^{12}$	95.66	P<0.0001
Time	$2.1X10^{14}$	5	$4.2X10^{13}$	2,206	P<0.0001
Colum Factor	$8.6X10^{13}$	1	$8.6X10^{13}$	218.9	P<0.0001
Subject	$1.6X10^{12}$	4	$3.9X10^{11}$	2.062	P=0.1240

Table 6. Colony Formation Assay in MCF7 and t-test analysis

Repeats	MCF7 Sh-CYB5D2	MCF7 Sh-Ctrl
Trial 1	188	144
Trial 2	179	159
Trial 3	212	150

Two-Tailed t-test	
D.F	4
C.V	2.45
α	0.05
t-Value	3.90
t-Test	0.0175

APPENDIX V


TRE Vector
pRevTRE
GFP
IRES
CYB5D2
(*tetO*)
P_{minCMV}
pLHCX Vector
pLHCX
GFP
IRES
CYB5D2
CMV promoter