Universita degli Studi di Cagliari

DOTTORATO DI RICERCA

MEDICINA MOLECOLARE E TRANSLAZIONALE

Ciclo XXXI

TITOLO TESI

Star-related lipid transfer protein 10 (STARD10): a novel key player in alcohol-induced breast cancer progression

Settore scientifico disciplinare di afferenza MED03

Presentata da: ANDREA FLORIS

Coordinatore Dottorato Prof. AMEDEO COLUMBANO

Tutor Prof. CARLO CARCASSI

Esame finale anno accademico 2017 – 2018

Tesi discussa nella sessione d'esame Gennaio-Febbraio 2019

INTRODUCTION

Breast cancer is the most common invasive cancer in females worldwide. It accounts for 16% of all female cancers, 22.9% of invasive cancers in women and 18.2% of all cancer deaths worldwide[1]. Breast cancer is a complex heterogeneous disease including a range of pathologies and patterns of gene expression with more the 70 gene signatures implicated with, creating a variable clinical behavior and response to therapy. The predictive biomarkers in breast cancer are the estrogen (ER), progesterone (PR) receptors and human epidermal growth factor receptor HER2 (erbB2/neu)[2] whose overexpression is associate with a lower probability of response to tamoxifen and trastuzumab[3]. Current therapy decision-making is increasingly governed by the molecular classification of breast cancer (luminal, basal-like, HER2+). Breast cancer rates are much higher in developed nations compared to developing ones. There are several reasons for this, with possibly life-expectancy being one of the key factors, in fact breast cancer is more common in elderly women, the different lifestyles and eating habits of females are also contributory factors. Currently the endogenous

and environmental factors that contribute to its etiology remain elusive, tobacco use, unregulated diet and alcohol consumption are the three major human cancer risk factors[4]. Epidemiological evidence and experimental studies supporting a positive association between alcohol consumption and breast cancer risk in a concentration- and duration-dependent manner, showing that alcohol drinking increases breast cancer risk 10-20 % for each 10g (~1 drink) consumed daily by adult. Woman who had 2-3 alcoholic drinks per day had a 20% higher risk of breast cancer compared to women who didn't drink alcohol[5,6]. Research consistently shows that ethanol is a tumor promoter and stimulates migration/invasion as well as proliferation of breast tumor cells and enhances epithelial-mesenchymal transition[7]. A higher frequency of drinking has been reported to be positively associated with increased risk for developing regional-distant tumor and frequency of late-stage breast cancer diagnosis and in addition of breast cancer carcinogenesis, alcohol may also enhance the growth of existing breast tumor and his capability to invade and metastasize[8]. Data from animal studies and cell culture experiments support the role of ethanol in breast carcinogenesis, where ethanol intake can individually stimulate the initiation and progression breast cancer. Oxidation of ethanol

to acetaldehyde or formation of free radicals could be involved in ethanol-mediated breast cancer promotion, through inhibition of repair of carcinogen-induced DNA damage. ROS include superoxide anion radical (O2), singlet oxygen (1O2), hydrogen peroxide (H2O2) and the highly reactive hydroxyl radical (OH-) can act as a second messengers in signaling cascades that are vital for cellular responses to external stimuli[9]. Generation of free radicals by oxidative metabolism of ethanol could induce tumorigenesis and progression possibly by expression modulating, degradation and post-translational modifications. Acetaldehyde is also genotoxic and may interact to and interfere with the function of other important cell macromolecules[10]. The molecular mechanism underlying ethanol action, however, remain to be determined. Ethanol triggers various intracellular signal pathways in cell overexpressing ErbB2 protein, activating members of mitogen-activated protein kinase (MAPKs). The ErbB protein family is a receptors kinase group that includes four closely related members: epidermal growth factor receptor (EGFR/ErbB1), ErbB2/neu, ErbB3 and ErbB4. ErbB2 tyrosine kinase receptor plays a critical role in the pathogenesis of breast cancer, amplification and/or overexpression of ErbB2 occurs in 20-30% of human breast cancers and correlates with poor

prognosis[11]. MAPKs play an active role in the transmission of signals from cell surface receptors and environmental stimuli to diverse biological activities. Three major MAPKs have been identified: ERK, JNK and p38. ERKs protein are mainly activated by growth factors and are involved in the regulation of cell proliferation and stimulated cell invasion. In Human breast cancer and mammary epithelial cells with high expression of ErbB2, ethanol induces ErbB2 expression and its auto-phosphorylation that activates the mitogen-activated protein kinase (MAPK) signaling members, extracellular signal-regulated kinase (ERK), c-Jun NH2 terminal protein kinase (JNK1/2), p38 mitogen-activated protein kinase (p38 MAPK), PI3-kinase (Phosphatidyl inositol 3 kinase) and Akt (AK strain trasforming), well-know to be dowstream targets of ErbB2[12]. The steroidogenic acute regulatory protein (StAR)-related lipid transfer (StarD) domain is a protein module of 210 residues that binds lipids. In human, StarD domain was found in fifteen distinct proteins and these can be grouped in six subfamilies with diverse expression patterns and can be found free in the cytoplasm, attached to membrane or in the nucleus[13]. StarD10 is a member of the StarD protein family and lipid transfer protein with selective binding site to phosphatidylcholine (PC) and

phosphatidylethanolamine (PE), two potential precursors for lipid metabolism and a major constituent of cell membranes. StarD10 expression is abundant in the liver where a putative function is the delivery of phospholipids in the canalicular membrane for secretion into bile[14], while in the mammary gland, StarD10 expression is developmentally regulated for the lipids milk enrichment[15]. Cellular growth and apoptosis may also be influenced by the PC to PE ratio as a reduction in this ratio can result in a loss of membrane integrity that could predispose to cell cancer transformation. Hi choline transport was suggested as the cause for elevated levels of PC and it is one of the earliest metabolic events associated with the initial stimulation of cell growth and proliferation in normal cells. The intracellular choline metabolism in mammary cells is divided into two major ways: synthesis of PC and oxidation to produce methyl donor betaine. Choline metabolism and its derived metabolites can produce extensive alterations as a result in phospholipid change composition can occur before the morphological tumor changes. Phosphatidylcholines are generally the most abundant phospholipid class in membranes of eukaryotic cells which are composed principally of four distinct classes of glycerophospholipids: phosphatidylcholines,

phosphatidylethanolamines, phosphatidylserines (PS) and phosphatidylinositols (IP), It's also constitute the major phospholipid class contained in lipoproteins and biliary lipid aggregates. Belong to glycerolphospholipids family are composed of two fatty acids covalently linked to a glycerol moiety by ester bonds in the sn-1 and sn-2 positions. Its fatty acyl composition plays a critical role in regulating the physical properties of membranes with more unsaturated fatty acids functioning to increase fluidity. PC is involved in structural and biological functions as membrane trafficking process and cellular signaling, it is able to induce direct activation of the MEK-ERK 1/2 pathway protein, increase cell viability and induce proliferation[16]. The biological effects correlated with PC concentration changes in biological membranes are due to an altered cellular localization of membrane enzymatic proteins and its activation status[17]. The dynamic properties of the cell membrane as well as the dynamic movement of lipids in micelles and vesicles is of importance in such diverse areas as activation of membrane enzyme system and the specific assembly or mobilization of microtubules and microfilaments. The role of StarD10 as key player on subcellular lipid transfer and cellular signaling regulation has not been clarified yet. Post-translational

modification by phosphorylation is a common mechanism to regulate the activity of protein, increasing their local negative charge to promote conformational changes or influencing interaction with protein partners. StarD10 protein is well known to be regulated via phosphorylation on Serine 284 by Casein Kinase II (CKII)[18]. StarD10 is highly expressed at protein level in mouse mammary tumor, in 35% of primary breast carcinoma and 64% of human breast cancer cell lines. This data supports the role of StarD10 as lipid binding protein in deregulated cell growth and tumorigenesis. StarD10 was found to be co-expressed with ErbB2 in many breast carcinoma cell lines, suggesting a selective growth advantage and cellular transformation for tumor expressing both protein[19]. Although StarD10 expression alone was not sufficient to transform cells, it potentiated cellular transformation when co-expressed with ErbB2 epidermal growth factor receptor by a yet unknown mechanism[19]. The aim of this study was to investigate the role of StarD10 and ErbB2 cross-talk in breast cancer as consequence of ethanol administration, and elucidate the molecular mechanisms.

MATERIALS AND METHODS

Cell culture and treatments

All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Specifically, MCF-7 and SKBR-3 cells (human breast cancer cell lines) were grown according to instructions provided by ATCC, while MCF12-A (human breast epithelial cells) were maintained in DMEM/F12 medium (Corning) containing epidermal growth factor (EGF) (20ng/mL) (Thermo Fisher, Waltham, MA), hydrocortisone (0.5mg/mL), cholera toxin (100ng/mL), insulin (10μg/mL) (Sigma, Saint Louis, MO) and supplemented with 5% horse serum (Thermo Fisher, Waltham, MA), penicillin (100 U/ml)/streptomycin (100 U/ml) at 37°C with 5% CO2. In this study, cells were exposed to ethanol (Sigma Aldrich, St. Louis, MO) at pharmacologically relevant concentration of 100mM for 48 hours[20].

Human breast tissue specimens

Five breast tissue and thirteen breast cancer tissues samples were obtained during surgical reductive mastoplasty and surgical resection for primary breast cancer respectively by Dr. Biancolella (University of Tor Vergata, Rome, Italy) and Dr. Orrù (University of Cagliari, Cagliari, Italy) (**Table 1**). These tissues were immediately frozen in liquid nitrogen for subsequent RNA and protein extraction. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by Cedars Sinai Medical Center's human research review committee.

MMTV-neu transgenic mice model

Mice mammary adenocarcinoma tissues was provided by Dr. Jia Lou (University of Kentucky College of Medicine, Lexington, KT). FVB MMTV-neu transgenic mice were purchased from Jackson Laboratory (Bar Harbor, MA). Twelve

weeks old mice were divided into two groups, the (treat group) were fed with ethanol liquid diet at concentration 6.6% v/v, while the other (control group) were put on an alcohol-free liquid diet. Both groups were monitored weekly to observe growth and development of tumor. The mice with the tumor that increased its size and go beyond 20mm were euthanized and the tumor mass was analyzed as described by (Luo, 2016)[7].

Transient cell transfection

MCF-7 and SKBR-3 cells were transfected with the following overexpression vectors: StarD10 (Myc-DDK-tagged), ErbB2-EGFP, pCMV4-p65, CMV6-c-Myc-DDK, pMIEG3-c-Jun, pLX304-Fos-V5. All plasmids and the corresponding negative control empty vectors were purchased from Origene (Rockville, MD) and Addgene (Cambridge, MA). MCF-7 and SKBR-3 cells were cultured in 6-well plates (0.5×106 cells/well) and transfected using 5 μl of JetPRIME from Polyplus (New York, NY) with 2 μg of target plasmid per well. After 4 hours, the transfection medium was changed with normal medium and the cells were cultured for additional 44 hours. Ethanol (100mM)

was administrated every 4 hours and mRNA and protein expression analysis were performed as indicated.

STARD10 and ERBB2 promoter reporter assays

The STARD10 and ERBB2 promoter-luciferase reporter plasmids (GeneCopoeia, Rockville, MD), p65, c-Jun, c-Fos and c-Myc were co-transfected as indicated into MCF-7 and SKBR-3 cells (0.5×106 cells/well, 6-well plates) as described above for 24 hours and ethanol (100mM) was added as indicated for 48 hours. Gaussia luciferase (GLuc) and secreted Alkaline Phosphatase (SEAP) activities were measured following the manufacturing's instruction (GeneCopoeia, Rockville, MD).

ChIP assay

ChIP assays were performed using Imprint Chromatin Immunoprecipitation kit (Sigma, St. Louis, MO). Sonicated chromatin was immunoprecipitated with 2 μg of antibody against p65 (Proteintech, Rosemont, IL) reverse cross-linked and PCR amplified for 35 cycles with the following murine STARD10

promoter primer sequences: part 1. chr11:72791657-72796391) Forward: 5'-TCCTAATATCCAGAGGAGCAC-3'; Reverse: 5'-TCTGGAAGTTAACTGACAGCC-3'; part 2. (chr11:72791657-72792196) Forward: 5'-GGCTCTCAGTTAACTTCCAGA-3'; Reverse: 5'-GCACAACTAACTCAGCAGCAA-3', and murine ERBB2 promoter primer sequences: part 1. (chr11:98411386-98411757) Forward: 5'-GAAAGTAGATTAAGAGAGGGCC-3'; Reverse: 5'-GTTCTGACTTTACCCAGTTCTC-3' (Ambion, Austin, TX). Human STARD10 promoter primer sequences are: Forward 5'-CTTGAGCTCCTGAGAAATGTAGT-3'; Reverse 5'-GAGGGTCATTCCTTGTAATCAT-3', while human ERBB2 promoter primer sequences are: Forward 5'-CACAAGGTAAACACAACACATCC-3'; Reverse 5'-GTAAAGGGCCCCGTGGGAA-3'.

RNA interference

To perform the RNAi experiments, five different predesigned small interfering RNAs (siRNAs) targeting human STARD10 (#1 sense sequence: 5'-GGCCAUGAAGAAGAUGUACtt-3', antisense: 3'-GUACAUCUUCUUCAUGGCCtt-5'), (#2 sense sequence 5'-

GGCCAUGAAGAAGAUGUACtt-3' and antisense: 3'-GUACAUCUUCUUCAUGGCCtt-5'), (Ambion, Austin, TX),and human RELA (#1 sense sequence: 5'-GCCCUAUCCUUUACGUCAtt-3', antisense: 3'-UGACGUAAAGGGAUAGGGCtg-5'), (#2 sense sequence: 5'-GGAGUACCCUGAGGCUAUAtt-3', antisense: 3'-UAUAGCCUCAGGGUACUCCat-5') and negative control siRNA were purchased from Ambion (Austin, TX), while two human ERBB2 siRNAs were obtained from Qiagen (Hilden, Germany) (#1 catalog no. SI02223571; #2 catalog no.SI00300195). MCF-7 and SKBR-3 cells were cultured in 6-well-plate (0.5x106 cells/well) and transfected using RNAiMax (5μl/well) (Invitrogen, Carlsbad, CA) with STARD10 siRNA (10nM), ERBB2 siRNA (10nM), RELA siRNA (10nM) or negative control siRNA for 48 hours for mRNA or protein expression analysis. For combined overexpression and silencing, overexpression was performed in the last 24 hours of STARD10, RELA or ERBB2 silencing.

Real-time PCR analysis

Total RNA was isolated using Quick-RNA Kits (Zymo Research, Irvine, CA), according to the manufacturer's protocol, subjected to reverse transcription (RT) by M-MLV Reverse transcriptase (Invitrogen, CarlsBad, CA). Two μl of RT product was subjected to real-time PCR analysis. TaqMan probes for human STARD10, ERBB2, RELA, c-Myc, c-Fos and c-Jun and the Universal PCR Master Mix were purchased from ABI (Foster City, CA). Hypoxanthine phosphoribosyl-transferase 1 (Hprt1) was used as housekeeping gene. The delta Ct (ΔCt) obtained was used to find the relative expression of genes according to the formula: relative expression=$2^{-\Delta\Delta Ct}$, where ΔΔCt= ΔCt of respective genes in experimental groups – ΔCt of the same genes in control group.

Western blots

Proteins from MCF-7, SKBR-3 cells and animal breast tissues were prepared using RIPA buffer containing protease inhibitor cocktail (Sigma, St. Louis, MO) and resolved on 10% SDS- polyacrylamide gels following standard protocols (Amersham BioSciences, Piscataway, NJ). Membrane were

blotted with STARD10, ERBB2, ERK, phospho-ERK, c-MYC, p65, c-JUN, c-FOS (Proteintech, Rosemont, IL), control β-actin and Histone 3 (Sigma, St. Louis, MO) antibodies. Membranes were developed by chemiluminescence ECL detection system (Amersham BioSciences, Pittsburgh, PA) and blots were quantified using the Quantity OneTM densitometry program (Bio-Rad laboratories, Hercules, CA).

Immobilized Metal Affinity

Ethanol treatment at 100mM was performed 48 hours after seeding, on typically ~60-80% confluent cultures in 75cm2 Flask (Corning, NY), detached cells from the culture plate using 0.25% Trypsin-EDTA (Fisher Scientific, Hampton, NH), pellet cells by centrifugation at 1000 RPM x 2min. The total protein extracted as described above and were subjected to immobilized metal affinity chromatography using the PhosphoCruz Protein Purification Columns (Santa Cruz Biotechology, Dallas, TX) according to the manufacturer's protocols. The phosphoenriched lysates were subjected to immunoblotting using STARD10 monoclonal antibody.

Cell proliferation and viability

The MTT assay (Roche Molecular Biochemicals, Indianapolis, IN) was performed to determine the number of viable cells in culture as described (Sun, 2002). MCF-7 and SKBR-3 cells were plated into 96-well-plates (4x103cells/well). 1/10 volume of MTT labeling reagent was added to each well and incubated at 37°C for 4 hours until the color turned orange. Plate reader was used to measure absorbance of formazan product at 570nm, with a reference wavelength of 750nm.

Cell migration assays

5×10^4 of breast cancer cells were seeded on each side of culture insert 2-Well for live cell analysis (Ibidi, Munich, Germany), with a 500 μM separation between each side of the well, and allowed to grow for 8 h before to remove the insert. After removal of the insert cells were transfected with specifics plasmids for every condition and treated with ethanol as described above . At several time points, at insert removal (0 h) and following 24h and 48h, the plates were removed from the incubator and place it under an inverted microscope (EVOSTM FL Imaging System with SONY ICX445 monochrome CCD

camera 1300x1024 pixel), to take a snapshot pictures and to check for a wound closure. The results of a snapshot pictures were analyzed to measure the wound area using microscope image processing software (imagej.nih.gov/ij/). The migration capacity of all conditions was arbitrarily designated as one and every alterations were expressed as an arbitrary value relative to not treated controls.

Measurement of intracellular calcium

Intracellular calcium levels were determined with a colorimetric calcium detection kit from Abcam (Cambridge, MA). Briefly, cells grown on 10 mm dishes and breast tissues from animal model were lysate and centrifuged at 15,000 RPM for 15 min at 4□. The supernatant was collected and reacted with chromogenic reagent. The absorbance of formed chromophore was measured at 575 nm using the SPECTROstar Omega reader (BMG Labtech, Ortenberg, Germany).

Membrane Fluidity Assay

Approximately $5x10^5$ MCF-7 and SKBR-3 cells have been seeded into 4-well Chamber slides (Thermo Scientific Nunc product, number 154526), treated with ethanol and transient transfected with StarD10 plasmid as described above. The cells were analyzed using the membrane fluidity kit (M0271, Marker Gene Technologies, Inc., Eugene, OR). Untreated cells were used as positive control. The slides were treated with 200 µl of perfusion buffer with 20 µm fluorescent lipid reagent (pyrene decanoic acid) and 0.08% of pluronic F127, provided with the kit. After incubation for 1 h at RT, the cells were then washed with PBS and subsequently was read the fluorescence at both 400 and 450-470nm using the Multifrequency Fluorometer with exitation filter at 360nm. (FLUOstar Omega, BMG Labtech, DU). The samples were excited at 341 nm, the emission spectra were recorded at 372–400nm monomer and 450-470nm excimer. The normalized fluorescence was calculated as ratio excimer to monomer. Typical epifluorescence microscopic analysis was performed using an excitation filter (350nm), a dichroic filter (370nm) and emission filters for monomer fluorescence (405nm interference filter) and excimer fluorescence (470nm cut-on filter) (Omega Optical Co., Brattleboro,VT).

Casein Kinase II activity assay

Casein Kinase II activity was measured in MCF-7, SKBR-3 breast cancer cells (1x106 cells) and 10mg of mice breast tissues lysate using the CycLex CK2 Kinase assay kit (Woburn, MA) according to the manufacturer's recommended protocol.

Statistical analysis

Data are expressed as mean±SEM. Statistical analysis was performed using ANOVA and Fisher's test. For mRNA and protein levels, ratios of genes and proteins to respective housekeeping densitometric values were compared. Significance was defined by $p<0.05$.

RESULTS

STARD10 expression in normal human breast and cancer tissues

Because STARD10 expression appears to be deregulated in several types of cancer including breast cancer[19], we examined STARD10 mRNA level in 38 independent breast cancer microarray datasets from the GEO database (**Table 1)**. *STARD10* mRNA levels were at least 5- and 10-fold higher in DCIS and IDC, respectively, than normal breast tissues (**Figure 1A**). Consistent with these results, all 13 ERBB2-positive breast tumors (**Table 2**) that we tested had 3- and 4-fold higher levels of *STARD10* and *ERBB2* mRNA, respectively, compared to normal breast tissue (**Figure 1B,** left panel). In normal breast cancer tissues, STARD10 expression was not detectable at protein level, while in ERBB2 positive human breast cancer tissue, it was expressed in 30% of samples (**Figure 1B,** right panel). To confirm that high level of ERBB2 expression correlates with its downstream targets, we next measured ERK and p-ERK protein levels in the same human tissue samples that indicated both proteins levels increased compared to control (**Figure 1B,** right panel). This data supports the literature which states that STARD10 is overexpressed in 35% of primary human breast cancers and positively correlates with ERBB2 overexpression[18,19]

.

Table 1. STARD10 expression in human breast cancer databases

Sample	Title	Value	Rank	x value	value	median	ttest
GSM535613	healthy breast 1, biological rep. 1	36.844	63	1.01	36.844	111.81	
GSM535614	healthy breast 2, biological rep. 2	133.67	75	1.02	133.67		
GSM535615	healthy breast 3, biological rep. 3	123.11	74	0.99	123.11		
GSM535616	healthy breast 4, biological rep. 4	40.552	64	0.98	40.552		
GSM535617	healthy breast 5, biological rep. 5	100.52	73	0.97	100.52		
GSM535613	healthy breast 1, biological rep. 1	33.46	62	0.96	33.46		
GSM535614	healthy breast 2, biological rep. 2	154.74	77	1.15	154.74		
GSM535615	healthy breast 3, biological rep. 3	160.01	77	1.21	160.01		
GSM535616	healthy breast 4, biological rep. 4	85.25	71	1.03	85.25		
GSM535617	healthy breast 5, biological rep. 5	180.94	79	1.09	180.94		
GSM535604	DCIS 1, biological rep. 1	94.224	71	3.51	94.224	415.01	0.0095294
GSM535605	DCIS 2, biological rep. 2	355.97	86	3.65	355.97		
GSM535606	DCIS 3, biological rep. 3	560.8	91	3.61	560.8		
GSM535607	DCIS 4, biological rep. 4	137.59	75	3.52	137.59		
GSM535608	DCIS 5, biological rep. 5	1382.6	96	3.25	1382.6		
GSM535609	DCIS 6, biological rep. 6	318	85	3.33	318		
GSM535610	DCIS 7, biological rep. 7	384.32	87	3.6	384.32		
GSM535611	DCIS 8, biological rep. 8	479.93	89	3.35	479.93		
GSM535612	DCIS 9, biological rep. 9	90.014	71	3.39	90.014		
GSM535604	DCIS 1, biological rep. 1	184.14	79	3.62	184.14		
GSM535605	DCIS 2, biological rep. 2	501.82	89	3.39	501.82		
GSM535606	DCIS 3, biological rep. 3	698.15	92	3.54	698.15		
GSM535607	DCIS 4, biological rep. 4	143.77	76	3.21	143.77		
GSM535608	DCIS 5, biological rep. 5	1702.6	96	3.98	1702.6		
GSM535609	DCIS 6, biological rep. 6	445.71	88	3.87	445.71		
GSM535610	DCIS 7, biological rep. 7	517.82	89	3.67	517.82		
GSM535611	DCIS 8, biological rep. 8	732.75	92	3.99	732.75		
GSM535612	DCIS 9, biological rep. 9	158.52	78	3.97	158.52		
GSM535618	IDC 1, biological rep. 1	915.54	94	6.2	915.54	961.98	1.624E-08
GSM535619	IDC 2, biological rep. 2	1218.2	95	6.09	1218.2		
GSM535620	IDC 3, biological rep. 3	967.15	94	6.15	967.15		
GSM535621	IDC 4, biological rep. 4	1360	96	6.1	1360		
GSM535622	IDC 5, biological rep. 5	611.36	91	6.05	611.36		
GSM535618	IDC 1, biological rep. 1	956.81	94	6.01	956.81		
GSM535619	IDC 2, biological rep. 2	1035.7	94	6.03	1035.7		
GSM535620	IDC 3, biological rep. 3	793.12	93	6.12	793.12		
GSM535621	IDC 4, biological rep. 4	1190.8	95	6.06	1190.8		
GSM535622	IDC 5, biological rep. 5	474.61	89	6.21	474.61		

STARD10 and ERBB2 expression in human breast cell lines

All cell lines that overexpressed *ERBB2* mRNA were found to have high STARD10 levels. STARD10 expression, however, was also detected in cell lines that did not overexpress ERBB2 (Olayioye 2004). Here, we confirmed that STARD10 was highly expressed independently of ERBB2 level (**Figure 1C**). Specifically, both MCF-7 and SKBR-3 cells exhibited a gain of *STARD10* protein level even though its mRNA level appeared to be upregulated only in MCF-7 cells, compared to normal MCF-12A cells (**Figure 1C**). This finding confirmed the immunohistochemical analysis that show STARD10 expression was negligible in normal breast tissue[18]. Presumably, we presumed that alterations in hormone homeostasis during breast cancer transformation could be responsible for the induction in STARD10 expression even though no evidence is presented yet.

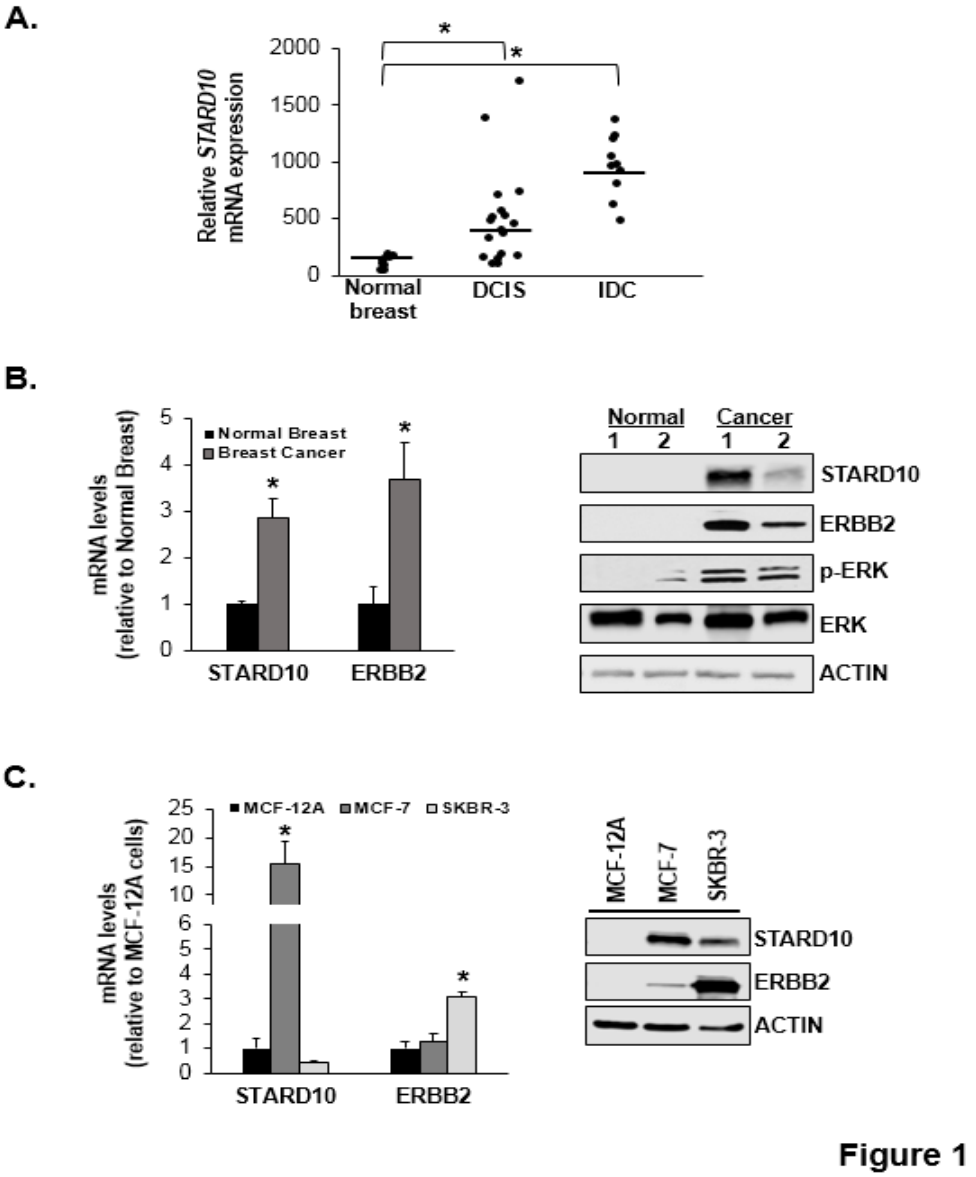

Figure 1

FIGURE 1. Expression of STARD10 in breast cells and tissues. (A) *STARD10* mRNA level from 28 independent breast cancer microarrays of DCIS and IDC (GEO database) compared to normal breast tissue. The database normalized STARD10 expression by RMA (robust multi-array average) method. *p<0.001 *vs.* normal breast. **(B)** RT-PCR analysis of STARD10 and ERBB2 expression in 10 primary ERBB2-positive human breast tumors compared to normal breast tissues. Western blotting analysis of STARD10, ERBB2, pERK, and ERK (right panel). Results represent mean ± SE from 15 samples. *p<0.04 *vs.* normal breast. **(C)** mRNA levels of *STARD10* in MCF-7 and SKBR-3 cells were compared to mRNA expression in MCF-12 cells using RT-PCR. Western blotting was performed to measure STARD10 and ERBB2 protein levels. Results are expressed as a fold relative to control (mean±SE) from 10 experiments. *p<0.002 *vs.* STARD10 MCF-12 cells.

							Pathologic (PN)		ER		PR		ErbB2			
n	age	T	size	grade	M0	N0	grade	LN	+/-	grade	+/-	grade	+/-	grade	CK	Ki67
Tumor breast tissue 1	47	DCIS	pT1 (1.2cm)	2	M0	N0	pN0		+	90%	+	90%	+	3	-	30%
Tumor breast tissue 2	66	DCIS	pT1 (1.5cm)	2	M0	N0	pN+	N/A	+	90%	+	90%	+	2	-	10%
Tumor breast tissue 3	64	DCIS	pT1 (1.7cm)	2	M0	N0	pN0		+	85%	+	80%	+	2	-	5%
Tumor breast tissue 4	70	DCIS	pT2 (3cm)	3	M0	N0	pN+	LN 2/4	+	90%	+	80%	+	2	+	20%
Tumor breast tissue 5	52	DCIS	pT1 (1.5cm)	2	M0	N0	pN0		+	90%	+	20%	+	2	-	10%
Tumor breast tissue 6	68	DCIS	pT1 (1.5cm)	2	M0	N0	pN+	LN 1/6	+	90%	+	65%	+	2	+	5%
Tumor breast tissue 7	84	IDC	pT1 (1cm)	2	M0	N0	pN0		+	90%	+	1%	+	2	-	18%
Tumor breast tissue 8	56	IDC	pT2 (2.8cm)	3	M0	N0	pN+	LN 7/14	+	90%	+	20%	+	3	+	40%
Tumor breast tissue 9	66	DCIS	pT1 (0.8cm)	2	M0	N0	pN0		+	90%	+	80%	+	3	-	5%
Tumor breast tissue 10	43	IDC	pT1 (2cm)	2	M0	N0	pN+mic	LN 1 mic /8	+	90%	+	80%	+	3	+	5%
Normal breast tissue 1	41															
Normal breast tissue 2	48															
Normal breast tissue 3	32															
Normal breast tissue 4	39															
Normal breast tissue 5	52															

Table 2. Characteristics of breast cancer tissues from ErbB2-positive patients.

Alcohol administration enhances STARD10 protein level in MMTV-neu transgenic mice and in breast cancer cell lines

Luo's laboratory demonstrated that alcohol feeding in the FVB MMTV Neu transgenic mice, that express high levels of neu (ERBB2 in human), increased cancer metastasis activating ErbB2/p38γ MAPK signaling pathway (Xu 2016). Here we investigated whether alcohol influences STARD10 expression in

the above animal model. Alcohol administration increased STARD10, ERBB2 and p-ERK protein levels by 6.8-, 4.8- and 1.5-fold compared to control tumor tissues (**Figure 2A**). Ethanol treatment raised *STARD10 and ERBB2* mRNA levels by 1.6 and 1.9-fold respectively, in MCF-7 cells (**Figure 2B**) and by 2.2 and 2.6-fold respectively, in SKBR-3 cells (Figure 2C). A comparable induction in STARD10 and ERBB2 protein levels was observed upon ethanol exposure in MCF—7 and SKBR3 cells (Figure 2B and 2C). Activated ERK (p-ERK) regulates growth factor-responsive targets in the cytosol and it is well-known function downstream of ERBB2 (Roskoski 2014). Hence, we evaluated its activation status in vitro. p-ERK was found significantly increased by 2-fold after alcohol treatment compared to control (**Figure 2B and 2C**) as we previously found in the in vivo model (**Figure 2A**).

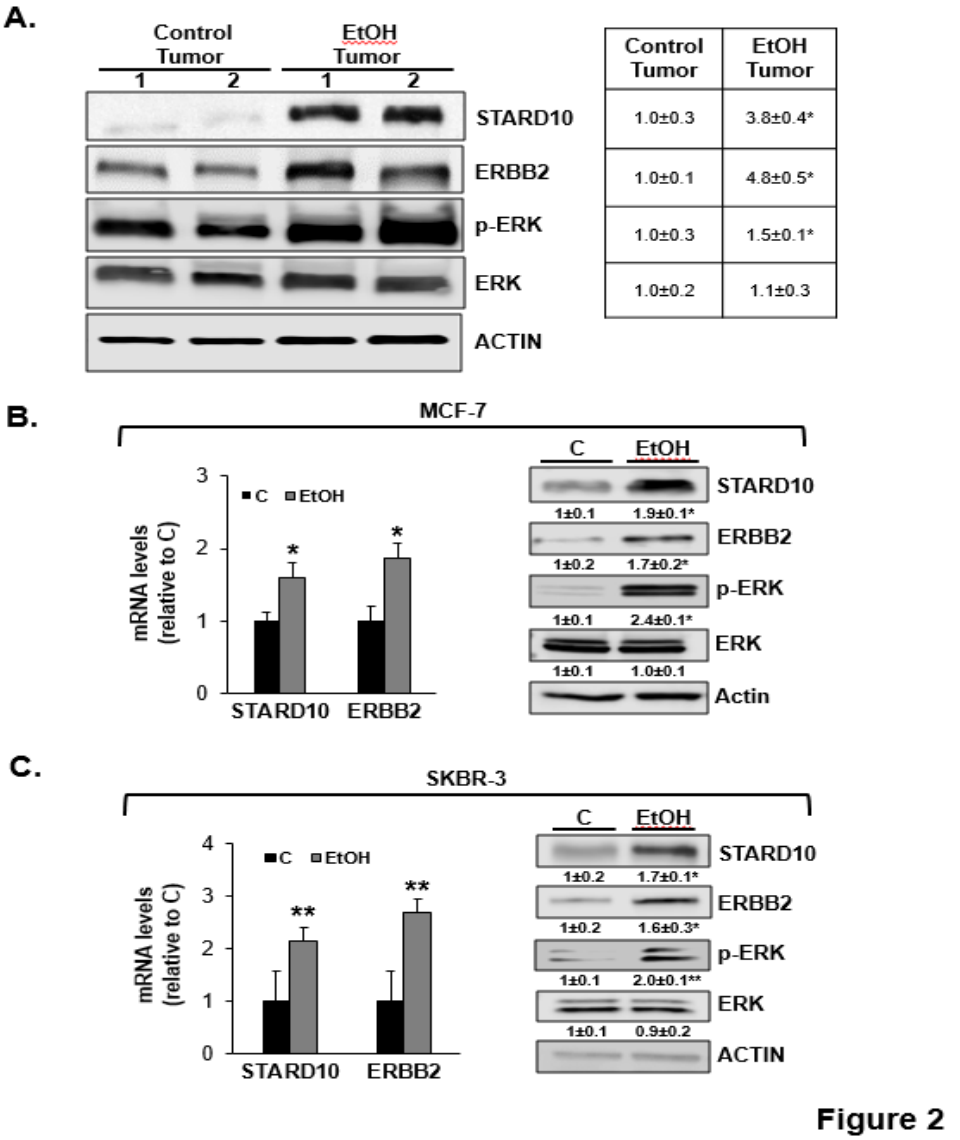

Figure 2

FIGURE 2. Ethanol induces STARD10 expression in vivo and in vitro. (A) Western blotting analysis of STARD10 and ERBB2 expression in ethanol-fed MMTV-neu transgenic mice. Densitometric ratios normalized to actin are shown in the right panel. Results are expressed as fold relative to control (mean±SE) from 4 mice per group. *p<0.05 *vs.* control group. **(B)(C)** MCF-7 and SKBR-3 cells were plated at density of 0.4 x 10^6 cells in 6-well/plates and treated with 100mM ethanol for 48 hours. The mRNA levels of *STARD10* and *ERBB2* in ethanol-treated MCF-7 and SKBR-3 cells were measured by RT-PCR and compared to control. STARD10, ERBB2, pERK, and ERK were analyzed using Western blotting. Results are expressed as fold relative to control (mean±SE) from 5 experiments. . *p<0.02 *vs* control left panel; *p<0.01 *vs.* control rigth panel.

STARD10-ERBB2 crosstalk in ethanol-promoted mammary tumor

Since our preliminary data proves that ethanol treatment leads STARD10 and ERBB2 upregulation in vivo and in vitro (**Figure 2**), we further explored the role of STARD10 in ethanol-induced tumor promotion to test the hypothesis that STARD10 and ERBB2 cooperate in ethanol induced breast cancer. We overexpressed *STARD10* for 24 hours in vitro which caused 3- and 1.6-fold induction of *ERBB2* mRNA levels in MCF-7 and SKBR-3 cell lines, respectively, when compared with empty vector control (**Figure 3A**), while forced expression of *ERBB2* caused a 2- and 1.8-fold increase in *STARD10* mRNA level in MCF-7 cells (**Figure 3A**) and SKBR-3 (**Figure 4A**), compared to control vector. Similar results were found at protein levels. Specifically, STARD10 and ERBB2 overexpression raised the level of ERBB2 protein by 1.6- and 1.8-fold in MCF-7 cells (**Figure 3A**) and SKBR-3 cells (**Figure 4A**), respectively, compared to empty vector control. In contrast, STARD10 and ERBB2 co-overexpression caused 10- and 30-fold induction of *STARD10* and 10- and 50-fold induction of *ERBB2* mRNA levels, while at

protein levels we had not the corresponding change in both cell lines, compared to single overexpression alone (**Figures 3A** and **Figure 4A**). Moreover, we found that 48 hours *ERBB2* silencing prevented ethanol-induced STARD10 overexpression in both MCF-7 and SKBR-3 cell lines (**Figure 3A** and **Figure 4B**). This finding suggests that STARD10 and ERBB2 positively regulate each other's expression in breast cancer cells.

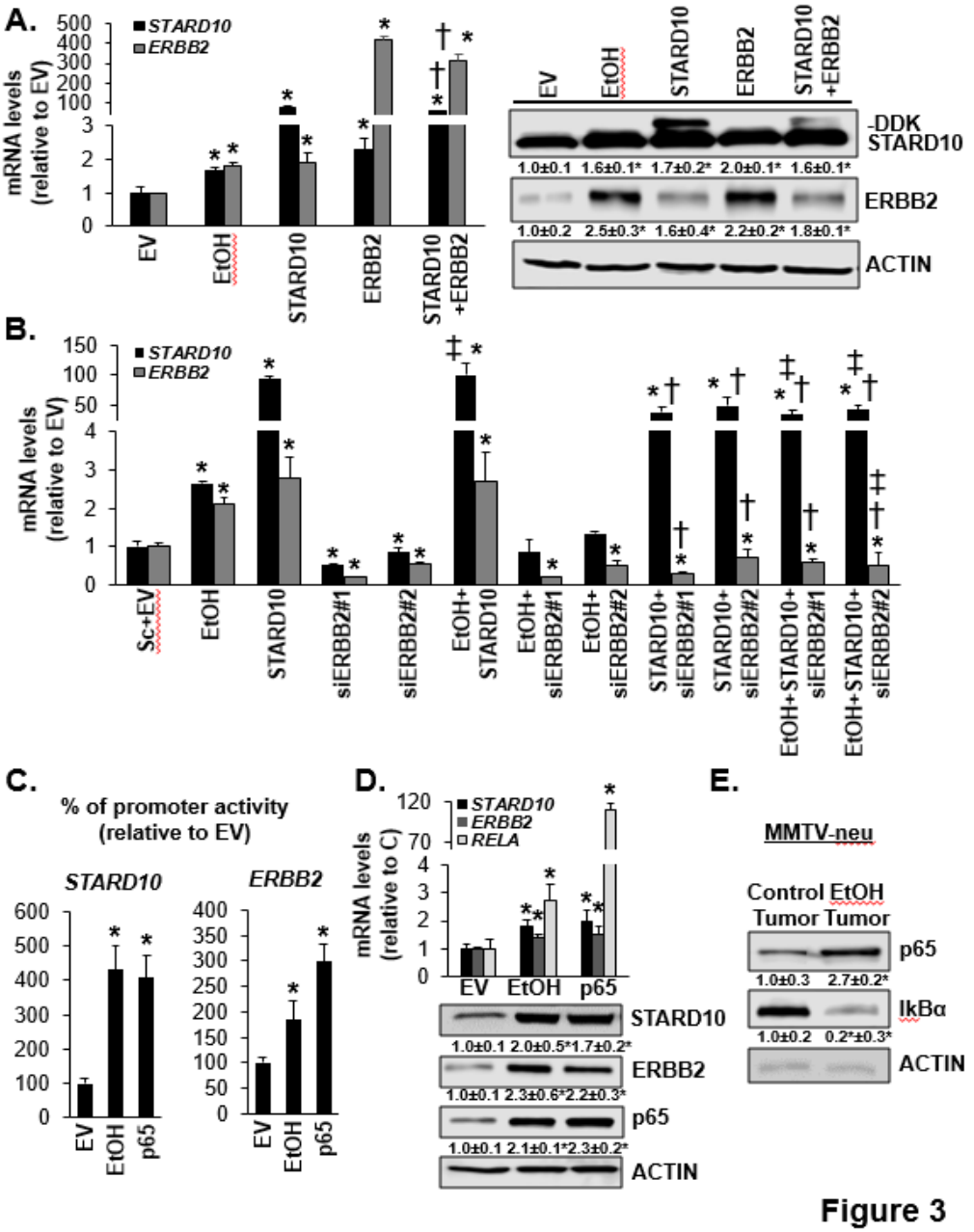

Figure 3

FIGURE 3. Ethanol-induced p65 positively regulates STARD10 and ERBB2 expression in MCF-7 and MMTV-neu. (A) mRNA analysis of *STARD10* and *ERBB2* was accomplished using RT-PCR in MCF-7 cells. 0.4 x 10^6 cells were transfected and treated with 100mM ethanol for 48 hours. Western blotting analysis was performed to measure STARD10 and ERBB2 compared to control from 4 indipendent experiments. *p<0.04 *vs.* EV. †p<0.05 *vs* STARD10 or ERBB2. **(B)** mRNA levels of STARD10 and ERBB2 in MCF-7 cells treated with ethanol (100mM) and transfected with STARD10 overexpression vector and ERBB2 siRNA (10nM) for 48 hours. Results are expressed as fold relative to Sc+EV (mean ± SE) from 3 independent experiments. *p<0.04 vs.Sc+EV; †p<0.04 vs. STARD10; ‡p<0.03 vs. EtOH. **(C)** *STARD10* and *ERBB2* promoter activity analysis in MCF-7

cells using reporter assay from 4 indipendent experiments. *P<0.04 *vs.* EV STARD10 promoter. **P<0.05 *vs.* EV ERBB2 promoter. **(D) upper panel**. RT-PCR analysis of STARD10 and ERBB2 expression. Cells were treated with 100mM ethanol or p65 transfection. *p<0.04 *vs.* EV. **Lower panel**. Western blotting analysis of STARD10, ERBB2, p65. Results are expressed as fold relative to EV (mean ± SE) from 3 independent experiments. *p<0.05 *vs.* EV. **(E)** Western blotting analysis of p65 and IkBα in ethanol-treated MMTV-neu mice. Results are expressed as fold relative to control (mean±SE) from 4 mice per group. *p<0.05 *vs.* control tumor.

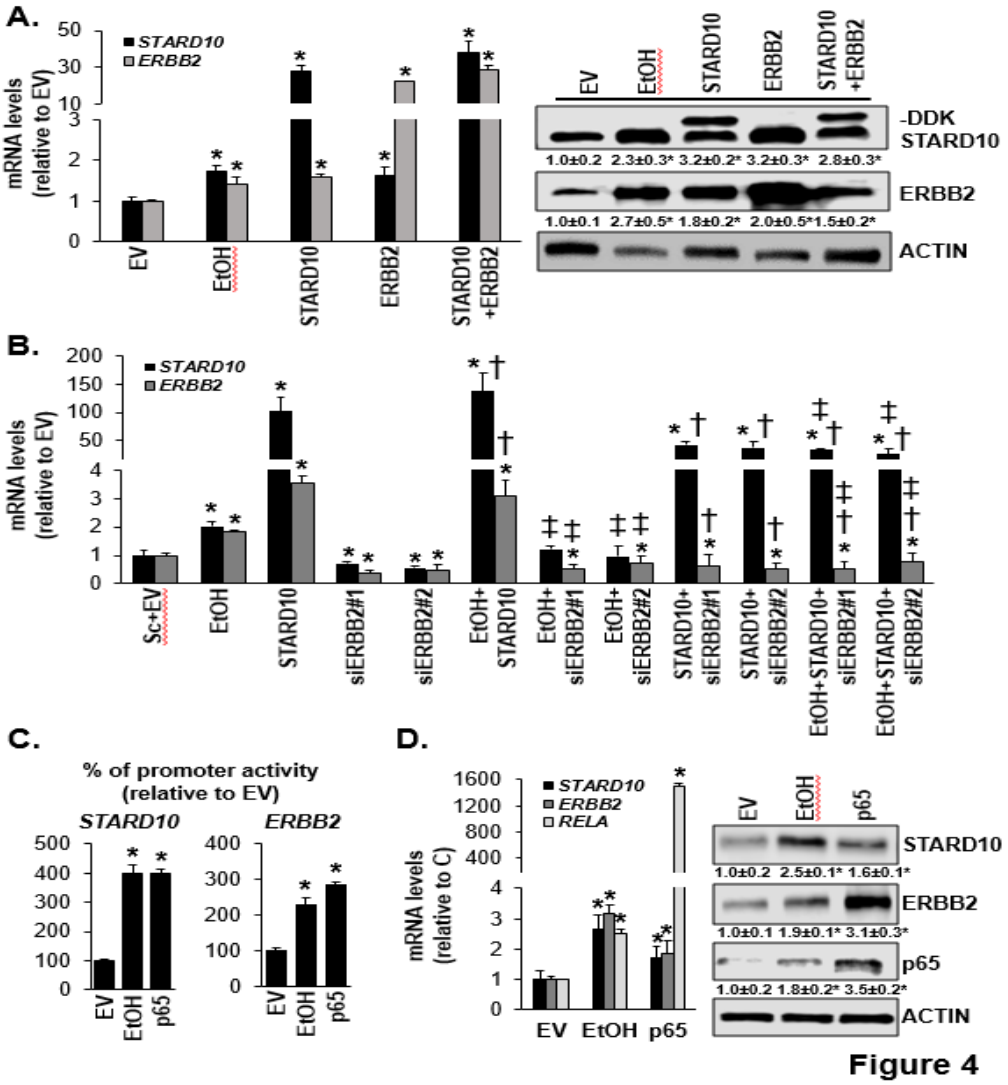

Figure 4

FIGURE 4. Ethanol-induced p65 that increases STARD10 and ERBB2 expression in SKBR-3 cells. (A) SKBR-3 cells were treated with 100mM ethanol and transfected with STARD10 and/or ERBB2 for 48 hours. STARD10 and ERBB2 mRNA and protein levels were accomplished using RT-PCR and Western blot analysis, respectively, compared to control from 4 independent experiments. *p<0.05

vs. EV. **(B)** mRNA levels of STARD10 and ERBB2 in SKBR-3 cells treated with ethanol (100mM) and transfected with STARD10 overexpression vector and ERBB2 siRNA (10nM) for 48 hours. Results are expressed as fold relative to Sc+EV (mean ± SE) from 3 independent experiments. *p<0.02 vs.Sc+EV; †p<0.01 vs. STARD10; ‡p<0.05 vs. EtOH. **(C)** *STARD10* and *ERBB2* promoter activity analysis was performed using reporter assay from 4 independent experiments. *p<0.02 *vs.* EV STARD10 promoter; *p<0.04 *vs.* EV ERBB2 promoter. **(D)** Cells were treated with 100mM ethanol or transfected with p65 plasmid for 48 hours. STARD10 and ERBB2 expression was analyzed by RT-PCR and Western Blotting analysis to measure their mRNA and protein levels. Results are expressed as fold relative to EV (mean ± SE) from 3 independent experiments. *p<0.05 *vs.* EV mRNA; *p<0.03 *vs.* EV proteins.

Ethanol-induced p65 expression promotes STARD10 and ERBB2 expression in vivo and in vitro

The stress-responsive transcription factor NF-κB is activated by a variety of cytotoxic conditions and it is considered to be the major downstream event of ERBB2 overexpression[23]. In order to investigate whether p65 was involved in ethanol-induced STARD10 and ERBB2 expression, PROMO^{TM} software[24] was used to predict the transcriptional factors (TFs) that could potentially bind and regulate both STARD10 and ERBB2 promoters. We provided evidence that in human *STARD10* promoter, p65, c-MYC, c-FOS and c-JUN are the predominant TFs that co-occupy this region (chr11:72791657-72795657) (**Figure 5A**). All the above overexpressed TFs positively regulated STARD10 expression (**Figure 5B and 5C**) in MCF-7 cells except

c-JUN even though several binding sites were found in *STARD10* promoter sequence (**Figure 5A**). One of the more interesting finding was that p65 had the stronger induction at protein level of STARD10 compared to the other TFs (**Figure 5D)**. Alcohol consumption is associated with higher expression of NF-kB p65 that stimulates tumor growth and aggressiveness[25]. Indeed, p65 overexpression had similar effect as ethanol treatment on STARD10 and *ERBB2* promoter activities that were induced by 4- and 3-fold in both MCF-7 and SKBR-3 cell lines, respectively, compared to empty vector. This was associated with a corresponding increase in *STARD10* and *ERBB2* expression levels (**Figure 3C-D-E** and **Figure 6C-D**). This finding was also confirmed analyzing the p65 protein level in the FVB MMTV Neu transgenic mice, where it was strongly induced by 2.7-fold in ethanol-fed mouse tumor compared to control tumor, in contrast, the inhibitory protein IkappaB-alpha (IkBα)[26] for p65 nuclear translocation decreased by 0.8-fold (**Figure 3E**).

In order to demonstrate that ethanol positively regulates both STARD10 and ERBB2 expression via p65 involvement, we performed the *RELA* gene silencing in vitro to test our hypothesis. **Figure 6A** and **Figure 7A** clearly show that ethanol required p65 to induce *STARD10* and *ERBB2* promoter activities in both MCF-7

and SKBR-3 cell lines, in addition this trend was confirmed by measuring the mRNA levels of these two genes (**Figure 6B** and **Figure 7B**). This data also confirmed previously published findings showing the ability of p65 to bind and regulate ERBB2 promoter[27].

Ethanol promotes p65 nuclear translocation and its binding to STARD10 and ERBB2 promoter sequences

Since NF-κB is also an important redox-sensitive TF and ethanol increased intracellular reactive oxygen species (ROS) level[28,29], we postulated that ethanol activates NF-κB signaling. NF-κB activation is associated with nuclear translocation of the p65 component of the complex and IκBα phosphorylation and degradation[30]. As show in **Figure 6C** and in **Supplementary Figure 3C**, ethanol induced both nuclear and cytoplasmic p65 NF-κB protein levels by 2.9- and 1.5-fold in MCF-7 and by 1.6- and 1.3-fold in SKBR-3, respectively, indicating that ethanol stimulated also total p65 in addition of nuclear translocation of p65 NF-κB. Ethanol also enhanced IκB-α decreased the levels of IκB-α. This finding was also confirmed in vivo ethanol treated MMTV-neu mice (**Figure 6E**). To support this hypothesis, we also

demonstrated that ethanol treatment strongly induces p65 binding to both *STARD10* and *ERBB2* promoter sequences in MCF-7 cells by 2.4- and 2.2-fold, and in MMTV-neu mice by 2.5- and 1.9-fold, respectively (**Figure 6D** and **6F**). These results indicated that ethanol exposure activated NF-κB signaling.

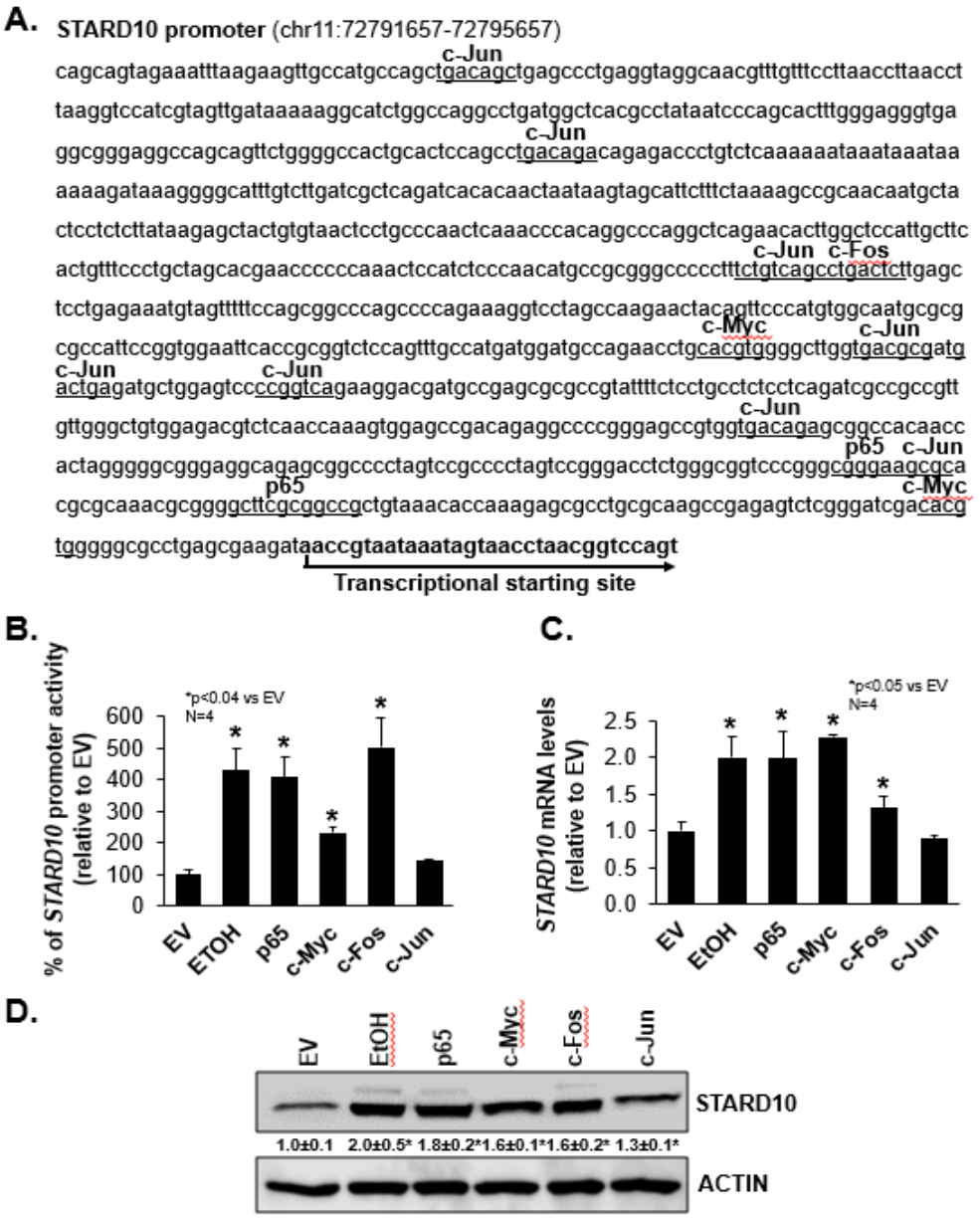

Figure 5

FIGURE 5. ERBB2 and its downstream targets overexpression positively regulate STARD10 expression in MCF-7 cells. Cell were treated with 100mM ethanol for 48 hours. **(A)** ERBB2 downstream targets binding sites on human *STARD10* promoter sequence. **(B)** *STARD10* promoter activity by reporter assay. **(C)** RT-PCR of StarD10 mRNA level. Results are expressed as percentage relative to EV for promoter analysis and as fold relative to EV for mRNA level. Statistically significant in four independent experiments. *p<0.04 *vs* EV promoter; *p<0.05 *vs* EV mRNA. **(D)** Protein levels were examined by Western blotting using an anti-STARD10 antibody. Results were expressed as fold relative to EV. Data are

expressed as (mean ± SE) from triplicate of four independent experiments. *p<0.05 *vs.* EV.

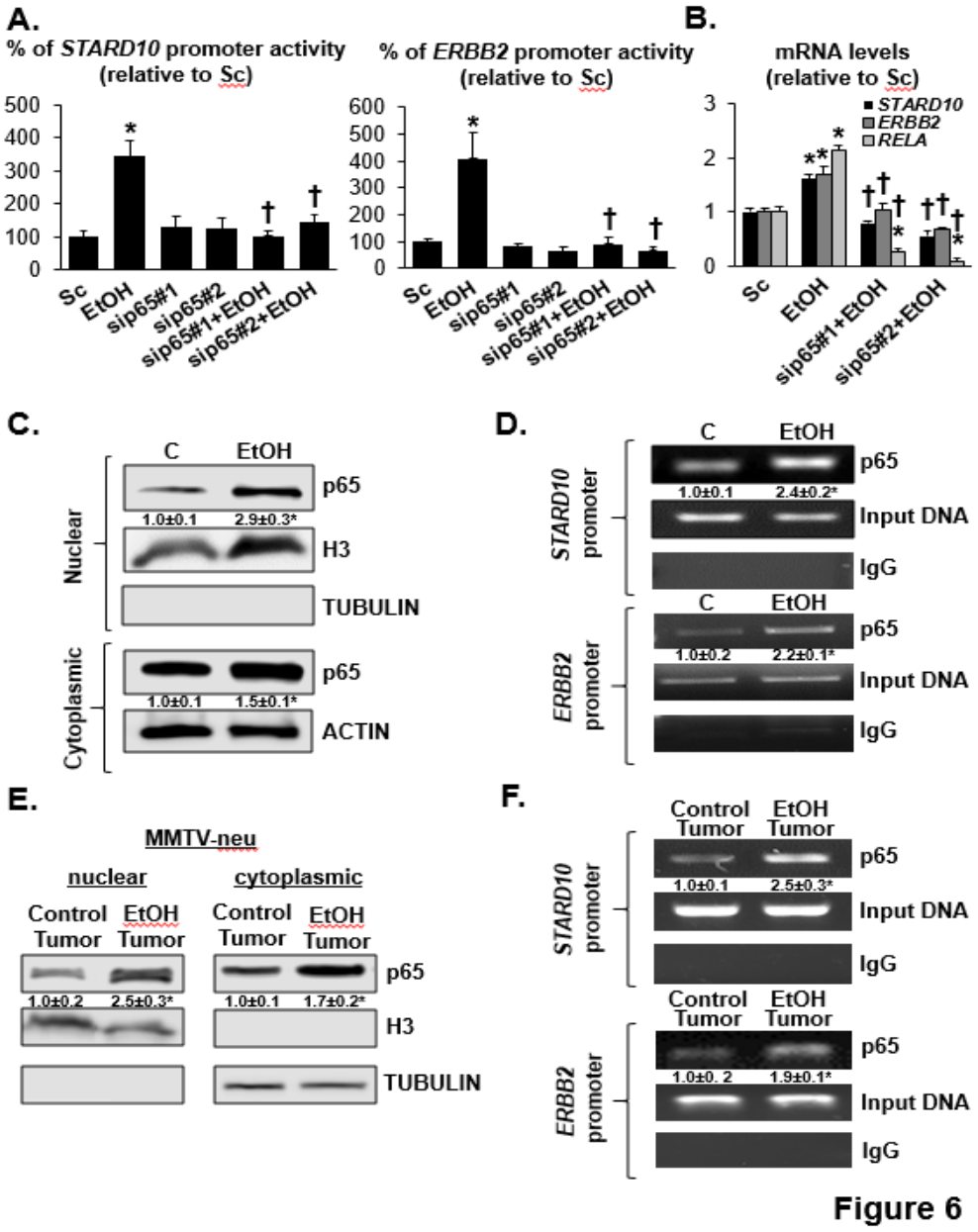

Figure 6

FIGURE 6. p65 positively regulates ethanol-induced STARD10 and ERBB2 expression binding their promoter sequences in MCF-7 cells. (A) *STARD10* and *ERBB2* promoter activity assay and RT-PCR for STARD10 and ERBB2 mRNA levels were measured in MCF-7 cells treated with 100mM ethanol and transfected with sip65 (10nM) for 48 hours.. *p<0.03 *vs.* Sc. †p<0.04 *vs.* EtOH for STARD10 promoter; *p<0.04 *vs.* Sc. †p<0.04 *vs*. EtOH for ERBB2 promoter. *p<0.004 *vs.* Sc; †P<0.03 *vs*. EtOH for mRNA levels. **(B)** mRNA levels of *STARD10*, *ERBB2* and

RELA measure by RT-PCR from 3 indenpendet experiments. *p<0.004 vs. Sc; †p<0.03 vs. EtOH. **(C)** Western blotting analysis of p65 in nuclear and cytoplasmic fractions. Nuclear marker (H3) and cytosolic marker (tubulin) were immunoblotted to demonstrate fraction purity. Data are expressed as (mean ± SE) from triplicate of 4 independent experiments. *p<0.05 *vs.* control. **(D)** ChIP analysis for p65 binding to *STARD10* and *ERBB2* promoters. Input genomic DNA (Input DNA) was used as a positive control, while non-specific antibody IgG was used as a negative control. Results are summarized as the densitometric changes as fold of control after normalizing to input DNA. *p<0.03 *vs.* control. **(E)** Western blotting analysis of p65 from nuclear and cytoplasmic fractions from ethanol-fed MMTV-neu transgenic mice tumor. Results are expressed as fold relative to control tumor (mean±SE) from 4 mice per group. *p<0.05 *vs.* control tumor. **(F)** ChIP analysis of p65 binding to ERBB2 and STARD10 promoters in MMTV-neu ethanol-fed transgenic mice tumor. Results are summarized as the densitometric changes as fold of control after normalizing to input DNA. *p<0.04 vs. control.

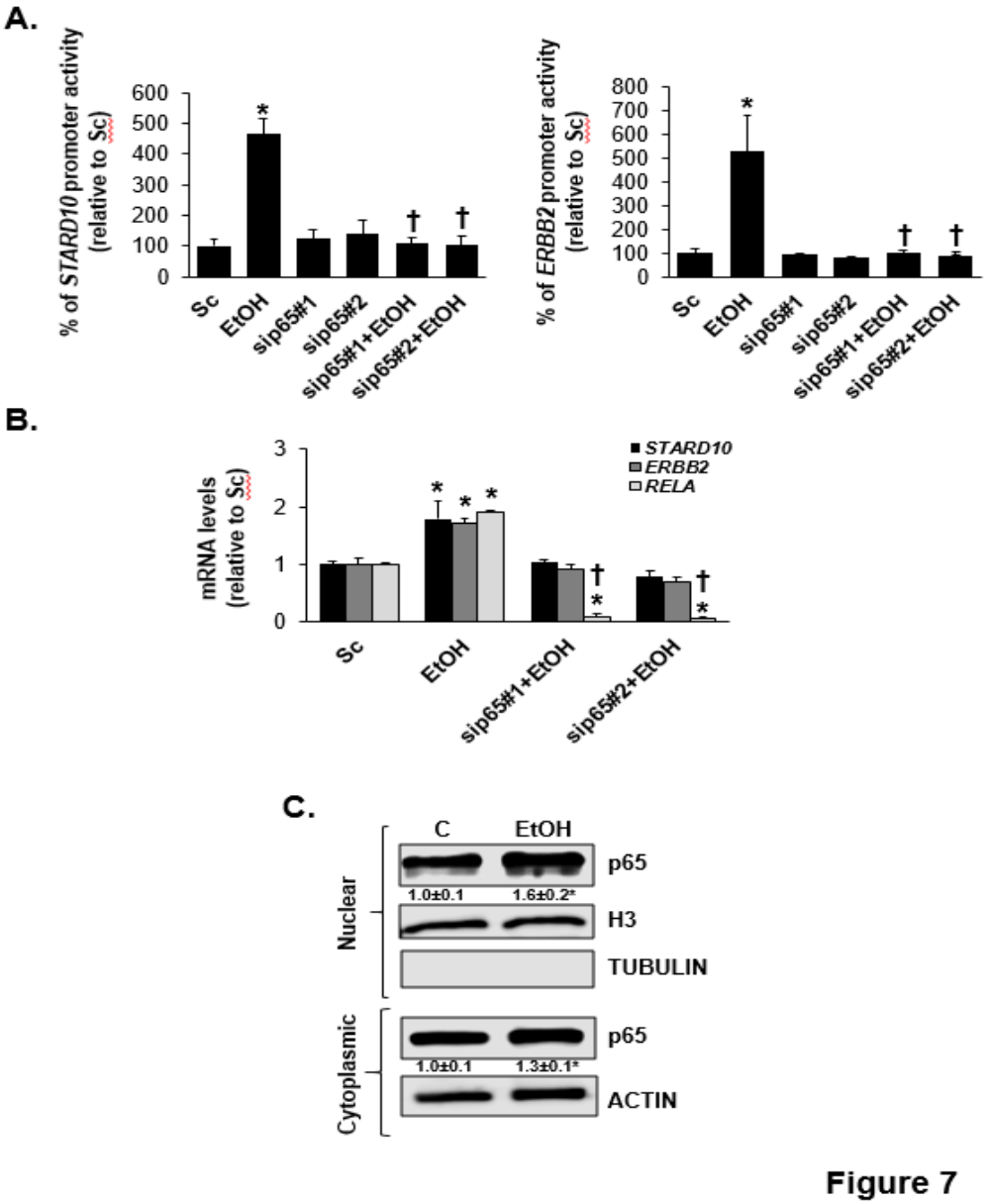

Figure 7

FIGURE 7. p65 positively regulates ethanol-induced STARD10 and ERBB2 expression binding their promoter sequence in SKBR-3 cells. Cells were treated with 100mM ethanol and transfected with p65 siRNA (10nM) for 48 hours (**A)** *STARD10* promoter activity assay. *p<0.003 *vs.* Sc. †p<0.003 *vs.* EtOH. ERBB2 promoter activity assay. *p<0.04 *vs.* Sc. †p<0.04 *vs*. EtOH. **(B**) Relative expression of *STARD10*, *ERBB2*, and *RELA* mRNA and the efficiency p65 silencing were determined by qRT-PCR; *p<0.05 *vs.* Sc. †p<0.0003 *vs.* EtOH. **(C)** Nuclear and cytoplasmid p65 protein level were analyzed by Western blotting. Nuclear marker (H3) and cytosolic marker (tubulin) were immunoblotted to demonstrate fraction purity. Data are expressed as (mean ± SE) from triplicate of four independent experiments. *p<0.05 *vs.* control.

Ethanol lowers CKII activity in breast cancer

CKII has been described to be a key negative regulator of STARD10 modulating is phosphorylation status[31]. In order to explore the role of ethanol on STARD10 phosphorylation/activation, MCF-7 and SKBR-3 cell lines were exposed to 100mM of ethanol for 48 hours and then phospho-fraction was separated by column chromatography as described in material and methods. The results show that ethanol increased the STARD10 unphosphorylated fraction by 5-fold compared to the control and correspondingly decreased the STARD10 phosphorylated fraction by 90% compared to the control (**Figure 8A**). Even though, we found that ethanol has no effects on CKII expression (**Figure 8B** and **8C**), its enzymatic activity decreased after ethanol administration by 40% and 20% in MCF-7 and SKBR-3, respectively (**Figure 8D**). These results have been confirmed in vivo MMTV-neu transgenic mice tissues (**Figure 8E** and **8F**).

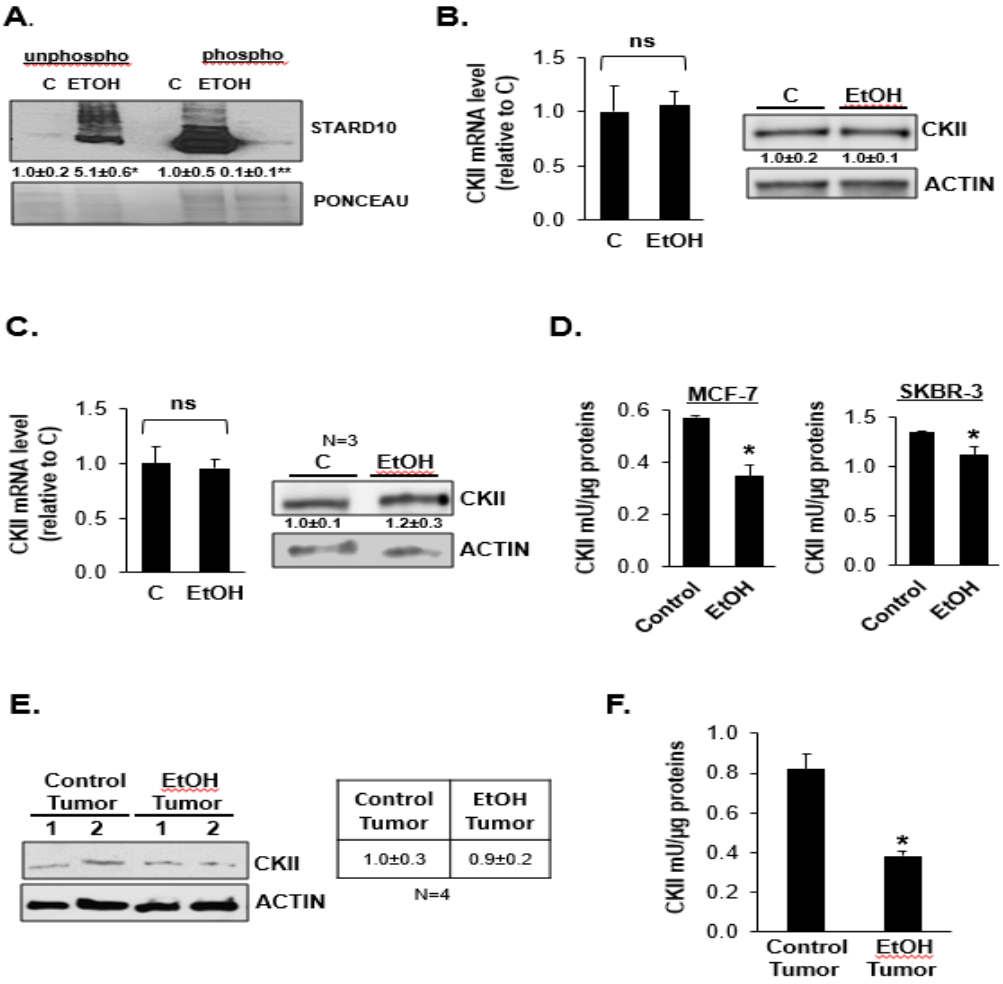

Figure 8

FIGURE 8. Effect of ethanol on CKII expression and/or activity in vitro and in vivo. (A) Phosphorylated and unphopshorylated STARD10 in ethanol-treated MCF-7 cells protein extracts (100mM for 48 hours). STARD10 protein levels were analyzed by Western Blotting. The results are expressed as fold relative to control from 3 independent experiments and normalized with red pounceau. **(B) (C)** CKII mRNA and protein levels were measured by RT-PCR and Western blotting, respectively in MCF-7 and SKBR-3 cell lines. CKII protein was normalized with β-actin expression. Three independent experiments were performed in triplicates. **(D)** The CKII enzymatic activity was evaluated as absorbance at 450nm of cell lysate in modified kinase reaction buffer and normalized with standard in MCF-7 and SKBR-3 cells treated with 100mM ethanol for 48 hours. Data are expressed as (mean ± SE) from 4 independent experiments performed in triplicates. *p<0.01 *vs.* control. **(E)** Western blotting analysis of CKII in ethanol-fed MMTV-neu mice tumors. Densitometric ratios normalized to actin is shown in the right panel. Four mice per group were used.

(F) CKII enzymatic activity assay in MMTV-neu was normalized with standard from 4 mice per group. *p<0.005 *vs.* control tumor.

Forced expression of STARD10 and ethanol administration increase membrane fluidity in MCF-7 and SKBR-3 cell lines

It is well-know that ethanol can influence cell migration and invasion in vitro that modulates cellular viability, proliferation, migration, and invasion in cancer cells[21,10]. For this reason, membrane fluidity was assayed on live MCF-7 and SKBR-3 cell lines treated with 100mM ethanol or transiently transfected with STARD10 for 48 hours using a fluorescent probe. The use of lipophilic pyrene probes, that undergo excimer formation upon spatial interaction, is considered one of the best systems to study membrane fluidity[32]. Measuring the ratio of excimer (EM 470nm) to monomer (EM 372) fluorescence, a quantitative monitoring of the membrane fluidity was attained. The confocal microscopy images showed that ethanol increased the membrane fluidity by 1.4-fold in both cell lines (**Figure 9A**), and STARD10 forced expression resulted in 1.4- and 1.5-fold increases in fluidity (**Figure 9A** lower panel).

Ethanol and STARD10 mediate calcium transport that increases cytoplasmic calcium concentration

Previous reports have established the fact that increases in cell membrane fluidity cause an increase in calcium ion permeability[33]. For the first time, we confirmed that ethanol administration increases cytoplasmic calcium concentration by 2.2- and 1.2-fold in MCF-7 and SKBR-3 cell lines, respectively (**Figure 9B**). Also, we provide evidence that STARD10 overexpression enhanced membrane permeability, leading to increased calcium ion uptake by 2.5- and 1.3-fold in MCF-7 and SKBR-3 cell lines, respectively (Figure 6B). These results were confirmed in MMTV-neu transgenic mice that showed a 1.6-fold increase in calcium concentration in the ethanol group compared to control group (**Figure 9B** right panel).

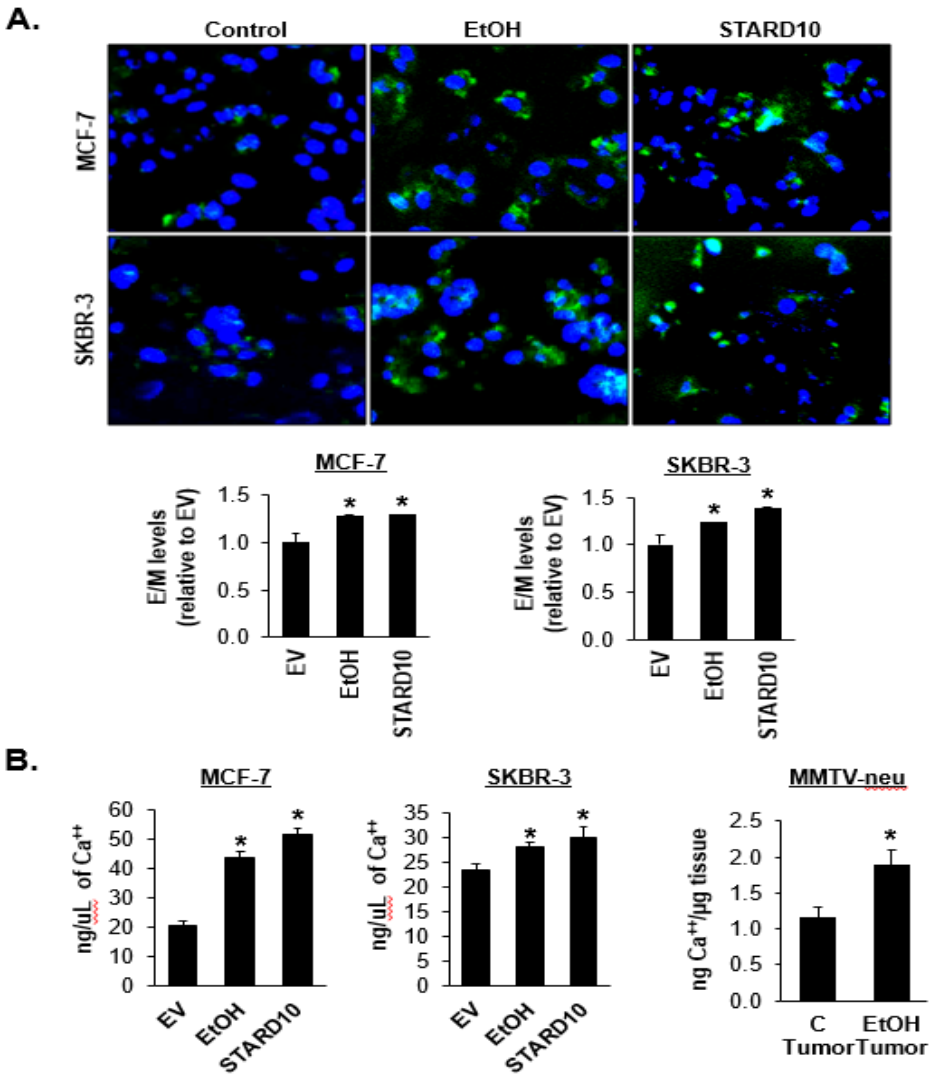

Figure 9

FIGURE 6. Ethanol and STARD10 overexpression increase membrane fluidity and intracellular calcium concentration in vitro and in vivo. (A) Cells were treated with ethanol (100mM) for 48 hrs or transfected with StarD10. Membrane fluidity assay in MCF-7 and SKBR-3 cells. Typical epifluorescence microscopic analysis was performed using an excitation filter (350nm), a dichroic filter (370nm) and emission filters for monomer fluorescence (405nm interference filter) and excimer fluorescence (470nm cut-on filter). The normalized fluorescence was calculated as a ratio of excimer to monomer. **(B)** Calcium ion concentrations in cell lysates from ethanol-treated or STARD10 transfected MCF-7 and SKBR-3 cells (100mM for 48 hours). Calcium ion concentration in breast tumor cell lysates from 4 ethanol-fed MMTV-neu transgenic mice. All data are expressed as (mean ± SE) from 3 independent experiments performed in triplicates. *p<0.05 *vs.* EV MCF-7 and SKBR-3; *p<0.04 vs control tumor MMTV-neu.

Mechanism of action of ethanol, ERBB2 and STARD10 in breast cancer cell growth and migration

Several reports demonstrated that ethanol stimulates both cell proliferation and migration of breast cancer cells[10]. Also, increased ERBB2 expression seems to be correlated with ethanol stimulation[21]. In order to demonstrate that ethanol promotes cell growth and migration via induced-expression of STARD10 and ERBB2, MCF-7 and SKBR3 cell lines were treated for 48 hours with 100mM ethanol. MTT assay was performed to determine the effect of STARD10, ERBB2 and ethanol on cell proliferation, which revealed that STARD10 overexpression enhanced the viability of the mammary tumor cells compared to control in a manner similar to ethanol administration and ERBB2 overexpression (**Figure 10A** and **Figure 11B**). We also proved that ethanol requires ERBB2 expression to sustain STARD10-induced cell growth (**Figure 10B** and **Figure 11B**). Wound-healing assay clearly show that ethanol exposure promoted cell migration by 20% compared to control in both MCF-7 and in SKBR-3 cell lines (**Figure 10C** and **Figure 11C**). Similarly, the ectopic expression of STARD10 and ERBB2 markedly enhanced the cells' migration ability compared to the control **(Figures 10C** and **Figure 11C)**.

STARD10 and ERBB2 co-overexpression caused an induction of migration level, without a corresponding change in viability in both cell lines, compared to single overexpression alone (**Figures 10** and **Figure 11**) suggesting us that ERBB2 promoted this migratory event because STARD10 overexpression. To further investigate whether inhibiting STARD10 expression could prevents ethanol-induced cell growth and migration via ERBB2 expression, we performed loss-of-function studies by silencing *STARD10* by siRNA in MCF-7 and SKBR3 cell lines. The transfection efficiency was determined by qRT-PCR. The efficiency of *STARD10* siRNA#1 was higher than *STARD10* siRNA#2 (**Figure 12A** and **12D**). Surprisingly the results indicated that inhibition of STARD10 significantly increased the growth rate of both cell lines compared to scramble siRNA (**Figure 12B** and **12E**). Similar results were observed in migration capability of MCF-7 and SKBR-3 cells (**Figure 12C** and **12F**). The results suggest that the steadiness of STARD10 is important for regulating the proliferative activity in breast cancer and its dysregulation leads to an increase in cell proliferation and migration with consequent increase in neoplastic progression. The findings and proposed scheme of events are summarized in **Figure 10D**.

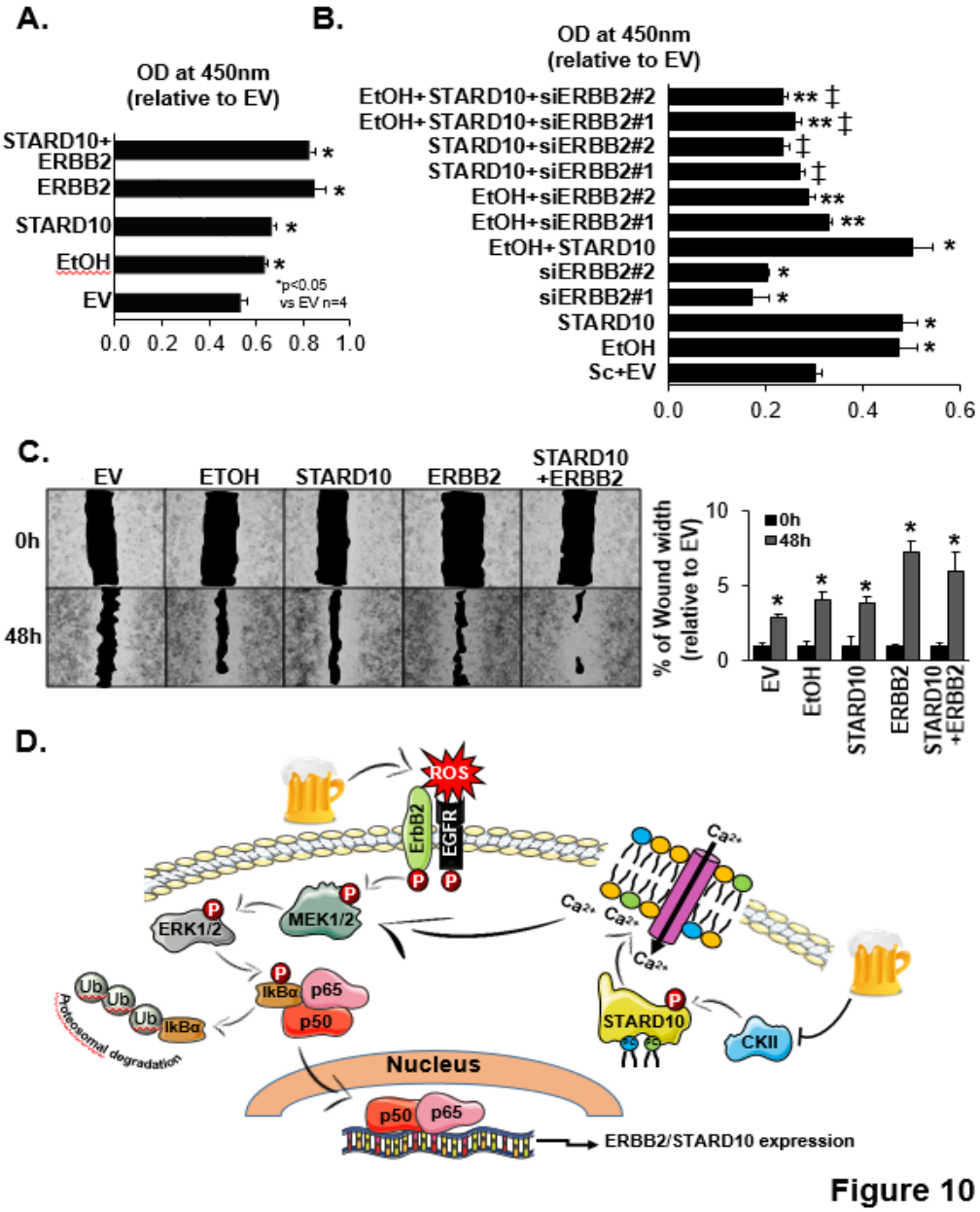

Figure 10

FIGURE 10. Ethanol administration, STARD10 and ERBB2 overexpression promote breast cancer cell malignancy in MCF-7 cells. MCF-7 cells were treated with 100mM ethanol and transfected with STARD10 and/or ERBB2 for 48 hours **(A) (B)** MTT assay showing data are expressed as (mean ± SE) from 3-4 independent experiments performed in triplicates. *p<0.05 *vs.* EV. **(C)** Migration assay. Results are shown as total wound area at 0 hrs and 48 hours. Data are expressed as (mean ± SE) from 4 independent experiments performed in triplicates. *p<0.05 *vs.* EV 48 hours. **(D)** Pathway schematic depicting known intracellular signaling mechanisms activated downstream of the ethanol administration proposed to mediate cell proliferation as well as cell migration through STARD10 and ERBB2 activation.

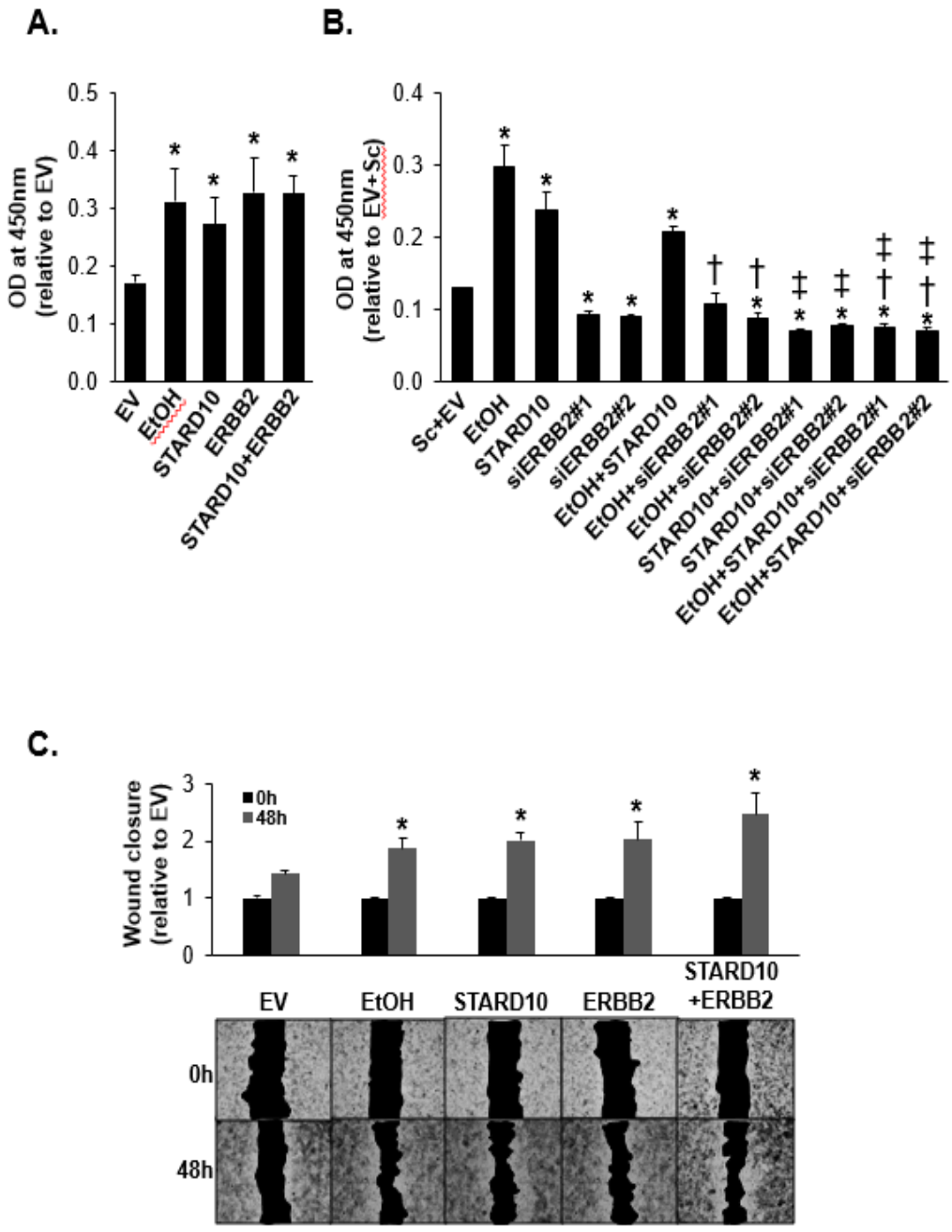

Figure 11

FIGURE 11. Ethanol administration, STARD10 and ERBB2 overexpression promote cell malignancy in SKBR-3 cells. Cells were treated with 100mM ethanol and transfected with STARD10 and ERBB2 plasmids or *ERBB2* siRNA (10nM) for 48 hours **(A) (B)** MTT assay. Data are expressed as (mean ± SE) from 4-5 independent experiments performed in triplicates. *p<0.04 *vs.* EV; *p<0.01 vs. Sc+EV; †p<0.02 vs. EtOH; ‡p<0.02 vs. STARD10. **(C)** Migration assay. Results are

shown as total wound area at 0 hrs and 48 hrs. Data are expressed as (mean ± SE) from 5 independent experiments performed in triplicates. *p<0.05 *vs.* EV 48 hrs.

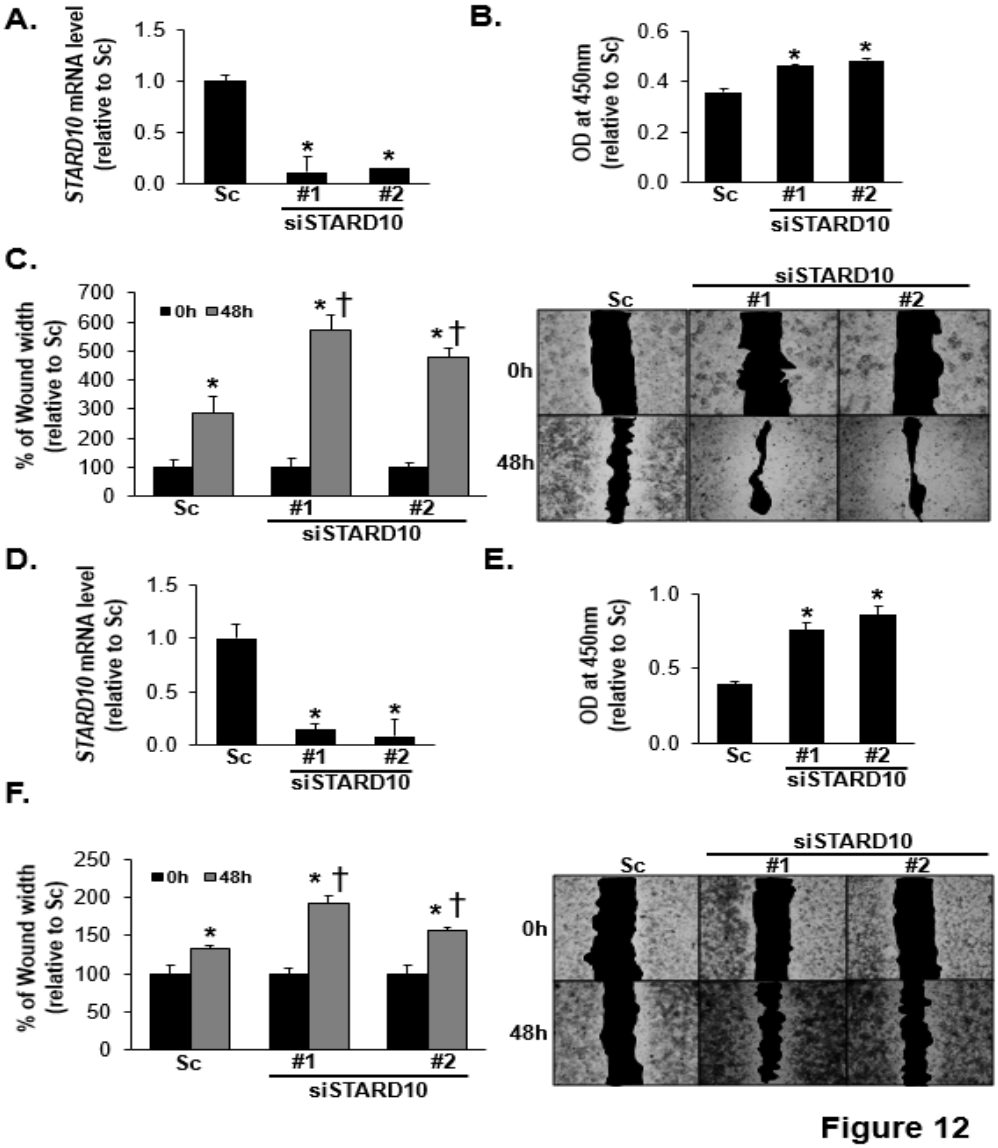

FIGURE 12. STARD10 silencing increases cell malignancy in vitro. MCF-7 and SKBR-3 cells were transfected with two different *STARD10* siRNAs (10nM) for 48 hours. **(A)(D)** Efficiency of *STARD10* silencing was determined by qRT-PCR. Data are expressed as (mean ± SE) from 3 independent experiments performed in triplicates.*p<0.001 *vs*. Sc MCF-7 cells; *p<0.001 *vs*. Sc SKBR-3 cells **(B)(E)** MTT assay showing viability of MCF-7 cells transfected with two different StarD10 silencers. Data are expressed as (mean ± SE) from 3 independent experiments performed in triplicates. *p<0.04 *vs.* Sc MCF-7 cells; *p<0.03 *vs.* Sc SKBR-3 cells. **(C)(F)** Graphs of migration assay. Data are expressed as (mean ± SE) from 4 independent experiments performed in triplicates. 48 hrs. Results are shown as percent of wound with compared to Sc at 0 hrs and 48 hrs. Data are expressed as

(mean ± SE) from 3 independent experiments performed in triplicates. *p<0.04 *vs.* Sc 0 hours MCF-7 cells; †p<0.05 *vs*. Sc 48 hours MCF-7 cells. *p<0.05 *vs.* Sc 0 hours; †P<0.05 *vs*. Sc 48 hours SKBR-3 cells.

DISCUSSION

Alcohol abuse has been reported to promote mammary tumorigenesis enhancing cell growth in vitro and in vivo[21,34]. In addition to its carcinogenic effect, alcohol abuse is associated with progression and aggressiveness of existing mammary tumors[35]. Mammary tissues and breast cancer cells normally metabolize alcohol by CYP2E1, ADH, xanthine oxidoreductase (XOR), and NOX which produces ROS, causing oxidative stress[36,37]. Human breast cancer cells or mammary epithelial cells with a high expression of receptor tyrosine-protein kinase ERBB2 exhibited an enhanced response to ethanol-stimulated cell invasion in vitro[7], therefore ethanol stimulates ROS production in mammary epithelial cells in an ERBB2-dependent manner[38]. ERBB2 belongs to the epidermal growth factor receptor (EGFR) family and plays an important role in cell proliferation and transformation through formation of heterodimers with EGFR and HER3[39]. No known ligand has been identified for ERBB2, ethanol induces its

phosphorylation that activates the mitogen-activated protein kinase MAPK signaling members, extracellular signal-regulated kinase ERK and other several important signaling cascades well-known to be downstream target of ERBB2 that play a key role in the carcinogenesis and aggressiveness of breast cancer[40]. STARD10 is a specific lipid carrier for PC and PE, is well-known to be overexpressed in Neu/ErbB2-induced mammary tumors in transgenic mice, in several human breast carcinoma cell lines, and in 35% of primary human breast cancers[18]. It was found to be co-expressed with ERBB2 in Neu tumors and human breast carcinoma cell lines and was demonstrated to cooperate with ErbB pathway in cellular transformation[19]. In this paper we tried to elucidate the mechanism by which ERBB2/STARD10 crosstalk promotes ethanol induced cell growth and migration in breast cancer cells. We also provide evidence that the common transcription factor p65 is involved in mediating co-expression of STARD10 and ERBB2. Our results indicate a mutual induction of STARD10 and ERBB2 that positively regulates ethanol-induced malignancy/aggressiveness phenotype. This is supported by the finding that MCF-7 and SKBR-3 cell lines are more susceptible to cell growth and migration when treated with ethanol, which induces both STARD10 and ERBB2 and also overexpressing these

key players. In resting cells, NF-kB is cytoplasmic sequestered as a latent complex bound to one or more members of the IkB protein family (IkBa, IkBb, IkBe, IkBg). Ethanol stimuli through ERBB2 phosphorylation activates the mitogen activated protein kinase (MAPK) signaling members than induce phosphorylation via activation of the IkB kinase complex, IKK) and subsequent proteasomal degradation of IkB inhibitory proteins, activating NF-kB for nuclear translocation. In the nucleus the p65/p50 heterodimer binds *ERBB2* promoter-specific consensus DNA elements[27] and for the first time we provide evidence that p65 also binds to *STARD10* promoter positively regulating its transcription. STARD10 transfers PC and PE between membranes, replenishing membranes with lipids metabolized by phospholipases. Lipids are delivered via monomeric exchange between the cytosolic membrane surfaces of different organelles. Monomeric exchange requires desorption of the lipid from the donor membrane, passage through the aqueous phase, and subsequent insertion into the acceptor membrane[41]. This is the first report demonstrating that the increased STARD10 protein amount can change the membrane fluidity with a consequent increase in membrane permeability to calcium ions (Ca^{2+}). It is well known that elevated intracellular Ca^{2+} triggers numerous signaling pathways including protein

kinases such as the calmodulin-dependent kinases (CaMKs) and the extra-cellular signal-regulated kinases (ERKs)[42]. These results support the novel hypothesis is that STARD10 is a key mechanism to induce ERBB2 via its function as a lipid transporter.

REFERENCES

1. Chlebowski RT, Luo J, Anderson GL, Barrington W, Reding K, Simon MS, Manson JE, Rohan TE, Wactawski-Wende J, Lane D, Strickler H, Mosaver-Rahmani Y, Freudenheim JL, Saquib N, Stefanick ML. Weight loss and breast cancer incidence in postmenopausal women. *Cancer* **2018**. doi: 10.1002/cncr.31687.

2. Krishnamurti U, Silverman JF. HER2 in breast cancer: a review and update. Adv *Anat Pathol* **2014**;21:100-7. Review.

3. Llombart-Cussac A, Cortés J, Paré L, Galván P, Bermejo B, Martínez N, Vidal M, Pernas S, López R, Muñoz M, Nuciforo P, Morales S, Oliveira M, de la Peña L, Peláez A, Prat A. HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. *Lancet Oncol* **2017**;18:545-54.

4. Khan N, Afaq F, Mukhtar H. Lifestyle as risk factor for cancer: evidence from human studies. *Cancer Lett* **2010** ; 293:133–43.

5. McDonald JA, Goyal A, Terry MB. Alcohol Intake and Breast Cancer Risk: Weighing the Overall Evidence. *Curr Breast Cancer Rep* **2013**;5(3). doi: 10.1007/s12609-013-0114-z.

6. Kwan ML, Chen WY, Flatt SW, Weltzien EK, Nechuta SJ, Poole EM, Holmes MD, Patterson RE, Shu XO, Pierce JP, Caan BJ. Post-diagnosis alcohol consumption and breast cancer prognosis in the after breast cancer pooling project. *Cancer Epidemiol Biomarkers Prev* **2013**;22:32–41. doi: 10.1158/1055-9965.EPI-12-1022

7. Xu M, Wang S, Ren Z, Frank JA, Yang XH, Zhang Z, Ke Z, Shi X, Luo J. Chronic ethanol exposure enhances the aggressiveness of breast cancer: the role of p38γ. *Oncotarget* **2016**;7:3489–505.

8. Roswall N, Weiderpass E. Alcohol as a Risk Factor for Cancer: Existing Evidence in a Global Perspective. *J Prev Med Public Health* **2015**;48:1–9.

9. Wang J, Heng YJ, A. Eliassen H, Tamimi RM, Hazra A, Carey VJ, Ambrosone CB, de Andrade VP, Brufsky A, Couch FJ, King TA, Modugno F, Vachon CM, Hunter DJ, Beck AH, Hankinson SE. Alcohol consumption and breast tumor gene expression. *Breast Cancer Res* **2017**;19:108.

10. Gelfand R, Vernet D, Bruhn K, Vadgama J, Gonzalez-Cadavid NF. Long-term exposure of MCF-12A normal human breast epithelial cells to ethanol induces epithelial mesenchymal transition and oncogenic features. *Int J Oncol* **2016**;48:2399–414.

11. Sidhanth C, Manasa P, Krishnapriya S, Sneha S, Bindhya S, Nagare RP, Garg M, Ganesan TS. A systematic understanding of signaling by ErbB2 in cancer using phosphoproteomics. *Biochemistry and Cell Biology* **2018**,96:295-305

12. Merlin JL, Harlé A, Lion M, Ramacci C, Leroux A. Expression and activation of P38 MAP kinase in invasive ductal breast cancers: correlation with expression of the estrogen receptor, HER2 and downstream signaling phosphorylated proteins. *Oncol Rep* **2013**;30:1943-8.

13. Alpy F, Tomasetto C. Give lipids a START: the StAR-related lipid transfer (START) domain in mammals. *Journal of Cell Science* **2005**;118:2791-2801

14. Ito M, Yamanashi Y, Toyoda Y, Izumi-Nakaseko H, Oda S, Sugiyama A, Kuroda M, Suzuki H, Takada T, Adachi-Akahane S Disruption of Stard10 gene alters the PPARα-mediated bile acid homeostasis. *Biochim Biophys Acta* **2013**;1831:459-68.

15. Olayioye MA, Hoffmann P, Pomorski T, Armes J, Simpson RJ, Kemp BE, Lindeman GJ, Visvader JE.The phosphoprotein StarD10 is overexpressed in breast cancer and cooperates with ErbB receptors in cellular transformation. *Cancer Res* **2004**:15;64:3538-44.

16. Gándola YB, Pérez SE, Irene PE, Sotelo AE, Miquet JG, Corradi GR, Carlucci AM, Gonzalez L. Mitogenic Effects of Phosphatidylcholine Nanoparticles on MCF-7 Breast Cancer Cells. *Biomed Res Int* **2014**;2014:687037.

17. Dawaliby R, Trubbia C, Delporte C, Noyon C, Ruysschaert JM, Antwerpen PV, Govaerts C. Phosphatidylethanolamine Is a Key Regulator of Membrane Fluidity in Eukaryotic Cells. *J Biol Chem* **2016**; 291(7): 3658–667.

18. Olayioye MA, Murphy NC, Biankin AV, Millar EK, McNeil CM, O'Toole SA, Segara D, Crea P, Olayioye MA, Lee CS, Fox SB, Morey AL, Christie M, Musgrove EA, Daly RJ, Lindeman GJ, Henshall SM, Visvader JE, Sutherland RL. Loss of STARD10 expression identifies a group of poor prognosis breast cancers independent of HER/Neu and triple negative status. Int J Cancer. **2010** Mar 15;126(6):1445-53. doi: 10.1002/ijc.24826

19. Olayioye MA, Vehring S, Müller P, Herrmann A, Schiller J, Thiele C, Lindeman GJ, Visvader JE, Pomorski T. StarD10, a START domain protein overexpressed in breast cancer, functions as a phospholipid transfer protein. *J Biol Chem* **2005** 22;280:27436-42.

20. Luo J, Miller MW. Basic fibroblast growth factor- and platelet-derived growth factor-mediated cell proliferation in B104 neuroblastoma cells: effect of ethanol on cell cycle kinetics. *Brain Res* **1997**:3;770:139-50.

21. Xu M, Ren Z, Wang X, Comer A, Frank JA, Ke ZJ, Huang Y, Zhang Z, Shi X, Wang S, Luo J. ErbB2 and p38γ MAPK mediate alcohol-induced increase in breast cancer stem cells and metastasis. *Mol Cancer* **2016**;15:52.

22. Roskoski R Jr. ErbB/HER protein-tyrosine kinases: Structures and small molecule inhibitors. *Pharmacol Res* **2014**;87:42-59. Review.

23. Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene* **2001**;20:1287-99.

24. Farré D, Roset R, Huerta M, Adsuara JE, Roselló L, Albà MM, Messeguer X. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res* **2003**;31:3651-3.

25. Wang F, Yang JL, Yu KK, Xu M, Xu YZ, Chen L, Lu YM, Fang HS, Wang XY, Hu ZQ, Li FF, Kan L, Luo J, Wang SY. Activation of the NF-κB pathway as a mechanism of alcohol enhanced progression and metastasis of human hepatocellular carcinoma. *Mol Cancer* **2015**;14:10. doi: 10.1186/s12943-014-0274-0.

26. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **1995**;9:2723-35.

27. Cao N, Li S, Wang Z, Ahmed KM, Degnan ME, Fan M, Dynlacht JR, Li JJ. NF-kappaB-mediated HER2 overexpression in radiation-adaptive resistance. *Radiat Res* **2009**;17:9-21.

28. Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal* **2005**;7:395-403.

29. Wu D, Zhai Q, Shi X. Alcohol-induced oxidative stress and cell responses. J *Gastroenterol Hepatol* **2006**;21:S26-9.

30. Kanarek N, Ben-Neriah Y. Regulation of NF-κB by ubiquitination and degradation of the IκBs. *Immunol Rev* **2012**;246:77-94.

31. Olayioye MA, Buchholz M, Schmid S, Schöffler P, Hoffmann P, Pomorski T. Phosphorylation of StarD10 on serine 284 by casein kinase II modulates its lipid transfer activity. *J Biol Chem* **2007**;282:22492-8.

32. Dix JA, Verkman AS. Pyrene eximer mapping in cultured fibroblasts by ratio imaging and time-resolved microscopy. *Biochemistry* **1990**;29:1949-53.

33. Los DA, Murata N. Membrane fluidity and its roles in the perception of environmental signals. *Biochim Biophys Acta* **2004**;1666:142-57. Review.

34. Lu Y, Ni F, Xu M, Yang J, Chen J, Chen Z, Wang X, Luo J, Wang S. Alcohol promotes mammary tumor growth through activation of VEGF-dependent tumor angiogenesis. *Oncol Lett* **2014**;8:673-678.

35. Williams LA, Olshan AF, Tse CK, Bell ME, Troester MA. Alcohol intake and invasive breast cancer risk by molecular subtype and race in the Carolina Breast Cancer Study. *Cancer Causes Control* **2016**;27:259-69.

36. Triano EA, Slusher LB, Atkins TA, Beneski JT, Gestl SA, Zolfaghari R, Polavarapu R, Frauenhoffer E, Weisz J. Class I alcohol dehydrogenase is highly expressed in normal human mammary epithelium but not in invasive breast cancer: implications for breast carcinogenesis. *Cancer Res* **2003**;63:3092-100.

37. El-Rayes BF, Ali S, Heilbrun LK, Lababidi S, Bouwman D, Visscher D, Philip PA. Cytochrome p450 and glutathione transferase expression in human breast cancer. *Clin Cancer Res* **2003**;9:1705-9.

38. Mohan N, Shen Y, Endo Y, ElZarrad MK, Wu WJ. Trastuzumab, but Not Pertuzumab, Dysregulates HER2 Signaling to Mediate Inhibition of Autophagy and Increase in Reactive Oxygen Species Production in Human Cardiomyocytes. *Mol Cancer Ther* **2016**;15:1321-31.

39. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* **2003**;11:495-505.

40. Sirkisoon SR, Carpenter RL, Rimkus T, Miller L, Metheny-Barlow L, Lo HW. EGFR and HER2 signaling in breast cancer brain metastasis. *Front Biosci* **2016**;8:245-63.

41. Levine T. Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions. *Trends Cell Biol* **2004**;14:483-90.

42. Schmitt JM, Wayman GA, Nozaki N, Soderling TR. Calcium activation of ERK mediated by calmodulin kinase I. *J Biol Chem* **2004**;279:24064-72.