High Concentration Calcitriol Induces Endoplasmic Reticulum Stress Related Gene Profile in Breast Cancer Cells

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Title: High Concentration Calcitriol Induces Endoplasmic Reticulum Stress Related Gene Profile in Breast Cancer Cells

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ABSTRACT

Calcitriol, the active form of vitamin D, is known for its anti-cancer properties including induction of apoptosis, inhibition of angiogenesis and metastasis. Understanding the mechanism of action for calcitriol will help development of novel treatment strategies. Since vitamin D exerts its cellular actions via binding to its receptor and by altering expressions of a set of genes, we aimed to evaluate the effect of calcitriol on transcriptomic profile of breast cancer cells. We previously demonstrated that calcitriol alters ER stress markers, therefore in this study we have focused on ER-stress related genes to reveal calcitriol's action on these genes in particular.

We have treated breast cancer cell lines MCF-7 and MDA-MB-231 with previously determined IC_{50} concentrations of calcitriol and evaluated transcriptomic alterations via microarray. During analysis only genes altered by at least 2 fold with a p value less than 0.05 were taken into consideration.

Our findings revealed an ER stress associated transcriptomic profile induced by calcitriol. Induced genes include genes with pro-survival function (NUPR1, DNAJB9, HMOX1, LCN2 and LAMP3) and with pro-death function (CHOP, DDIT4, NDGR1, NOXA and CLGN). These results suggest that calcitriol induces an ER-stress like response inducing both pro-survival and pro-death transcripts of the process.

Key Words: Calcitriol, Vitamin D, Cancer, ER stress, Unfolded Protein Response

INTRODUCTION

Calcitriol (1,25-dihydroxyvitamin D₃), the active form of vitamin D, is a secosteroid hormone responsible for regulation of calcium and phosphate metabolism (Adams and Hewison 2010). Calcitriol exerts its actions by binding to its receptor and acting as a transcription factor regulating various genes (Cheskis and Freedman 1994; Deeb et al. 2007). Calcitriol has potent anti-cancer properties such as inhibition of tumor growth, angiogenesis and metastasis, and induction of apoptosis shown in various types of cancer both *in vitro* and *in vivo* (Tsang et al. 2010). Apoptosis among these properties is known to be associated with ER stress (Schröder and Kaufman 2005).

Endoplasmic Reticulum (ER) is a membrane bound organelle responsible for several cellular functions including post-translational folding of proteins and calcium storage (Sitia and Braakman 2003). Accumulation of unfolded or misfolded proteins due to dysfunctional folding mechanisms is known as ER stress (Sitia and Braakman 2003). Cellular response to ER stress is referred as unfolded protein response (UPR) in which translation is slowed down, protein degradation is induced and folding capacity of ER is increased to maintain ER homeostasis (Sitia and Braakman 2003). UPR may act both as a survival pathway or apoptotic pathway depending on the severity of ER stress. If increased size and folding capacity of ER, effective elimination of unfolded proteins via endoplasmic reticulum-associated degradation (ERAD) and inhibition of protein production overcomes ER stress, homeostasis becomes restored and cell survives (Tsang et al. 2010). However excessive unfolded protein load can overwhelm UPR-associated survival mechanisms, initiating apoptotic cell death via signaling pathways such as CHOP and JNK (Szegezdi et al. 2006).

Microarray is a platform to evaluate variations in whole-genome expression profiles. We used this platform to analyze the effects of calcitriol on transcription profile of the breast cancer cells. We have showed a special interest in ER-stress related genes as we have previously determined that calcitriol alters common markers of UPR including XBP-1 splicing and BIP expression (Haddur et al. 2015).

MATERIALS AND METHODS

Cell Culture – All studied cell lines were kindly provided by Ege University Medical School Department of Medical Oncology. Breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in RPMI medium (Lonza) with 10% fetal bovine serum (Lonza) and 1% penicillin-streptomycin (Sigma) supplement. All cell lines were maintained in CO₂ incubator with standard incubation conditions. 10⁴ cells per well were seeded onto 96-well plates for viability experiments and 20x10⁴ cells per flask were seeded onto 25 cm² for other experiments. Cells were treated with different concentrations of calcitriol (Cayman) reconstituted in dimethyl sulfoxide (DMSO-Sigma) at 20 mM and cytotoxic concentration of calcitriol and appropriate concentration of vehicle (DMSO) were used.

Microarray Studies – 3 replicates of cells were treated with IC₅₀ concentrations of calcitriol (40 µM for MCF-7 and 50 µM for MDA-MB-231) which has been previously determined by us (Haddur et al. 2015), or appropriate concentrations of the vehicle (DMSO) for 24 hours. Cells were collected and total RNA was isolated with RNeasy Mini Kit (Qiagen). Microarray studies were carried out following instructions of WT Expression Kit (Ambion), GeneChip WT Terminal Labeling and Controls Kit (Affymetrix), and GeneChip Human Exon 1.0 ST Array Chip (Affymetrix). Hybridization of the chip was carried by using GeneChip Hybridization, Wash and Stain Kit (Affymetrix), and scanning was carried out with Scanner 3000 7G system (Affymetrix). Scanning results were evaluated by Partek Genomic Suite software. During analysis calcitriol treated cells are compared with vehicle (DMSO) treated cells (3 replicates) and statistical evaluation were carried out with one-way ANOVA analysis. Genes with at least 2-fold increase or decrease in expression and with p values less than 0.05 were included in final analyses. Raw data and analysis files are uploaded to GEO database (accession number: GSE53975).

RESULTS

Calcitriol Induces ER Stress Related Gene Profile – To determine the effects of calcitriol on other ER stress related genes we have carried out profiling studies in MCF-7 and MDA-MB-231 cells

after treatment with calcitriol for 24 hours. Only genes with at least 2 fold increase or decrease in expression and with p values less than 0.05 (according to one-way ANOVA results) were included in tables (tables 1-5). Table 1 and table 2 list upregulated and downregulated genes (respectively) in MCF-7 cell line. Table 3 and table 4 list upregulated and downregulated genes (respectively) in MDA-MB-231 cell line. Lastly, table 5 lists altered genes common in both cell lines.

Calcitriol treatment altered expression of 34 MCF-7 specific, 41 MDA-MB-231 specific and 10 shared genes (figure 1). CYP24A1 (1,25-dihydroxyvitamin D3 24-hydroxylase), an indicator of vitamin D activity and responsiveness (Lemay et al. 1995), elevated in both cell lines significantly as expected (table 3) proving the effect of calcitriol on both cell lines.

Investigation of elevated genes revealed an ER stress related alteration in transcriptomic level for both cell lines (figure 1). 11 of 38 (28.9%) significantly upregulated genes in MCF-7 and 7 of 41 (17.1%) significantly upregulated genes in MDA-MB-231 were previously reported to be regulated by or associated with ER stress (tables 1 and 3). This enrichment is even more evident in genes altered in both cell lines: 4 of 10 (40.0%) genes altered by calcitriol in both cell lines were ER stress associated. List of ER stress related genes altered by calcitriol in our study include: NUPR1, HMOX1, LCN2, LAMP3, DNAJB9, DDIT3, DDIT4, STC2, C4orf34, FTH1, NDRG1, PMAIP1, TRIB3 and CLGN.

Genes associated with survival/cell death pathways also altered by calcitriol. 9 of 38 (23.7%) upregulated genes in MCF-7 and 10 of 41 (24.4%) upregulated genes in MDA-MB-231 play a role in these processes (tables 1 and 3). Furthermore 1 of 10 downregulated genes (SGK1, 10.0%) in MDA-MB-231 cell line is known to play a role in apoptosis (table 4). List of survival/cell death associated genes altered by calcitriol include: NUPR1, LCN2, DNAJB9, NDRG1, DDIT3, DDIT4, GPR56, GDF15, PMAIP1, RGCC, TRIB3, G0S2, PDCD4, SGK1, MAPK13 and SNX33. It is important to point out many of the ER stress related genes induced by calcitriol also have functions in apoptosis or survival pathways.

We have also evaluated other functional pathways enriched by calcitriol such as angiogenesis and metastasis. 6 of 44 altered genes in MCF-7 and 6 of 51 altered genes are known to have a role in metastasis (tables 1-4). List include: HMOX1, LCN2, LAMP3, NDGR1, GPR56, GDF15, RPSA, MMP7, INHBA, IL8 and MAPK13. 4 of 38 upregulated genes in MCF-7 and 5 of 51 altered genes in MDA-MB-231 have been reported to be involved in angiogenesis (tables 1, 3 and 4). List include: HMOX1, LCN2, NDGR1, GPR56, GDF15, RGCC, THBS1 and IL8.

DISCUSSION

Even though calcitriol is known to inhibit cell viability at nanomolar concentrations (Goeman et al. 2014; Lee et al. 2006; Swami et al. 2003), IC_{50} of calcitriol in MCF-7 and MDA-MB-231 cell lines were determined respectively as 40 μ M and 50 μ M in our previous study (Haddur et al. 2015). Since IC_{50} concentrations depend on the number of seeded cells, incubation time, concentration and content of the serum used to supplement the media, the carrier of choice as well as other factors; it is best to determine an IC_{50} value specific to the laboratory and/or experimental design. Therefore instead of using established inhibitory concentrations of calcitriol we have decided to use a higher concentration determined in our laboratory in a previous study with similar conditions (Haddur et al. 2015).

Calcitriol is known for its the growth inhibitory (James et al. 1996), anti-angiogenic (Mantell et al. 2000) and anti-metastatic (Flanagan et al. 2003) effects on breast cancer cells. However, the role of calcitriol in different physiological processes such as ER stress is not very well known. We have previously demonstrated that calcitriol treatment alters XBP-1 splicing and BIP expression, two common markers of ER stress and UPR (Haddur et al. 2015). In this study we further analyzed this association by evaluating the effects of the calcitriol treatment on transcription profile of the cells. Treatment altered several ER stress related genes in both MCF-7 and MDA-MB-231 cell lines. It is known that ER stress may serve as a cell death pathway or a survival pathway for cancer cells under stress (Rutkowski et al. 2006; Tsang et al. 2010) and effects of calcitriol on ER stress related genes may be discussed in a similar manner. We have determined expressional alterations in genes involved

both in survival and apoptotic downstream of ER stress. Several of the genes induced by calcitriol (including NUPR1, DNAJB9, HMOX1, LCN2 and LAMP3) were pro-survival genes while others (including DDIT3, DDIT4, NDGR1, PMAIP1 and TRIB3) had pro-death functions. The most altered gene in MCF-7 was NUPR1 (nuclear protein 1), an ATF4 inducible transcription factor associated with ER stress (Jin et al. 2009). Its expression was 7.45 fold increased ($p=2.91 \times 10^{-5}$) in MCF-7 cells but remained unchanged in MDA-MB-231 cells (table 1). NUPR1 is a bad prognostic factor for breast cancer progression because of its role in metastasis, drug resistance, and protection of cells from stress induced cell death (Chowdhury et al. 2009). In our case NUPR1 expression was increased most probably due to calcitriol induced ER stress to protect cells from a possible apoptotic fate. DNAJB9 (DnaJ (HSP40) homolog subfamily B member 9) is an ER localized regulator of heat shock proteins and it protects cells from ER associated apoptosis (Kurisu et al. 2003). Calcitriol induced DNAJB9 expression significantly in MDA-MB-231 cells (2.27 fold, $p=0.034$) and slightly in MCF-7 cells (table 3). HMOX (heme oxygenase 1), another pro-survival ER stress related gene (Liu et al. 2005), was also elevated with calcitriol treatment, this time both in MCF-7 (3.94 fold increase, $p=1.44 \times 10^{-3}$) and in MDA-MB-231 (3.50 fold increase, $p=2.43 \times 10^{-3}$) cells (table 5). HMOX1 plays a role in angiogenesis (Sunamura et al. 2003), metastasis (Tsuji et al. 1999) and known to be induced by hypoxia (Lee et al. 1997). Similarly LCN2 (Lipocalin-2), an oncogenic gelatinase which plays a role in breast cancer progression (Yang et al. 2009), was significantly upregulated in MCF-7 (2.41 fold increase, $p=0.044$) and also increased in MDA-MB-231 (1.82 fold increase, $p=0.143$) cells after calcitriol treatment (table 1). As a metastatic (Leng et al. 2011) and angiogenic (Leng et al. 2009) ER stress response gene (Mahadevan et al. 2011), it would be logical to assume it was also induced by calcitriol as a part of pro-survival downstream of ER stress. It is important to note that a dual role for lipocalin-2 in cell death was proposed (Kehrer 2010) as the protein shows both anti-apoptotic and pro-apoptotic properties in a manner similar to ER stress. Finally, expression of another pro-survival gene LAMP3 (lysosomal-associated membrane protein 3) is induced in MCF-7 cells (2.39 fold increase $p=4.08 \times 10^{-3}$) but remained unchanged in MDA-MB-231 cells. LAMP3 is a hypoxia inducible UPR gene known to play a role in metastasis (Mujcic et al. 2009). Similar to LCN2, LAMP3 is also a bad prognostic factor for breast cancer (Nagelkerke et al. 2011). Taken together, induction of these stress induced pro-

survival genes in mRNA level may serve as a protection mechanism in cells treated with calcitriol, assuming increased transcription increases the activity of mentioned proteins.

Calcitriol also induced pro-apoptotic ER stress genes. DDIT3 (DNA-damage-inducible transcript) also known as CHOP (C/EBP homologous protein) is a well-known pro-apoptotic, ER stress responsive transcription factor (Okuda et al. 2012). The expression of DDIT3 significantly increased in MCF-7 (3.00 fold increase, $p=0.043$) and MDA-MB-231 (3.12 fold increase, $p=0.038$) cells in response to calcitriol (table 5). Similarly DDIT4 (DNA-damage-inducible transcript 4), another pro-apoptotic ER stress responsive transcription factor (Okuda et al. 2012), also upregulated by calcitriol in MCF-7 (2.71 fold increase, $p=0.038$) and MDA-MB-231 (4.90 fold increase, $p=0.042$) cells (table 5). Both genes are previously reported to be regulated by calcitriol (Campos et al. 2012; Lisse et al. 2011; Zhang et al. 2013). Another ER stress related gene induced by calcitriol in both cell lines was NDRG1 (N-myc downstream regulated 1) (MCF-7: 2.12 fold increase, $p=3.91 \times 10^{-3}$; MDA-MB-231: 1.69 fold increase, $p=0.023$) (Tables 3). NDRG1 is an anti-metastatic tumor suppressor and have a role in ER stress associated apoptosis (Segawa et al. 2002). NDRG1 is also previously reported to be regulated by calcitriol and believed to be important in anti-metastatic properties of the vitamin (Krishnan et al. 2004). Another tumor suppressor induced by calcitriol was PMAIP1 (Phorbol-12-myristate-13-acetate-induced protein 1) which is also known as NOXA. NOXA is a p53 inducible protein activated during ER stress to trigger apoptosis (Wang et al. 2009) and its expression was increased 2.51 fold ($p=1.22 \times 10^{-4}$) in MCF-7 cells (table 1). TRIB3 (Tribbles homolog 3), a gene involved in ER stress associated apoptosis (Szegezdi et al. 2006) was induced by calcitriol insignificantly in MCF-7 (2.20 fold increase, $p=0.122$) and significantly in MDA-MB-231 (2.01 fold increase, $p=0.021$) cells (table 3). TRIB3 is an inhibitor of AKT and has a role in sensitizing cells to apoptosis (Du et al. 2003). Induction of all these apoptotic ER stress inducible genes may explain most if not all of growth inhibitory effects of vitamin D. However, it is important to note that the data obtained represents variations in mRNA profile and since most of the signaling pathways are also regulated in protein or post translational level, these claims should be further investigated.

Certainly apoptotic or tumor suppressor genes induced by calcitriol were not limited to ER stress associated genes. While tumor suppressor GPR56 (G protein-coupled receptor 56) was induced

in MCF-7 cells (table 1), pro-apoptotic (growth differentiation factor 15) was upregulated in both cell lines (table 5). Effect of calcitriol on GDF15 is previously reported in prostate cells (Krishnan et al. 2004). Genes with similar function and induced by calcitriol in MDA-MB-231 cells include: pro-apoptotic G0S2, pro-apoptotic tumor suppressor PDCD4, p53 inducible inhibitor of cell cycle RGCC. The inducing effect of calcitriol on G0S2 was previously reported but only on smooth muscle cells (Wu-Wong et al. 2007). SGK1 (Serum and glucocorticoid-regulated kinase 1), an anti-apoptotic, proliferative protein activated by mTOR and PI3K signaling pathways, was inhibited by calcitriol in MDA-MB-231 cells (table 3). Similarly, expression of oncogenic ANP32D (Acidic leucine-rich nuclear phosphoprotein 32 family member D) was also inhibited by calcitriol (table 3). If represented in protein level, these alterations may explain growth inhibitory effects of calcitriol; however, associations of these proteins to ER stress response are yet to be determined. Interestingly expressions of three cancer related survival genes SNX33 (sorting nexin 33), MAPK13 (Mitogen-activated protein kinase 13) and GEM (GTP-binding protein) were also increased only in MDA-MB-231 cells after calcitriol treatment (table 3). Among these; only GEM has been previously reported to be regulated by calcitriol in breast cancer cells (Vanoirbeek et al. 2009). These inductions might be due to a cell lines specific stress response since MDA-MB-231 cells are known to be more aggressive and resistant compared to MCF-7 cells.

Anti-cancer effects of calcitriol also include inhibition of angiogenesis and metastasis. Some of the previously mentioned ER stress response genes are known to be involved in metastasis and angiogenesis. While induced stress response survival genes such as HMOX1, LCN2 and LAMP3 promote metastasis, ER stress associated anti-cancer protein NDGR1 inhibits the process. Angiogenesis is no different: while HMOX1 and LCN2 promote angiogenesis, NDGR1 inhibits it. These results suggest that the dual role of calcitriol induced ER stress may extend beyond cell death/survival decision to activation/inhibition of metastasis and angiogenesis. Growth related genes regulated by calcitriol also have a role in these processes.

Other than previously mentioned genes, several others involved in these processes were altered by calcitriol treatment. While metastatic RPSA (ribosomal protein SA) and INHBA (Inhibin,

beta A) were inhibited by calcitriol in MDA-MB-231 cells (table 4), expression of MMP7 (matrix metalloproteinase-7), a well-known metastatic protease, was downregulated in MCF-7 cells (table 2). IL8 is a pro-inflammatory cytokine with carcinogenic, metastatic and angiogenic activities. Calcitriol treatment downregulated IL8 expression in MDA-MB-231 cells (table 4). THBS1 (Thrombospondin 1), a glycoprotein playing a role in cell to cell and cell to matrix interactions, is known to be involved in tumor angiogenesis and downregulated by calcitriol in MDA-MB-231 cells (table 4). Inhibition of these proteins might be crucial for anti-angiogenic and anti-metastatic properties of calcitriol.

There are also significant alterations in amino acid metabolism (CTH, SLC7A11, SLC1A4, PDK4, PHGDH, ASNS, SERINC2, IDH1, CPS1 and SLC3A2). Amino acid metabolism is known to be regulated during UPR by AFT4 (Harding et al. 2003) and an important aspect of cancer. However, their importance in ER stress related cellular effects of calcitriol are yet to be defined.

Our findings suggest a strong association between calcitriol and ER stress, however to understand the importance of this association for the cellular actions of the calcitriol, further studies evaluating altered genes in protein and post-translational level are required.

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Table 1. List of genes upregulated by calcitriol in MCF-7 cells. ER stress related genes (if any) marked with relevant reference.

Gene Symbol	MCF-7		MDA-MB-231		ER Stress
	p-value	Fold change	p-value	Fold change	
NUPR1	1.027E-04	7.542	5.791E-01	1.179	(Jin et al., 2009)
AKR1C1	5.868E-03	4.834	6.931E-01	1.189	
AKR1C2	9.426E-05	4.793	7.647E-01	-1.070	
ULBP1	1.125E-03	4.406	9.026E-02	-1.781	
HMOX1	1.411E-03	3.939	2.432E-03	3.497	(Liu et al., 2005)
SLFN5	3.027E-05	3.534	2.612E-03	1.906	
PRY	3.337E-02	3.340	8.641E-01	1.087	
GDF15	1.710E-02	3.259	1.502E-02	3.371	
SLC7A11	4.691E-03	3.013	2.775E-02	2.146	
DDIT3	4.304E-02	3.001	3.773E-02	3.120	(Okuda et al., 2012)
ASNS	7.463E-03	2.963	5.677E-03	3.140	
PCK2	2.361E-04	2.929	4.086E-02	1.516	
CTH	2.451E-02	2.926	2.254E-02	2.988	
SULT1C2	1.113E-04	2.899	3.150E-05	3.563	
CLGN	4.101E-04	2.857	2.755E-01	1.236	(Schröder, 2008)
CHAC1	2.441E-03	2.745	2.366E-01	1.346	
DDIT4	3.821E-02	2.708	4.225E-03	4.896	(Okuda et al., 2012)
PHGDH	2.296E-05	2.635	1.669E-01	-1.184	
BEX2	9.224E-04	2.529	3.827E-01	1.183	
CYP1A1	3.509E-05	2.524	4.504E-05	2.446	
PMAIP1	1.225E-04	2.511	1.867E-01	-1.212	(Wang et al., 2009)
GPR56	3.979E-05	2.502	1.452E-01	-1.200	
STC2	5.791E-04	2.460	4.366E-01	-1.144	(Ito et al., 2004)
WARS	1.687E-03	2.413	2.926E-02	1.656	
LCN2	4.407E-02	2.413	1.433E-01	1.820	(Mahadevan et al., 2011)

TUBE1	7.295E-04	2.411	1.165E-01	1.339	
LAMP3	4.081E-03	2.394	7.193E-01	1.085	(Mujcic et al., 2009)
MTHFD2	3.488E-05	2.317	5.535E-01	1.065	
LCE2D	6.132E-03	2.313	4.710E-01	1.188	
GCNT3	1.759E-05	2.285	3.588E-01	1.093	
PKD4	3.667E-04	2.232	2.260E-01	1.196	
TRIB3	1.224E-02	2.195	2.112E-02	2.011	(Qian et al., 2008)
SLC1A4	1.873E-04	2.137	1.970E-01	1.179	
NDRG1	3.905E-03	2.115	2.338E-02	1.686	(Okuda et al., 2012; Segawa et al., 2002)
GTPBP2	3.117E-03	2.113	4.444E-02	1.533	
CARS	3.810E-04	2.055	3.762E-02	1.358	
LURAP1L	1.852E-02	2.031	3.271E-02	1.859	
ARHGEF2	3.611E-04	2.002	1.810E-03	1.713	

Table 2. List of genes downregulated by calcitriol in MCF-7 cells. There were no ER stress related genes.

Gene Symbol	MCF-7		MDA-MB-231		ER Stress
	p-value	Fold change	p-value	Fold change	
SEMA3D	6.957E-03	-2.035	4.999E-01	-1.149	
SDPR	2.677E-04	-2.064	2.004E-01	-1.178	
HIST1H2AB	8.418E-04	-2.170	1.868E-02	-1.553	
KLHL13	5.419E-06	-2.211	1.557E-01	-1.124	
CPS1	1.343E-05	-2.348	5.863E-01	1.053	
MMP7	3.361E-04	-2.541	4.659E-01	-1.127	

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Table 3. List of genes upregulated by calcitriol in MDA-MB-231 cells. ER stress related genes (if any) marked with relevant reference.

Gene Symbol	MDA-MB-231		MCF-7		ER Stress
	p-value	Fold change	p-value	Fold change	
CYP24A1	4.992E-10	99.231	5.818E-03	1.634	
DDIT4	4.225E-03	4.896	3.821E-02	2.708	
GEM	3.051E-07	3.942	1.624E-02	-1.309	
C4orf34	1.016E-02	3.767	2.446E-01	1.645	(Okuda et al., 2012)
SULT1C2	3.150E-05	3.563	1.113E-04	2.899	
HMOX1	2.432E-03	3.497	1.411E-03	3.939	(Liu et al., 2005)
GDF15	1.502E-02	3.371	1.710E-02	3.259	
ASNS	5.677E-03	3.140	7.463E-03	2.963	
DDIT3	3.773E-02	3.120	4.304E-02	3.001	(Okuda et al., 2012)
ATP6V0D2	3.725E-02	3.031	8.513E-01	1.090	
CTH	2.254E-02	2.988	2.451E-02	2.926	
EFTUD1	3.830E-05	2.978	1.828E-01	1.216	
SERINC2	1.903E-04	2.923	7.318E-01	1.060	
G0S2	7.399E-04	2.877	8.754E-01	1.033	
RGCC	3.701E-04	2.728	8.356E-01	1.037	
FTH1	2.738E-02	2.589	1.704E-01	1.703	(Oliveira et al., 2009)
C17orf81	1.230E-03	2.447	8.037E-01	-1.048	
CYP1A1	4.504E-05	2.446	3.509E-05	2.524	
ZFP36	9.911E-03	2.353	7.544E-01	1.086	
DNAJB9	3.415E-02	2.273	5.198E-01	1.242	(Kurusu et al., 2003)
CA2	4.249E-06	2.246	5.732E-03	-1.318	
SAT1	3.153E-02	2.246	7.132E-01	1.126	
SNORA70	4.798E-03	2.204	6.141E-01	-1.113	
TMEM120B	1.280E-03	2.196	6.921E-01	1.069	
SNX33	4.832E-03	2.188	7.806E-01	-1.060	
SLC7A11	2.775E-02	2.146	4.691E-03	3.013	
TUBA1A	5.959E-04	2.136	9.924E-01	1.001	
SLC3A2	1.569E-02	2.128	2.894E-01	1.324	
INSIG1	3.515E-04	2.126	9.407E-01	-1.010	
GOLPH3L	2.735E-03	2.119	1.104E-01	1.372	
WIPI1	1.777E-03	2.089	1.952E-01	1.255	
LY96	1.420E-02	2.089	6.767E-01	1.107	
AKR1C3	5.698E-03	2.060	7.750E-02	1.479	
SLC16A13	7.358E-03	2.050	8.056E-01	-1.053	
PDCD4	4.924E-03	2.045	1.217E-01	1.380	
SLCO4A1	5.863E-04	2.042	5.566E-01	1.083	
STARD4	1.404E-04	2.042	6.645E-01	-1.049	
CDA	3.345E-03	2.032	2.582E-01	1.233	
TRIB3	2.112E-02	2.011	1.224E-02	2.195	(Qian et al., 2008)
IDH1	1.074E-03	2.010	1.719E-02	1.521	
MAPK13	7.299E-04	2.008	5.759E-01	1.080	

Table 4. List of genes downregulated by calcitriol in MDA-MB-231 cells. There were no ER stress related genes.

Gene Symbol	MDA-MB-231		MCF-7		ER Stress
	p-value	Fold change	p-value	Fold change	
LINC00152	1.205E-02	-3.721	1.786E-01	-1.822	
THBS1	1.222E-04	-2.808	5.255E-01	1.104	
SERPINB2	4.947E-06	-2.795	1.964E-01	1.144	
SGK1	1.407E-05	-2.291	1.783E-01	-1.140	
RPSA	3.127E-02	-2.284	8.566E-01	1.061	
ANP32D	1.153E-02	-2.157	3.836E-01	1.243	
DPY19L2P2	2.012E-02	-2.121	3.641E-02	1.920	
OSTBETA	4.555E-02	-2.031	8.756E-02	1.791	
IL8	6.784E-03	-2.005	1.082E-01	1.415	
INHBA	2.968E-04	-2.003	7.297E-01	1.042	

Table 5. List of genes commonly altered by calcitriol in both cell lines. ER stress related genes (if any) marked with relevant reference.

Gene Symbol	Combined	MCF-7		MDA-MB-231		ER Stress
	p-value	p-value	Fold change	p-value	Fold change	
CYP1A1	1.308E-05	3.509E-05	2.524	4.504E-05	2.446	
SULT1C2	3.133E-05	1.113E-04	2.899	3.150E-05	3.563	
ASNS	4.219E-04	7.463E-03	2.963	5.677E-03	3.140	
HMOX1	4.229E-04	1.411E-03	3.939	2.432E-03	3.497	(Liu et al., 2005)
SLC7A11	1.376E-03	4.691E-03	3.013	2.775E-02	2.146	
GDF15	6.403E-03	1.710E-02	3.259	1.502E-02	3.371	
DDIT4	1.138E-02	3.821E-02	2.708	4.225E-03	4.896	(Okuda et al., 2012)
TRIB3	1.250E-02	1.224E-02	2.195	2.112E-02	2.011	(Qian et al., 2008)
CTH	1.257E-02	2.451E-02	2.926	2.254E-02	2.988	
DDIT3	4.464E-02	4.304E-02	3.001	3.773E-02	3.120	(Okuda et al., 2012)

Figure Caption:

Figure 1. A) Venn diagram showing MCF-7 specific (34 genes), MDA-MB-231 specific (41 genes) or shared (10 genes) transcriptomic alterations induced by calcitriol. MCF-7 and MDA-MB-231 cells were treated with calcitriol or DMSO for 24 hours. Gene expressions were evaluated by microarray and statistical analysis was carried out with one-way ANOVA. Genes altered at least by 2 fold and with a p value less than 0.05 were included in the Venn diagram. B) Hierarchical clustering of breast cancer cells treated with calcitriol or DMSO. 3 replicates of cells were treated with calcitriol and 3 replicates of cells treated with DMSO for 24 hours. Gene expressions were evaluated by microarray and statistical analysis was carried out with one-way ANOVA. All altered ER stress related (n=14) genes included in clustering. Lower color bar and values indicate gene expression in samples (red=high expression and blue=low expression). Genes are located in x-axis and samples are located in y axis. In samples two branches were identified: upper side covering all vehicle treated cells and one calcitriol treated MDA-MB-231 sample (lower expression of ER stress response genes), and lower side covering all remaining calcitriol treated samples (expression of ER stress response genes).

Figure 1