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In Human Breast Cancer Cells TR β Competes with ER α for Altering BCl2/Bax Ratio through SMP30-Mediated p53 Induction

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Abstract

Thyroid hormone and Estrogen regulate transcription(s) of target genes by binding to their nuclear receptors that interact with specific responsive elements -TRE and ERE, respectively. Recently, we have demonstrated that 3, 3'5 Triiodo L Thyronine (T_3) can induce apoptosis in ER positive breast cancer cells (MCF-7) through downregulation of Senescence Marker Protein-30 (*SMP30*) gene. SMP30, a novel age-associated protein which decreases during ageing is highly expressed in hepatocytes and in renal tubular epithelia. Earlier reports suggest that SMP30 too plays a diverse role in proliferation, survival and differentiation of the cells. SMP30 has also been reported to be downregulated by 17β-Estradiol (E_2) in prostate gland and mammary epithelial cells. Interestingly, Thyroid Receptors (TRs) and Estrogen Receptors (ERs) share a common consensus half site sequence. In this context; we hypothesize a possible competition between both the receptors in *SMP30* promoter under different types of hormonal signaling. To prove this hypothesis, gel retardation and luciferase assays were conducted by taking h*SMP30* promoter reporter constructs which validated our findings for the putative ERE site. Competition Chromatin Immunoprecipitation Assay (ChIP) in the above mentioned ERE showed differential TRβ binding upon thyroid/estrogen hormone treatment, while ERα showed binding mainly in control and estrogen treated sample. Although the *SMP30* promoter activity was almost same in response to E_2 and T_3, but the functional consequences of down regulation of SMP30 in human breast cancer cells post E_2/T_3 treatment were different in terms of apoptosis. To unravel the mechanism behind the differential consequences of E_2/T_3 treatment, in addition to looking at the expression of regular apoptotic markers such as Bax and Cleaved PARP, we have also tried to verify the possible involvement of p53, which has been already reported to be a downstream target of SMP30.

Keywords: 3,3'5 triiodo L thyronine; Ageing; Breast cancer; Apoptosis; Thyroid receptor; Estrogen receptor

Introduction

The development and growth of many human cancers including breast cancer are known to be influenced by steroid hormones [1,2]. Abnormal responsiveness of the cells especially to estrogen hormone has been a major cause of breast cancer development and progression [3]. Therefore better understanding and manipulation of the endocrine milieu may provide effective palliative treatment for patients with hormone-dependent cancers [4-6]. Thyroid hormone and estrogen share a common signaling pathway in regulation of proliferation and growth in the target cells, including cancer cells. So the aberrant signaling by these hormones needs to be evaluated in terms of regulated growth of normal cells *vs.* cancer cells.

Estrogen plays an important role in regulating the growth and differentiation of normal premalignant and malignant cells, i.e. typically breast epithelial cells through interaction with two nuclear estrogen receptors (ERα and ERβ) [7]. The action of thyroid hormone triiodothyronine (T_3) is mediated by TRs which belong to the super family of nuclear receptors [8] affecting the phenotype, proliferation and gene expression of cultured mammary epithelial cells [9-11]. The function of thyroid hormones and their receptors on cell proliferation varies depending upon the cell type, developmental state and pathophysiological condition [12] of the cells.

ERs and TRs belong to the nuclear hormone receptor superfamily sharing a basic structure consisting of a receptor specific amino terminal domain, central DNA binding domain and carboxyl terminal ligand binding domain [8]. These receptors share a common mechanism of action whereby hormone receptor complexes bound to cis-acting elements enhance or repress the target genes. ER homodimers and TR monomer or heterodimers along with Retinoid acid receptor (RXR), recognize E_2 (ERE) and T_3 response element (TRE) respectively [13]. TR and ER have the potential to bind to an identical half site consensus sequence, AGGTCA, although the number of spacing nucleotides between the half sites and their orientations may vary for ERE and TRE. T_3 bound TR can directly bind to ERE of the *PR* (progesterone receptor) promoter and stimulate its transcription [14]. When TR and ER both coexist, T_3 bound TR inhibits E_2 bound ERα-mediated transactivation of the *preproenkaphlin* [15,16] and *prolactin* gene [17] suggesting interference of estradiol signaling by thyroid hormone and *vice versa*. Previous studies suggested a crosstalk between ER and TR in neuroendocrine tissues leading to inhibition of estrogenic effects by thyroid hormone [18]. These crosstalks between E_2 bound ER and T_3 bound TR signaling pathways are postulated due to redundancy of DNA recognition and the common utilization of cofactors [19,16].

SMP30, a novel age-associated protein which decreases during ageing is highly expressed in hepatocytes and in renal tubular epithelia [20]. Earlier report suggests that SMP30 too plays a diverse role in

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proliferation, survival and differentiation of the cells [21]. On the contrary, SMP30 has also been shown to induce p53 expression [22] leading to p21 induction. Additionally, anti-apoptotic role of SMP30 is well documented [23-26]. In the context of the above background we wanted to question whether there is a competition between T_3 bound TR and E_2 bound ER for getting recruited in the common response element of *SMP30* gene. This is in line with our bigger goal to extend our study to confirm whether T_3 can be projected as an adjuvant therapy for ER positive breast cancer patient by unravelling the mechanism of crosstalk and/or competition between liganded TR and ER for manipulating the expression of SMP30. We already reported about down regulation of SMP30 in rat liver by thyroid hormone [27]. We also reported SMP30 down regulation by T_3 induced apoptosis in MCF-7 cells [28], which is in accordance with the fact that higher concentration of T_3 treatment can inhibit breast cancer cell proliferation [29]. It has also been reported that E_2 downregulates SMP30 in rat mammary and prostate gland [30]. However, E_2 is known to promote cell proliferation in both normal and transformed mammary epithelial cells by modifying the expression of hormone responsive genes involved in the cell cycle/programmed cell death. Estradiol induced apoptosis at basal level, may be referred to non-genomic steroid actions [31]. However, although SMP30 is down regulated by both T_3 and E_2, its functional consequences and cross-talk/or competition between T_3 liganded TR and E_2 liganded ER in SMP30 downregulation is yet to be unravelled. By unfolding the mechanistic insights, their role in development and progression of breast tumors can be established.

Materials and Methods

Cell culture

MCF-7, T-47D and MDA MB-231 human breast carcinoma cells were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) maintained in 5% CO_2 atmosphere at 37°C until 70-80% confluence. For stimulation with T_3 or E_2, culture medium was removed; the cells were rinsed twice with phosphate buffer saline (PBS) and incubated in medium containing 10% Charcoal-Stripped Fetal Bovine Serum (CS-FBS) for 3 days. T_3 (10 μM/10 nM) or E_2 (10 nM) (from Sigma) was diluted in medium containing 10% charcoal-stripped fetal bovine serum and cells were treated for the time-points indicated in figure legends.

Plasmid constructions

h*SMP30* promoter reporter construct was prepared by amplifying human *SMP30* promoter from MCF-7 genomic DNA by using h*SMP*Kpn4F primer and h*SMP*XhoR primer sequences which are shown in Table 1. Then the PCR product was purified using QIAquick Gel Extraction Kit (Qiagen). The fragments harboring ERE, having KpnI and XhoI restriction sites and pGL3-basic vector were digested with KpnI and XhoI enzymes. Ligation was performed using T4 DNA Ligase (USB) and cloned. The clones were then confirmed by PCR using vector specific universal RV3 and GL2 primers, cDNA fragments of hSMP30 were prepared from MCF-7 RT PCR products by using hSMP30 EcoRI F and XhoI R primers (Table 1). cDNA product was then ligated to digested pCMV 3T3A vector using T4DNA Ligase to make hSMP30 expression vector and cloned. All constructs were confirmed by manual sequencing.

Transfection and luciferase assay

For Dual Luciferase Assay, transient transfections were carried out using MCF-7 cells. 20 hrs before transfection, cells were plated in DMEM containing 10% CS-FBS, at a density of 1×10^5 cells per well, in 12 well plates. For transient transfection, 0.5 μg of reporter plasmid DNA, 0.25 μg of TRβ and TRα (as TRs), RXRα or 0.25 μg of ERα expression vector or pCMV vector and 50 ng of pRL-TK control vector were co-transfected using Fugene HD transfection reagent (Roche) as per manufacturer's instruction. After 24 hrs of transfection 10 μM T_3 or 10 nM E_2 hormones or DMSO was added for overnight treatment. The cells were harvested to prepare lysates and luciferase activities were measured in duplicates in three independent experiments.

Electrophoretic mobility shift assay

Electrophoretic Mobility Shift Assay (EMSA) was performed as described in Sar et al. [27]. Oligonucleotides (both strands) corresponding to h*SMP30* ERE sites were synthesized as shown in Table 2. For each site one strand was end labelled with γ^{32}P ATP using T4 poly nucleotide kinase and annealed to its complementary unlabelled strand. Nuclear extracts of MCF-7 (10 μg) were incubated with 20 fmoles of radiolabelled oligonucleotide duplex and 1 μg poly (dI-dC) in 30 μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mMNaCl, 1 mM DTT, 5% glycerol for 20 minutes at room temperature. In competition experiments, 100 fold molar excess of unlabeled self, consensus ERE oligos (Table 2) were added during incubation period. Then Gel Retardation Assay was performed in 5% non-denaturing polyacrylamide gel.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described [28]. Briefly, MCF-7 cells were grown to 90% confluence in DMEM supplemented with 10% charcoal stripped fetal bovine serum for 3 days. After addition of 10 μM T_3 or 10 nM E_2 or DMSO for 1hour, cells were cross-linked and lysed using ChIP Kit from Upstate Biotechnology Inc. Lysates containing DNA-protein complexes were sheared by sonication, precleared and immunoprecipitated according to manufacturer's protocol by antibodies for TRβ (Abcam), ERα (Abcam) and normal Rabbit IgG (Santacruz). DNA-protein-antibody complexes were pulled down with protein A-sepharose beads, washed and reverse-crosslinked for 4-5 hour at 65°C with 5M NaCl and subjected to Proteinase K digestion at 45°C for one hr. Then the purified DNA fraction was isolated by phenol-chloroform. *In-vivo* association of the protein complexes with h*SMP30* promoter EREs were demonstrated by performing PCR of ChIP-elutes using site-specific primers- h*SMP30*

	Sequence 5' - 3'
hSMP30 EcoR I F	ACAGAATTCCCTGCGACCATGTCTTCC
hSMP30 Xho I R	ACACTCGAGTCCCGCATAGGAGTAGGGA
hSMP30 Kpn4 F	CAATCTAGGCAAGAAATG
hSMP30 Xho R	CGACCTTCTTAGGACGTTTC

Table 1: Primers Used for Cloning.

	Sequence 5' - 3'
hSMP30 ERE 1 SS	ATGTTGGTCAGGCTGGTCTCAAACTCCTGACCTTAGG
hSMP30 ERE 1 AS	CCTAAGGTCAGGAGTTTGAGACCAGCCTGACCAACAT
hSMP30 ERE 2 SS	GAAGGACATTAAAGGGACAATTTCTATGACCTGGTG
hSMP30 ERE 2 AS	CACCAGGTCATAGAAATTGTCCCTTTAATGTCCTTC
ERE Consensus SS	TGGCTACAGGTCAGTCTGACCTCTGGCATG
ERE Consensus AS	CATGCCAGAGGTCAGACTGACCTGTAGCCA

Table 2: Oligonucleotide Used for EMSA.

ERE1 (h SMP PCR1F1 and Xho2 R), and non ERE region. These primer sequences were listed in Table 3.

Real-time PCR

MCF-7 or MDA MB-231 cells were transfected with hSMP30-pCMV 3Tag3A expression vector; after 24hrs of transfection, cells were treated with 10 μM of T_3 or 10 nM of E_2 for 24 hrs. Then cells were harvested and RNA was isolated using Trizol method. *BCL-2, BAX, SMP30* and *GAPDH* mRNA expression were determined by quantitative RT PCR using SYBR Green Jump-Start Taq Ready mix system (SIGMA) for quantitative PCR. The sequences of sense and antisense primers for *BCL-2, BAX, SMP30 and GAPDH* mRNA were given in Table 4. Relative values (mean ± SD) were normalized to *GAPDH* expression**.** Then PCR products were electrophoresed in 1.5% agarose gel.

Western blot analysis

For preparation of whole cell lysates, transfected or treated cells were washed with PBS and lysed in RIPA buffer at 4°C for 2 hr. Then, after 30 min centrifugation, the supernatant was isolated and quantification of protein was done by Bradford Method.

10%SDS-PAGE was performed with 50 (for p53-experiment)/120 μg protein in each well in Mini-PROTEAN Electrophoresis System (Biorad). PVDF membrane (Millipore) was used for overnight transfer of protein and transfer (at~30V) was performed with Biorad Mini Trans-blot Cells in methanol-containing Tris-Glycine transfer Buffer. Post-transfer blocking was done with non-fat dry milk (Santa Cruz) for 1hr. Each blot was the incubated with primary antibody overnight, washed with TBS-T and then respective HRP-conjugated secondary antibody incubation was done. Blot was subjected to chemilumenescent detection reagent (GE Healthcare) for visualisation of bands.

Antibody dilutions were as follows: anti-SMP30 antibody (Santacruz) 1:500 times, anti-cleaved PARP-amino-terminal (Cell Signalling) 1:1000 times, anti-TRβ (Abcam) 1:1000 times, anti-p53 (Imgenex) 1:5000 times, and their respective HRP conjugated secondary antibody (Santacruz) 1:5000 times, α-Tubulin antibody (Santa Cruz) 1:1000 times.

Western Blot Densitometric Analysis was performed by Biorad Quantity One Software.

	Sequence 5' - 3'
hSMP PCR 1F1	GGATTCAAGCAATTCTCCTGTCTCAGCC
hSMP XhO2 R	ACACTCGAGACAGTCTGGGCTTTCTCC
hSMP non ERE F	TGGAGAAAGCCCAGACTGTCAGAT
hSMP non ERE R	GGCTGGAAGAATCCTGCAAAG

Table 3: Primers Used in ChIP PCR.

	Sequence 5' - 3'
hSMP30 + 560 to + 580	GCCACCATTGGAACCAAGTT
hSMP30 +1105 to +1085	CCCTCCAAAGCAGCATGAAG
hGAPDH SS2	GATCATCAGCAATGCCTCCT
hGAPDH AS2	TTCCTCTTGTGCTCTTGCTG
Bcl2 SS	TGTGGATGACTGAGTACCTG
Bcl2AS	GGAGAAATCAAACAGAGGCC
Bax SS	CAGCTCTGAGCAGATCATGAAGACA
Bax AS	GCCCATCTTCTTCTTCCAGATGGTGAGC

Table 4: Primers Used for Real Time- PCR.

Flow cytometric analysis

To measure the extent of early or late induction of apoptosis flow cytometric analysis was performed. MCF-7 cells transfected with control vector (pCMV-3Tag3A) and expression vector containing *SMP30* gene (SMP30-pCMV3Tag3A) were incubated in presence or absence of 10 μM T_3 or 10 nM E_2 or DMSO for 24 hrs at 37°C. After 24 hrs of incubation cells were harvested and assayed for apoptosis using the Annexin V-FITC Apoptosis Detection Kit (Imgenex) according to manufacturer's instruction. Cells were analyzed in FACS Calibur (Becton Dickinson) by using Cell Quest Pro software.

Results and Discussions

SMP30 and TRβ expression in human breast cancer cells

We screened SMP30 expression in different human breast cancer cell lines by western blot analyses. Figure 1A shows SMP30 protein expression in all ER positive (MCF-7, ZR-75, and T47D), ER negative (MDA-MB 231) breast cancer cells and 3T3 fibroblast cells in 1st to 6th lane in upper panel. In middle panel, TRβ expression is shown in all the above described cell lines. Densitometric Analysis shows that SMP30 expression is differentially regulated in ER positive, ER negative breast cancer cells and in non-cancerous cells irrespective of TRβ expression status.

Down regulation of SMP30 in breast cancer cell line by β-Estradiol

We examined the effect of overnight treatment of estrogen (10 nM) on SMP30 expression at protein level. Figure 1B shows that overnight treatment with estrogen hormone down regulates SMP30 protein expression in MCF-7.

Identification of high affinity ER binding sites within *SMP30* Promoter

To determine whether there were any ER binding site within 2kb upstream of transcription start site of human *SMP30* (h*SMP30*) promoter, We scanned h*SMP30* promoter for EREs within 2kb from transcription start site. In h*SMP30* promoter we identified two ER binding half sites i.e. at 613 bp and 1.2 kbp from h*SMP30* transcription start site. The sequence of the former from -637 to -600 was found to be ATGTTGGTCAGGCTGGTCTCAAACTCCTGACCTTAGG and that of later from -1274 to -1235 was GAAGGACATTAAAGGGACAA TTTCTATGACCTGGTG. We performed Electrophoretic Mobility Shift Assay (EMSA) using MCF-7 nuclear extract. Electrophoretic mobility assay and competition with 100 fold molar excess of cold self and consensus ERE oligos confirmed the binding of ER to ERE site1 whereas ERE site 2 did not show any competition with 50 and 100 fold molar excess of cold consensus ERE as shown in Figure 2A and 2B. The above results clearly indicated that *SMP30* promoter has one specific binding site for ERs.

Recruitment/ Competition of TRβ and ERα to *SMP30* promoter after E_2 or T_3 treatment

We next investigated recruitment of ER on *SMP30* promoter by ChIP analysis (Figure 3A-3C). It was found that ERα can bind to h*SMP30* ERE1 of *SMP30* promoter irrespective of the presence or absence of E_2, but its binding affinity was decreased in the case of later. Our previous study confirmed the presence of two important putative TREs [28] in contrast to one putative ERE. TRβ could also bind to *hSMP30* ERE1 of *SMP30* promoter irrespective of the presence or absence of T_3, but

its binding affinity was decreased in the case of T_3 treatment. This is typically an established feature of negative thyroid response element. This may happen due to interaction of unknown thyroid receptor associated proteins on the negative thyroid response elements which eventually led to repression [28]. There are reports based on interference between estradiol and thyroid hormone signaling pathways. Previous studies suggest inhibition of estrogenic effects by thyroid hormone in neuroendocrine tissues [18]. Additionally, coexistence of ER and TR has been reported to inhibit E_2 bound ER mediated transactivation [15-17]. To unravel the above conflicting facts in relation to *hSMP30* gene, we did competition ChIP assays after treating MCF-7 cells with thyroid hormone as well as estrogen hormone.

On top of everything, percentage of TRβ bound to ERE/ TRE does overshadow the percentage of ERα bound to the same element under combinatorial treatment.

SMP30 Promoter activity in response to E_2 and T_3

We examined the response of E_2, T_3 and E_2 plus T_3 together on transcriptional activity of h*SMP30* promoter in MCF-7 cells by measuring luciferase activity. We transfected h*SMP30* promoter reporter construct along with expression vectors in MCF-7cells. Luciferase activity of h*SMP30* promoter (+ 66 to - 1252) having ERE1 (h*SMP30* Pro) showed slight repression in presence of E_2; however, the repression was further enhanced in presence of both E_2 and T_3 treatment in MCF-7 cells (Figure 4).

These results indicated that h*SMP30* promoter is negatively regulated by liganded ERα. However, T_3 bound TR was found to further inhibit E_2 bound ER transrepression of hSMP30 promoter in presence of both E_2 and T_3. Similar pattern of inhibitory effect of T_3 bound TR on E_2 bound ER has been reported on transactivation of *preproenkephalin* [15,16] and *prolactin* gene [17].

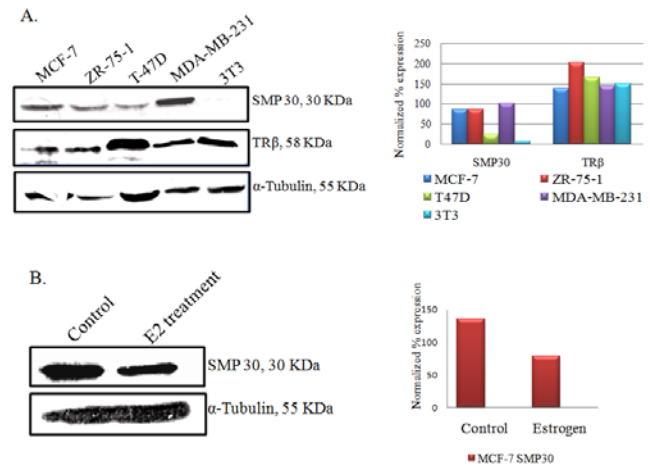

Figure 1: (A): SMP30 and TRβ expression in human breast cancer cells. SMP30 expression in different breast cancer cell-lines (MCF-7, ZR-75, T47D, MDA MB 231) and immortalized 3T3 fibroblast cell line (non-cancer cell-line) are shown by Western blot analyses and Densitometric histogram for protein band intensity. The histogram of % protein expression was prepared by subtracting the corresponding control.

(B): Down regulation of *SMP30* in human breast cancer cell line by 17-β-estradiol. Representative Western blot of total SMP30 protein in response to overnight treatment with 10nM E_2 in MCF-7 cells and Densitometric histogram for protein band intensity. The histogram of % protein expression was prepared by subtracting the corresponding control.

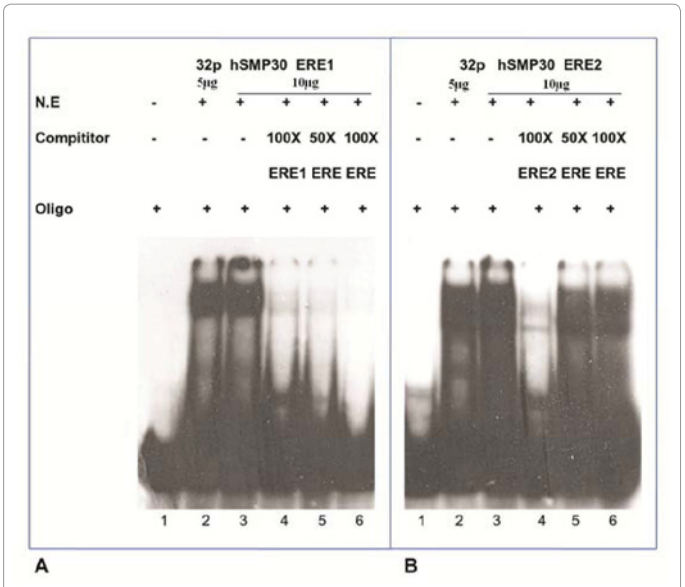

Figure 2: Identification of high affinity ER binding sites within h*SMP30* Promoter. (A) Electrophoretic mobility shift assay for h*SMP30* ERE1 to confirm the binding of ER. (2B) Electrophoretic mobility shift assay for h*SMP30* ERE2 to confirm the binding of ER.

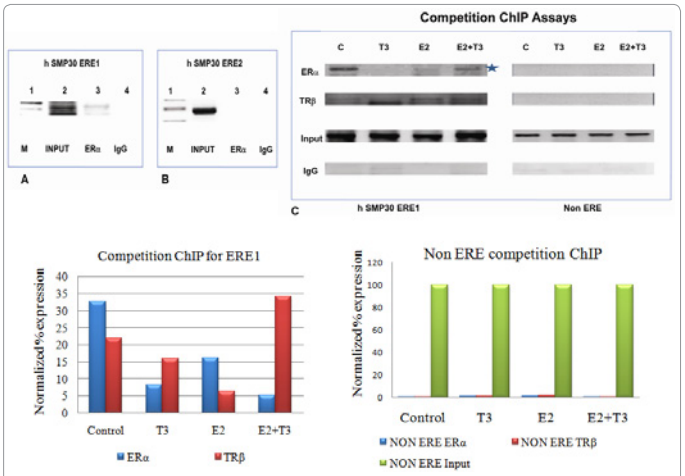

Figure 3: Recruitment/ Competition of TRβ and ERα to *SMP30* promoter after E_2 or T_3 treatment. (A,B) ERα binding to *SMP30* promoter is affected by E_2 treatment was shown in ChIP. (C) Recruitment/ Competition of TRβ and ERα to *SMP30* promoter after E_2 or T_3 treatment. Respective Band intensity was measured after subtracting the corresponding control (IgG) and normalized with respective positive control (Input).

T_3 is able to regulate Bcl2/Bax ratio through down regulation of SMP30, but this effect was not shown by E_2 mediated SMP30 down regulation

It is well established that Estradiol (E_2) enhances cell proliferation and inhibits apoptosis. As SMP30 is anti-apoptotic, to check the status of apoptosis as a result of SMP30 down regulation in response to E_2, we checked the expression of various pro- and anti-apoptotic genes. Estrogen (E_2) mediated up regulation of *BCL-2* and down regulation of *BAX* gene expression remained same before or after SMP30 over expression. Compared to estrogen, thyroid hormone treatment enhanced the proportion of MCF-7 cells undergoing apoptosis by

20-30% by down regulating anti apoptotic *BCL-2* and up regulating pro apoptotic *BAX* (Figure 5A).Over the past few years, increasing evidences have suggested the non genomic effects of thyroid hormone treatment induces apoptosis in lymphocytes and pro myeloleukemic HL-60 cells in dose dependent manner [32,33]. Sometimes, the effect of non physiological concentrations (about two orders of magnitude higher) of T_3 resulting in decreased proliferation, has been reported in breast cancer cells [29]. Similarly, we found there was maximal induction of early apoptosis in MCF-7 cells after 16 hr of 1-10 μM concentration of T_3 treatment and no further induction was found either by increasing the duration of T_3 treatment or by increasing the concentration of T_3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T_3 by up regulating *BCL-2* and down regulating *BAX* expression as shown by qRT-PCR (Figure 5C).

To further confirm the effect of estrogen hormone, we carried out the above experiments in ER negative MDA MB-231 cells (Figure 5B and 5D). Estrogen did not show any significant enhancement of apoptosis although there was no further enhancement of *BCL-2* expression (Figure 5D). On the other hand thyroid hormone was able to induce apoptosis in MDA MB-231 cells through reducing *BCL-2* and enhancing *BAX* gene expression. SMP30 over expression reversed the rate of apoptosis induction caused by T_3 (Figure 5B and 5D).

Taken together, the above results clearly suggested that down regulation of SMP30 has an important role during thyroid hormone induced apoptosis in MCF-7 as well as MDA MB-231 breast cancer cells.

Possible involvement of p53 in T_3 induced apoptosis

Since SMP30 has already been shown to induce p53 expression [22] leading to p21 induction, and in an important clinical study [34], hypothyroidism has been speculated to be associated with reduced evidence of primary breast carcinoma, it appeared interesting to us, to know, whether the physiological concentration of thyroid hormone, was unable to induce apoptotic stimuli even in presence of wild-type p53. To unravel the mechanism behind thyroid hormone induced apoptosis in human breast cancer cells through activated p53 molecule, we transfected wt p53 and phosphorylated form of p53 (p53-18D20D). On treatment of cells with E_2, T_3 and E_2 plus T_3, the expression of cleaved PARP was observed through western blot analysis. Since TR β has been already shown to interfere with p53-mediated signaling pathways in terms of apoptotic induction [35], the objective of our western was to check whether our p53-18D20D (phosphorylated p53) construct was resistant to T_3 (Figure 6).

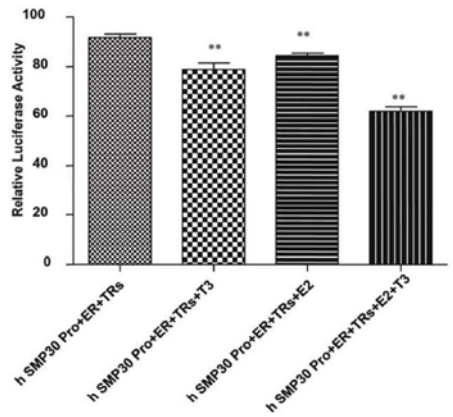

Figure 4: *SMP30* Promoter activity in response to E_2 and T_3. Transient transfections of h*SMP30* Promoter was carried out using MCF-7 cells. Values are the mean of three independent experiments ± SD normalized to Renilla activity. *P*<0.001difference from vehicle control using ANOVA.

Figure 6 shows effect of vehicle (A)/E_2 (B)/T_3 (C)/E_2+T_3 (D) over vector transfected, p53 and p53-18D20D transfected MCF-7 cells along with their respective densitometric analysis (E,F,G,H). Figure S2 (Supplementary data) shows decreased p53-mediated PARP induction upon any treatment versus almost stabilized PARP induction under any treatment in case of p53-18D20D. p53-18D20D construct expresses constitutively phosphorylated form of p53. The reduction of PARP induction of p53 under E_2 and T_3 seemed to be reversed by introduction of this construct. From our western blot results (Figure 6A and 6C), it was evident, that T_3 alone, at upper physiological level of 10 nM concentration, was unable to induce apoptosis in even MCF-7 cells which expresses wild type p53. But upon over-expression of p53, as expected, apoptotic signal was increased. So, it could be inferred, that p53 overexpression in ERα positive breast cancer cells was not enough to induce apoptotic stimuli even at upper physiological concentrations of T_3. Since, it has already been reported that the peak activity of E_2 occurs at 10 nm so we have decided to perform the competition assay between T_3 and E_2 at this concentration of E_2. But at the same physiological concentration; estrogen is able to induce basic level of apoptosis in same cells. This data supports the clinical evidence of hypothyroidism association with reduced occurrence of primary breast carcinoma and that of non-genomic effects of estrogen. TRβ, indeed have been shown to bind to p53-DNA-binding domain and thereby block its transcriptional activity [35] at physiological concentrations. By observing these opposing effects of T_3 and E_2 at physiological concentrations, we checked what happens if both the ligands are introduced in the same cell line. As per our western blot, the PARP-inducing capability of p53 is enhanced by phosphorylation of p53 at 18^{th} and 20^{th} position. In presence of physiological concentration of T_3, PARP inducing capability of p53-18D20D is higher than that of p53. This effect is probably due to inability of physiological concentration of T_3 to phosphorylate p53-18D20D (activated p53) at 15^{th} position with MAPK in contrast to wild type p53 [40]. p53 mutation or deregulation is the most common cause of neoplasia. However, the frequency of p53 mutation in breast cancer is significantly lower in terms of clinical cases [36]. Being the most thoroughly studied tumor-suppressor protein, p53 has been shown to induce growth arrest or apoptosis in response to cellular stresses including hypoxia, carcinogen exposure, nucleotide depletion or oncogenic signals. But the evidences of breast cancer in presence of wild-type p53 raise the question of deregulation in its balancing mechanisms.

Possible downstream mechanism behind differential regulation of SMP30 by E_2/T_3

SMP30, has been so far denoted as an anti-apoptotic protein, although it is an upstream positive regulator of p53 [34]. Hence it seems debatable, whether an inducer of tumor suppressor protein needs to be down regulated for induction of apoptosis, as is apparent from our results. Figure 7 shows possible mechanism behind differential effect of SMP30 regulation by T_3 or E_2. We hypothesize, T_3 or E_2 may regulate intracellular calcium levels apart from regulating SMP30 (subject to further study). SMP30 has been reported to inhibit activation of liver nuclear DNA fragmentation at about 0.5 to 2.0 μM Ca^{2+} [37]. This inhibition is reversed in presence of 25 to 50 μM Ca^{2+}, suggesting that

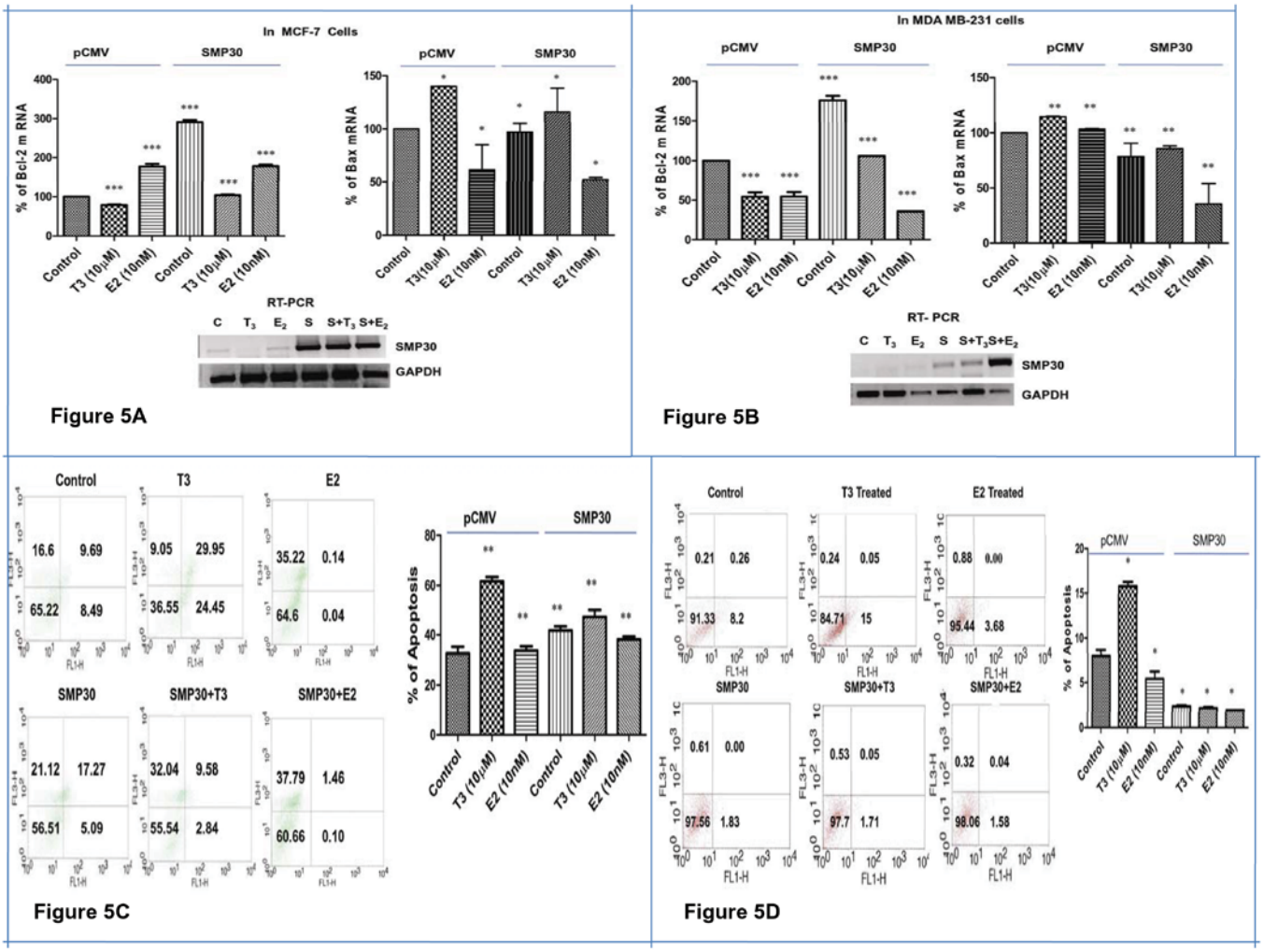

Figure 5: Induction of apoptosis as a result of T_3 Mediated repression of hSMP 30 in contrast to absence of any significant induction of apoptosis as result of E2 mediated repression of hSMP 30. (A,B) **A represen**tative qRT-PCR data for two apoptotic related genes *BCL-2* and *BAX* expression in MCF-7 and MDA MB-231 cells. Shown are the mean of triplicate samples **(mean ± SD)** normalized to *GAPDH*. C_T values obtained from the real time PCR was used to compare the expression label of treated sample from control assuming 100% amplification. Results were confirmed in three independent experiments. *P*<0.0001 difference from only vector transfected control using ANOVA for *BCL-2* gene, *P*<0.0232 difference from control using ANOVA for *BAX* gene expression in MCF-7 cells. *P*<0.0001 difference from only vector transfected control using ANOVA for *BCL-2* gene, *P*<0.001 difference from control using ANOVA for *BAX* gene expression in MDA MB-231 cells. Over expression of *SMP30* gene after transfection has been confirmed by RT-PCR. *SMP30* gene in (upper panel) and *GAPDH* gene in (lower panel) are shown using cDNA from MCF-7 and MDA MB-231 cells.

(C,D) Apoptosis was assessed post vector/SMP30 cDNA transfection and E2/T3 treatment by flow cytometry. Shown are the mean of triplicate samples ± SD normalized to control. Similar findings were found in two other experiments, $P < 0.001$ difference from only vector transfected control using ANOVA in Figure 5C and $P < 0.01$ difference from only vector transfected control using ANOVA in Figure 5D.

inhibition is dependent on intracellular Ca^{2+} level. On the other hand, concentration of intracellular Ca^{2+} has been reported to be a key element in apoptotic signalling [38]. SMP30 level as well as level of intracellular calcium, possibly regulates the fate of the cells in terms of Bax-mediated apoptosis or p21-mediated growth-check through differentially regulated p53 under thyroid hormone treatment/estrogen treatment. Remarkably, thyroid hormone concentration should have a definitive role in regulation of above mentioned intracellular calcium levels as being apparent from our study. In fact, Ca^{2+} has been reported to be the first messenger for action of thyroid hormone at level of plasma membrane [39]. Still, the mechanism of modulation of p53 activity by thyroid hormone/estrogen hormone signaling, through SMP30, which results into Bax-mediated PARP induction remains unclear.

Conclusion

Although E_2 and T_3 cause downregulation of SMP30 promoter but the functional consequences are different in terms of apoptosis. This study is aimed to unravel the underlying molecular mechanism of difference in functional consequences of E_2 and T_3 mediated downregulation of SMP30. We confirmed a putative ERE at -613 bp (h*SMP30* ERE1). ER and TR were shown to compete with each other for binding to this ERE in different hormonal treatments (estrogen and thyroid hormone). It was seen that the binding of TRβ was overshadowing ERα in combinatorial treatment. To ascertain the role of SMP30 in the estrogen hormone induced apoptosis of human breast cancer cells, we studied the effect of estrogen hormone after over expressing the *SMP30* gene in MCF-7 and MDA MB-231 cells respectively. Over expression

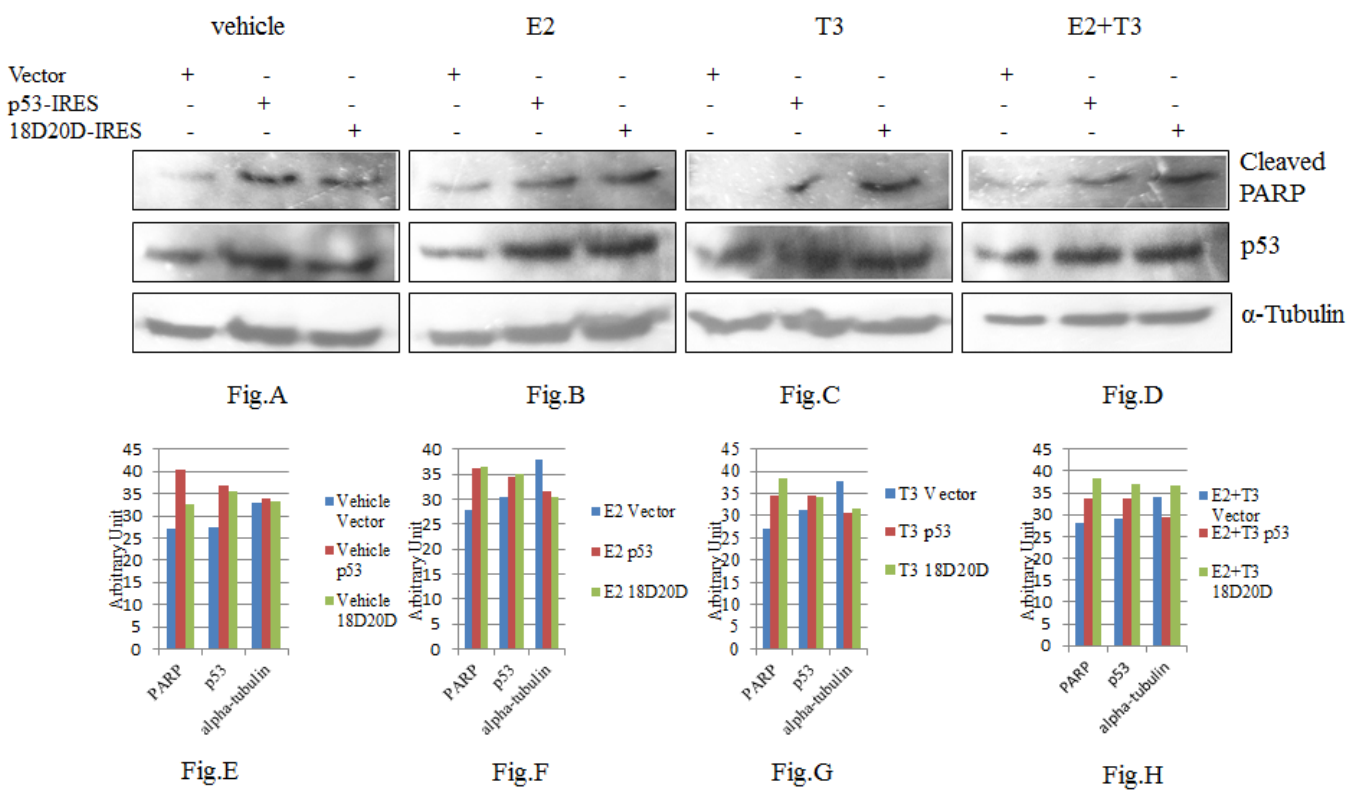

Figure 6: Western Blots showing effect of p53/phosphorylated p53 (p53-18D20D) over PARP under E2/T3 treatment. Figures 6A, B, C, D: Western Blots of vehicle treated (5A), 10 nM E_2 treated (5B), 10 nM T3 treated (5C), 10 nM E2+10 nM T_3 treated (5D) 50 µg MCF-7 whole cell lysates; cleaved PARP (amino terminal) was detected at 24 kDa; p53 was detected at 53 kDa; alpha-tubulin was used as loading control.

Figures 6E, F, G, H: Bar diagrams for Figures 5A, B, C, D respectively representing densitometric analysis performed with Biorad Quantity One software.

of SMP30 did not affect the effect of estrogen on *BCL-2* and *BAX* gene expression. At the same time over expression of SMP30 in our study resulted in reversal of thyroid hormone induced apoptosis of human breast cancer cells by increasing anti apoptotic *BCL-2* gene expression as well as down regulating proapoptotic *BAX* gene expression. These findings indicated anti-apoptotic role of SMP30 in breast cancer cells which is in accordance with earlier reports regarding the role of SMP30 in literature [23-26] in other systems. We found out that apoptosis can be induced by T_3 mediated down regulation of *SMP30* gene in human breast cancer cells. 10 µM of T_3 is able to induce significant amount of apoptosis [28] which is critically important for challenging metastatic invasion of breast cancer. SMP30, has been so far denoted as an anti-apoptotic protein, although it is an upstream positive regulator of p53 [34]. Hence it seems debatable, whether an inducer of tumor suppressor protein needs to be downregulated for induction of apoptosis, as is apparent from our results. Detailed study focussed on this area may indicate a possible treatment option of ER positive breast cancers through hormonal therapy to increase the survival rate.

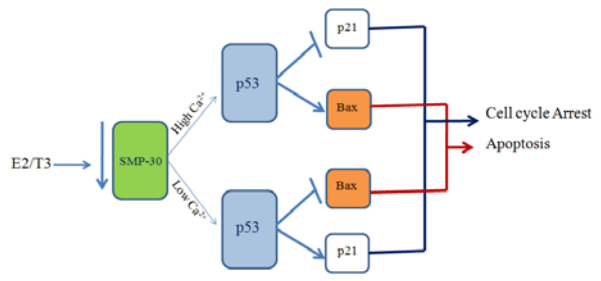

Figure 7: Putative Pathway involved in Regulation of Apoptosis/Growth check through SMP30. Downregulation of SMP30 leads to high intracellular Ca^{2+} resulting in p53 induced Bax and downstream PARP mediated apoptosis. Upregulation of SMP30 leads to decrease in intracellular Ca^{2+} resulting in p53 induced downstream p21 mediated growth check.

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