In Human Breast Cancer Cells TRβ Competes with ERα for Altering BCl2/Bax Ratio through SMP30-Mediated p53 Induction

Pramati Sar#, Dharmendra K Bhargava#, Debomita Sengupta#, Bandita Rath, Sanjib Chaudhary and Sandip K Mishra*

Cancer Biology Lab, Department of Gene Function and Regulation, Institute of Life Sciences (an Institute under Department of Biotechnology, Govt. of India), Nalco Square, Chandrasekharpur, Bhubaneswar, Odisha, India-751023

*Equal Contribution

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 (T3) 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 (E2) 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 for different types of hormonal signaling. To prove this hypothesis, gel retardation and luciferase assays were conducted by taking hSMP30 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 E2 and T3, but the functional consequences of down regulation of SMP30 in human breast cancer cells post E2/T3 treatment were different in terms of apoptosis. To unravel the mechanism behind the differential consequences of E2/T3 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 (ERs and ERβ) [7]. The action of thyroid hormone triiodothyronine (T3) 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 stage 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 (RXX), recognize E2 (ERE) and T3 response element (TRE) respectively [13]. TR and ER have the potential to bind to an identical half site consensus sequence, AGGGTCA, although the number of spacing nucleotides between the half sites and their orientations may vary for ERE and TRE. T3 bound TR can directly bind to ERE of the PR (progesterone receptor) promoter and stimulate its transcription [14]. When TR and ER both coexist, T3 bound TR inhibits E2 bound ERα-mediated transactivation of the preproenkaphlinin [15,16] and prolactin [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 E2 bound ER and T3 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...

*Corresponding author: Sandip K Mishra, Scientist D, Molecular Oncology, Gene Function & Regulation, Institute of Life Sciences, Nalco Square, Chandrasekharpur, Bhubaneswar, Odisha, India-751023. Tel: +91 0674 2300702; Fax: +91 674 2300728; E-mail: sandipkmishra@hotmail.com, sandipkmisra@ils.res.in

Received June 15, 2012; Accepted July 24, 2012; Published July 26, 2012


Copyright: © 2012 Sar P, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
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 T3 bound TR and E2 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 T3 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 T3 induced apoptosis in MCF-7 cells [28], which is in accordance with the fact that higher concentration of T3, treatment can inhibit breast cancer cell proliferation [29]. It has also been reported that E2 downregulates SMP30 in rat mammary and prostate gland [30]. However, E2 is known to promote cell proliferation in both normal and cancerous breast tissue [31]. E2 binds to ER and E2 bound ER for getting recruited in the common response element of p21 or p53 promoter EREs were demonstrated as described in Sar et al. [27]. Oligonucleotides (both strands) corresponding to hSMP30 ERE sites were synthesized as shown in Table 1. For each site one strand was end labelled with γ32P 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 (dl-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 unlabelled self, consensus ERE oligos (Table 2) were added during incubation period. Then Gel Retardation Assay was performed in 5% non-denaturing polyacrylamide gel.

**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% CO₂ atmosphere at 37°C until 70-80% confluence. For stimulation with T3 or E2, 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. T3 (10 µM/10 nM) or E2 (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**

hSMP30 promoter reporter construct was prepared by amplifying human SMP30 promoter from MCF-7 genomic DNA by using hSMKPknF and hSMPIxhoR 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 Xho I 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). The PCR 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 1×10⁵ 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 Eugene HD transfection reagent (Roche) as per manufacturer’s instruction. After 24 hrs of transfection 10 µM T3 or 10 nM E2, 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 hSMP30 ERE sites were synthesized as shown in Table 2. For each site one strand was end labelled with γ32P 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 (dl-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 unlabelled 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 T3 or 10 nM E2 or DMSO for 1 hour, 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 hSMP30 promoter EREs were demonstrated by performing PCR of ChIP-elutes using site-specific primers- hSMP30

[Table 1: Primers Used for Cloning.]

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSMP30 EcoRI F</td>
<td>ACAGAATTTCTGGACATGATGCTTC</td>
</tr>
<tr>
<td>hSMP30 XhoI R</td>
<td>ACACTCGAGTCCCCGATAGGAGTGGAGAGA</td>
</tr>
<tr>
<td>hSMP30 Kpn4 F</td>
<td>CAATCTAGCCAGAAGAAGT</td>
</tr>
<tr>
<td>hSMP30 Xho R</td>
<td>CGACCTCTTCTTACGACGTC</td>
</tr>
</tbody>
</table>

[Table 2: Oligonucleotide Used for EMSA.]

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSMP30 ERE 1 SS</td>
<td>ATGTGTGTCAGCTGGTCTCAATCCCTACCCAGTCTAGG</td>
</tr>
<tr>
<td>hSMP30 ERE 1 AS</td>
<td>CCTAAGTGGCAGGATTTGAGACCCAGCTGGACGAACAT</td>
</tr>
<tr>
<td>hSMP30 ERE 2 SS</td>
<td>GAAGGACATTTAAGGCCAATTTCTATCAAGGTCCTG</td>
</tr>
<tr>
<td>hSMP30 ERE 2 AS</td>
<td>CACCCAGCTGCAATAGTTCTCGCCATCTGTCCTC</td>
</tr>
<tr>
<td>ERE Consensus SS</td>
<td>TGGCTACAGTGGGACCTGCCATCTGTCCTCTATAGG</td>
</tr>
<tr>
<td>ERE Consensus AS</td>
<td>CATGCCAGAGTGGGACCTGCCATCTGTCCTCTATAGG</td>
</tr>
</tbody>
</table>
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 T3 or 10 nM of E2 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 (Santa Cruz) 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-PROTEAN Electrophoresis System (Biorad). PVDF membrane (Millipore) was used for overnight transfer (at~30V) was performed with Biorad Mini-PROTEAN Electrophoresis System. Trans-blot Cells in methanol-containing Tris-Glycine transfer Buffer. Post-transfer blocking was done with non-fat dry milk (Santa Cruz) 1:1000 times. Antibody dilutions were as follows: anti-SMP30 antibody (Santa Cruz) 1:5000 times, α-Tubulin antibody (Santa Cruz) 1:1000 times.

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 chemiluminescent detection reagent (GE Healthcare) for visualisation of bands.

Antibody dilutions were as follows: anti-SMP30 antibody (Santa Cruz) 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 incubation was done. Blot was subjected to chemiluminescent detection reagent (GE Healthcare) for visualisation of bands.

**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 T3 or 10 nM E2 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 (hSMP30) promoter, we scanned SMP30 promoter expression in different human breast cancer cell lines irrespective of the presence or absence of E2, but its binding affinity was decreased in the case of later. Our previous studies confirmed the presence of two important putative TREs [28] in contrast to one putative TREs [28] in human breast cancer cells.

### Recruitment/ Competition of TRβ and ERα to SMP30 promoter after E2 or T3 treatment

We next investigated recruitment of ER on SMP30 promoter by ChIP analysis (Figure 3A–3C). It was found that ERα could bind to hSMP30 ERE1 of SMP30 promoter irrespective of the presence or absence of E2, but its binding affinity was decreased in the case of later. Our previous studies confirmed the presence of the two important putative TREs [28] in contrast to one putative TRE. TRβ could also bind to hSMP30 ERE1 of SMP30 promoter irrespective of the presence or absence of T3.

---

**Sequence 5’ - 3’**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSMP30 + 560 to + 580</td>
<td>GCCACATTGGAAACACAGTT</td>
</tr>
<tr>
<td>hSMP30 + 1105 to +1085</td>
<td>CCTCTCGAAAGGCAGCATGAAG</td>
</tr>
</tbody>
</table>

**Sequence 5’ - 3’**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGDH AS2</td>
<td>TTTCTCTTTGCTTGTCCTT</td>
</tr>
<tr>
<td>Bax SS</td>
<td>CAGCTTGGACAGATAGACAGAAC</td>
</tr>
<tr>
<td>Bax AS</td>
<td>GCCCATCTTCTTTTCATGAGTGGAC</td>
</tr>
</tbody>
</table>

**Table 3: Primers Used in ChiP PCR.**

**Table 4: Primers Used for Real Time-PCR.**
its binding affinity was decreased in the case of T₃ 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 estrogentic effects by thyroid hormone in neuroendocrine tissues [18]. Additionally, coexistence of ER and TR has been reported to inhibit E₂ 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 E₂ and T₃**

We examined the response of E₂, T₃, and E₂ plus T₃ together on transcriptional activity of hSMP30 promoter in MCF-7 cells by measuring luciferase activity. We transfected hSMP30 promoter reporter construct along with expression vectors in MCF-7 cells. Luciferase activity of hSMP30 promoter (+66 to -1252) having ERE1 (hSMP30 Pro) showed slight repression in presence of E₂; however, the repression was further enhanced in presence of both E₂ and T₃ treatment in MCF-7 cells (Figure 4).

These results indicated that hSMP30 promoter is negatively regulated by liganded ERα. However, T₃ bound TR was found to further inhibit E₂ bound ER transrepression of hSMP30 promoter in presence of both E₂ and T₃. Similar pattern of inhibitory effect of T₃ bound TR on E₂ bound ER has been reported on transactivation of preproenkephalin [15,16] and prolactin gene [17].

T₃ is able to regulate Bcl2/Bax ratio through down regulation of SMP30, but this effect was not shown by E₂ mediated SMP30 down regulation

It is well established that Estradiol (E₂) 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 E₂, we checked the expression of various pro- and anti-apoptotic genes. Estrogen (E₂) 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 T3 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 T3 treatment and no further induction was found either by increasing the duration of T3 treatment or by increasing the concentration of T3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T3 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 induction caused by T3 (Figure 5B and 5D). However, the rate of apoptosis induction caused by T3 (Figure 5B and 5D). 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 T3 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 T3 treatment and no further induction was found either by increasing the duration of T3 treatment or by increasing the concentration of T3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T3 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 induction caused by T3 (Figure 5B and 5D). 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 T3 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 T3 treatment and no further induction was found either by increasing the duration of T3 treatment or by increasing the concentration of T3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T3 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 induction caused by T3 (Figure 5B and 5D). 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 T3 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 T3 treatment and no further induction was found either by increasing the duration of T3 treatment or by increasing the concentration of T3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T3 by up regulating BCL-2 and down regulating BAX expression as shown by qRT-PCR (Figure 5C).

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 T3 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 E2, T3 and E2 plus T3, the expression of p53 was up regulated through qRT-PCR (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 T3 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 T3 treatment and no further induction was found either by increasing the duration of T3 treatment or by increasing the concentration of T3 (data not shown). Over expression of SMP30 in MCF-7 cells lowered the proportion of apoptotic cells induced by T3 by up regulating BCL-2 and down regulating BAX expression as shown by qRT-PCR (Figure 5C).

**Possible downstream mechanism behind differential regulation of SMP30 by E2/T3**

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 E2/T3. SMP30 regulation by E2/T3 has been reported to inhibit activation of liver nuclear DNA fragmentation at about 0.5 to 2.0 µM Ca2+ [37]. This inhibition is reversed in presence of 25 to 50 µM Ca2+ suggesting that the possible differential effect is due to the difference in concentration of Ca2+.

Possible involvement of p53 in T3 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 E2, T3 and E2 plus T3, the expression of p53 was up regulated through qRT-PCR (Figure 5A).
Conclusion

Although E₂ and T₃ 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 E₂ and T₃ mediated downregulation of SMP30. We confirmed a putative ERE at -613 bp (hSMP30 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 ERa 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
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 T₃ mediated down regulation of SMP30 gene in human breast cancer cells. 10 µM of T₃ 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.

Acknowledgment

We acknowledge the support extended by the Director, ILS in terms of DBT core grant and core instrumentation facilities. We thank Dr. Ronald M. Evans, The Salk Institute for Biological Studies, San Diego, CA. for providing us pCMX-hRXR-α, pCMX-hTRβ and pCMX-rTRα plasmid constructs. pSG-mERalpha was received from Dr. Borja Belandia, Instituto de InvestigacionesBiomedicas, Spain. Prof. F. F. Lang, Department of Neuro-Oncology, UT MD Anderson Cancer Center provided us p53 p53-18D20D plasmid constructs. This work was funded by Department of Biotechnology, Govt. of India. PS and DS are supported by Research Fellowships from University Grant Commission, Government of India, New Delhi. DB and BR are supported by Institutional Research Fellowship from Institute of Life Sciences (an Institute under Department of Biotechnology, Govt. of India). SC is supported by Research Fellowship from Department of Biotechnology, Government of India, New Delhi. Part of this work has been presented in Annual Meeting of American Association for Cancer Research, 2012.

References


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 E2 treated (5B), 10 nM T3 treated (SC), 10 nM E2+10 nM T3 treated (SD) 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.

Figure 7: Putative Pathway involved in Regulation of Apoptosis/Growth check through SMP30. Downregulation of SMP30 leads to high intracellular Ca²⁺ resulting in p53 induced Bax and downstream PARP mediated apoptosis. Uprogulation of SMP30 leads to decrease in intracellular Ca²⁺ resulting in p53 induced downstream p21 mediated growth check.


39. Segal J (1990) Calcium is the First Messenger for the Action of Thyroid Hormone at the Level of the Plasma Membrane: First Evidence for an Acute Effect of Thyroid Hormone on Calcium Uptake in the Heart. Endocrinology 126: 2693-2702.