

PROTEOMICS OF TRANSFORMING GROWTH FACTOR β 1 (TGF β 1) SIGNALING IN 184A1 HUMAN BREAST EPITHELIAL CELLS SUGGESTS THE INVOLVEMENT OF CASEIN KINASE 2 α IN TGF β 1-DEPENDENT P53 PHOSPHORYLATION AT SER392

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Aim: Transforming growth factor β 1 (TGF β 1) is a potent regulator of breast tumorigenesis. It inhibits proliferation of carcinoma cells, but the strength of its inhibitory action varies for cells from benign, non-metastatic or metastatic tumors. The aim of this work was to generate a proteome profile of TGF β 1 action on non-tumorigenic human breast epithelial cells 184A1, and validate predicted involvement of casein kinase 2 α (CK2 α), p53 and structure-specific recognition protein-1 (SSRP1). **Materials and Methods:** Two-dimensional gel electrophoresis and mass spectrometry were used to identify TGF β 1-regulated proteins in 184A1 human breast immortalized non-tumorigenic cells. 184A1 cells may serve as a model of benign breast neoplasia. These cells were obtained from normal mammary tissue, were immortalized but are not malignant, and were obtained from the American Type Culture Collection. The systemic analysis was performed by using the Cytoscape tool. Transfection of cells with CK2 α construct and small interfering RNAs to CK2 α and SSRP1 were used to assess an impact of CK2 α and SSRP1 on phosphorylation of the p53 and cell proliferation. **Results:** Proliferation of 184A1 cells was transiently inhibited by TGF β 1. We identified 100 and 47 unique proteins which changed their expression and/or ³⁵S-incorporation, respectively, upon treatment with TGF β 1 for 2 h, 8 h or 24 h. Cell proliferation, death, migration, and metabolism were among the biological regulatory processes retrieved by the network analysis as affected by the identified proteins. The network analysis suggested that TGF β 1 may affect the phosphorylation of p53 at Ser392 by engaging CK2 α . This was confirmed by the immunoblotting and cell proliferation assays. **Conclusion:** We report here the list of 147 TGF β 1-regulated proteins in immortalized non-tumorigenic human breast epithelial cells, and show involvement of CK2 α in the regulation of p53 Ser392 phosphorylation.

Key Words: transforming growth factor β , proteomics, CK2 α , p53.

DOI: 10.32471/exp-oncology.2312-8852.vol-41-no-4.13853

Transforming growth factor β (TGF β) is a key regulator of cell proliferation, apoptosis, migration, and differentiation. TGF β consists of a family of 3 isoforms in mammals, TGF β 1, TGF β 2 and TGF β 3, which all act via type II and type I TGF β receptors [1–3]. TGF β 1 is the most common isoform [1–3]. Changes in responsiveness to TGF β 1 have been associated with tumorigenesis, suggesting that TGF β 1 may be a tumor suppressor or a promoter of metastasis, depending on type of cells [1–3]. The different impact on tumorigenesis has been explained by the variability of employed signaling mechanisms in different cells. As an example, TGF β 1-dependent inhibition of cell proliferation was found to vary from pronounced to almost negligible for breast epithelial cells [1, 4].

TGF β 1 binds first to a dimer of type II receptors, which then recruits two type I receptors. Activated heterotetrameric TGF β receptor complex phosphorylates Smad2 and Smad3 proteins, which form complexes with other proteins, including a common Smad4. A number of important so-called non-Smad

mechanisms can also be initiated by the activated receptors. These pathways include regulation of Erk1/2, p38 and c-Jun N-terminal kinase signaling, and involve modifications of proteins by acetylation and ubiquitylation [1–4]. An important component of TGF β 1 signaling is a direct impact on the protein synthesis via TGF β 1-dependent phosphorylation of eEF1A1 [5].

TGF β 1 inhibition of cell proliferation has been attributed to the effects of Smads, with modulation by non-Smad pathways, such as mitogen-activated protein kinases and protein synthesis. Cyclin-dependent kinases, their inhibitors, cyclins, and cdc25a have been proposed as targets of anti-cancer drugs acting on the cell cycle [6]. However, regulatory mechanisms from the TGF β receptors to these targets have been shown to be complex, and have a network-signaling features [7]. Recent proteomics studies of TGF β 1 signaling confirmed the complexity of signaling mechanisms initiated by TGF β 1 [4, 8–11]. These studies showed some similarities and differences in functional domains affected by TGF β 1 in different types of cells. The proteome profiling of cells representing various stages of tumorigenic transformation, e.g. from acquisition of immortalization only up to aggressive metastatic phenotype, may provide insights into differences of TGF β 1 action during tumorigenesis.

Submitted: June 26, 2019.

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Abbreviations used: CK2 α – casein kinase 2 α ; siRNA – small interfering RNA; SSRP1 – structure-specific recognition protein-1; TGF β – transforming growth factor β .

Here we report proteome profiling of TGF β 1 action on human immortalized non-malignant breast epithelial cells 184A1. Immortalization is one of the initial steps in carcinogenic transformation. We identified 100 and 47 unique proteins regulated by TGF β 1, and showed that casein kinase 2 α (CK2 α) is involved in TGF β 1-dependent modulation of p53 phosphorylation at Ser392.

MATERIALS AND METHODS

Cells and reagents. 184A1 human breast epithelial cells [12] were obtained from American Type Culture Collection, and were cultured in recommended by the American Type Culture Collection medium (Mammary Epithelial Growth Medium, complemented with penicillin/streptomycin, 5% horse serum, hydrocortisone, insulin, bovine pituitary extract, epidermal growth factor, gentamicin sulfate, amphotericin B and transferrin. 184A1 cells were obtained by immortalization with benzo(a)pyrene of cells obtained from normal mammary tissue of a normal reduction mammoplasty. The cells are immortal but not malignant.

Proteome profiling. For analysis of TGF β 1-regulated proteins, 184A1 cells were treated with human TGF β 1 at 10 ng/ml for 2 h, 8 h and 24 h (Fig. 1, a). In brief, cells were seeded in 10 cm dishes at 70% confluence, and the next day 10% fetal bovine serum containing medium was changed to the medium with 3% fetal bovine serum. TGF β 1 was added to cells to be treated for 24 h. For 8 h or 2 h incubation, TGF β 1 was added 8 h or 2 h before harvesting the cells, respectively. Control non-treated cells were cultured all the time period in 3% fetal bovine serum-containing medium. For ^{35}S -labeling, [^{35}S]methionine and [^{35}S]cysteine isotopes (Promega) were added to the medium during the last 2 h of incubation of cells, before harvesting. The final concentration of ^{35}S -label in culture medium was 10 $\mu\text{Ci}/\text{ml}$. Upon collection of proteins, cells were extensively washed with PBS and with 250 mM sucrose in 10 mM Tris-HCl, pH 7.2. Protein solubilization buffer was added directly to cells (8 M urea, 2.5% CHAPS, IPGPhor buffer (3.4 $\mu\text{L}/\text{ml}$), pH 3–10, and DL-Dithiothreitol (100 mM)), and proteins were extracted for 30 min at room temperature (18–20 $^{\circ}\text{C}$). The extract was centrifuged (13,000 rpm, 15 min), protein concentration was measured, and aliquots of the extracts were frozen at -70°C until use.

Extracted proteins (70 $\mu\text{g}/\text{gel}$) were subjected to isoelectrofocusing in an IPGPhor instrument (Amersham Biosciences/GE Healthcare, Uppsala, Sweden), in 18 cm IPG Drystrips, pH 3–10, linear. Isoelectrofocusing was performed as follows: 10 h passive rehydration, 3 h 50 V, active rehydration, 1 h 1,000 V, and 10 h 5,000 V, or until 50,000 Vh. Strips after isoelectrofocusing were equilibrated in sodium dodecyl sulfate-containing buffer, with DL-Dithiothreitol (100 mM) and then in the same buffer with iodoacetamide (200 mM), and were transferred onto 10% sodium dodecyl sulfate gels. Second dimension sodium dodecyl sulfate PAGE was performed in DaltSix, as follows: 1 W/gel, 20 min,

5 W/gel, 1 h, and 10 W/gel for 5–8 h. After electrophoresis, gels were fixed, stained with silver and dried, as described earlier [9]. To detect incorporation of ^{35}S -label, gels were exposed and scanned in a phosphor-imager FujiX-3000 to generate images of ^{35}S -labeled proteins. 2D gels were also scanned in a light scanner to generate images of silver-stained proteins. Images from the scanning and ^{35}S -exposure were up-loaded in Image Master Platinum version v 6.0 (Amersham-Biosciences/GE Healthcare, Uppsala, Sweden) for detection of differentially expressed spots. To ensure statistical significance of differential expression of spots, as normalized volumes Student's t-test was used to calculate probability by Image Master Platinum v 6.0. The threshold for significance was set at $p < 0.05$. Spots which showed changes of expression more than 50% between at least two experimental conditions were considered for identification.

Protein identification. Selected protein spots were cut from gels, and subjected to in-gel digestion, as described earlier [9]. In brief, dried gel-spot was rehydrated, de-stained, extensively washed in 0.1 M ammonium bicarbonate, then in 100% acetonitrile, and dried. Aliquot of activated trypsin (Promega) was added to the gel, and upon rehydration with trypsin solution, protein digestion was initiated. After 15–18 h incubation at 37 $^{\circ}\text{C}$, generated peptides were extracted, de-salted using ZipTips C18 μ and loaded with matrix (α -cyano-4-hydroxycinnamic acid) on a metal target for mass spectrometry. Mass spectra were collected on the Ultraflex MALDI TOF/TOF instrument (Bruker Daltonics) using FlexControl and FlexAnalysis software (Bruker Daltonics). Spectra were internally calibrated with tryptic peptides (842.51, 1045.56 and 2211.10 Da). Peptide mass fingerprinting was performed by searching NCBI nr database (RefSeq) with ProFound engine. One miscut, partial oxidation of methionine, alkylation of cysteine residues, tolerance less than 0.5 Da, and “mammalian” species were set for searches. No restrictions for pI, and a tolerance of 30 kDa to a migration position in 2D gels were set for molecular mass definition. Significance of identification was assessed by values of probability, Z-value and a number of matched peptides, and only significant (with $p < 0.05$) identifications were considered in our analysis.

Systemic analysis of TGF β 1-regulated proteins. Functional and pathway analysis was performed using Cytoscape tool for the network building and analysis [13]. Cytoscape operates with all significant databases of biomedical information. In our searches, we used only curated databases. Such stringent selection and analysis of experimental data is required to exclude the building of false-positive dependencies. Statistical significance of network nodes and edges (connections) was set to be at $p < 0.05$.

Transfections and immunoblotting. Cells were transfected in 6-well plates by LipofectAMINE 2000 reagent, as recommended by the supplier (Invitrogen, Carlsbad, USA). Small interfering RNA (siRNA) to CK2 α (cat. number sc-29918), structure

specific recognition protein 1 (SSRP1; cat. number sc-37877) and a control scrambled siRNA (cat. number sc-37007) were obtained from SantaCruz Biotechnology (Santa Cruz, USA). The control siRNA, siRNAs to CK2 α and SSRP1 were tested by the supplier for specificity and off-target effects. The medium was changed 6 h after transfection. CK2 α 1 expression vector pCMV6-XL5 (cat. number sc107027; Origene) was used. For immunoblotting, cell lysates were resolved on sodium dodecyl sulfate polyacrylamide gels and transferred onto Hybond P membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% (w/v) BSA and then incubated with a primary antibody against target proteins with dilutions, as recommended by the manufacturer, and followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The following antibodies were used: CK2 α (sc-9030, H-286, Santa Cruz, USA), and actin (sc-1615, C-11, broad range of actin isoforms, Santa Cruz, USA). The proteins were visualized using Luminol Reagents (Santa Cruz Biotechnology Inc.). For transfection with siRNA, cells were seeded in 24-well plates, and transfection procedure was performed the next day, as recommended by the siRNA supplier. After transfection, cells were cultured in a medium supplemented with 10% serum and used in assays within 24 hours of transfection.

Cell proliferation assay. Cell proliferation was measured by using [3 H]thymidine incorporation assay and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT assay) (Promega, Promega Biotech AB, Stockholm, Sweden). 184A1 cells were seeded in plates for proliferation assays. Cells were incubated with 0.1 μ Ci/ml of [3 H]thymidine for the last 24 h of the indicated time periods. Radioactivity incorporated into DNA was measured, as described earlier [9]. MTT assay was performed in parallel with [3 H]thymidine-incorporation test, except that no radioactivity was added. Cells were grown for the time periods indicated in the text, and MTT assay was performed according to the manufacturer's recommendations. Statistical significance of observed differences was evaluated by the Student's t-test.

Statistical analysis. Statistical significance of detection of protein spots in the 2D gels was set at $p < 0.05$ and was performed upon image analysis using Student's t-test by the Image Master Platinum v6.0 software. Statistical significance of protein identification by mass spectrometry was set at $p < 0.05$ and was evaluated by a probability, Z value, number of matched peptides and % of coverage. These values were obtained upon searches with ProFound search tool. Statistical significance of the network analysis was embedded in the network-building tool and was set to $p < 0.05$. This allowed incorporation in the network only curated and cross-confirmed nodes and edges. Statistical significance of paired comparisons in proliferation assays was evaluated by the Student's test, with $p < 0.05$ considered as a significant difference.

RESULTS

Proteome profiling of TGF β 1 action on 184A1 cells.

To explore TGF β 1 signaling in non-tumorigenic human breast epithelial cells, we performed proteome profiling of 184A1 cells. TGF β 1 inhibited cell proliferation and induced C-terminal phosphorylation of Smad2 protein, indicating that TGF β 1 signaling is intact in these cells (Fig. 1, b). We observed that the effect of TGF β 1 on cell proliferation was transient. TGF β 1 inhibited cell proliferation for up to 50%, after 24 h of treatment. However, after 48 h, the cell proliferation was restored to up to 80% of its original level (see Fig. 1, b). To monitor initiation of the intracellular signaling, phosphorylation of one of the substrates of type I TGF β receptor, Smad2, was analyzed. pS2 antibody to Smad2 C-terminus phosphorylation is a recognized tool to monitor activation of TGF β receptors. We observed that Smad2 was phosphorylated at its C-terminal serines after 2 h of treatment, but then its level of phosphorylation decreased

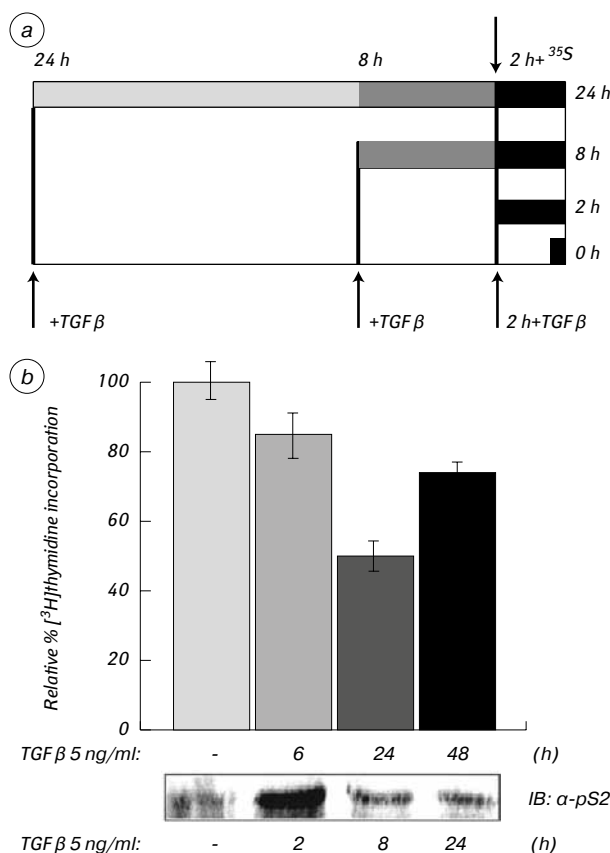


Fig. 1. Proliferation of 184A1 cells is inhibited by TGF β 1. *a* — scheme of the proteomics experiment. Time points when cells were seeded for the experiments, treated with human TGF β 1, labeled with [35 S]methionine and [35 S]cysteine, and harvested for extraction, are shown; *b* — 184A1 cells were treated with 10 ng/ml TGF β 1 for 6 h, 24 h and 48 h, as indicated. [3 H]thymidine incorporation was measured during the last 2 hours of incubation. Lower panel shows the scheme of treatment. As 100% is taken incorporation in cells not treated with TGF β 1. Smad2 is phosphorylated at the C-terminal serine residues upon treatment of 184A1 with TGF β 1. The whole cell extracts from cells treated as indicated were subjected to immunoblotting with pS2 antibodies. Migration position of phosphorylated Smad2 is indicated by the arrow. Representative experiments out of 3 performed are shown for (*b*)

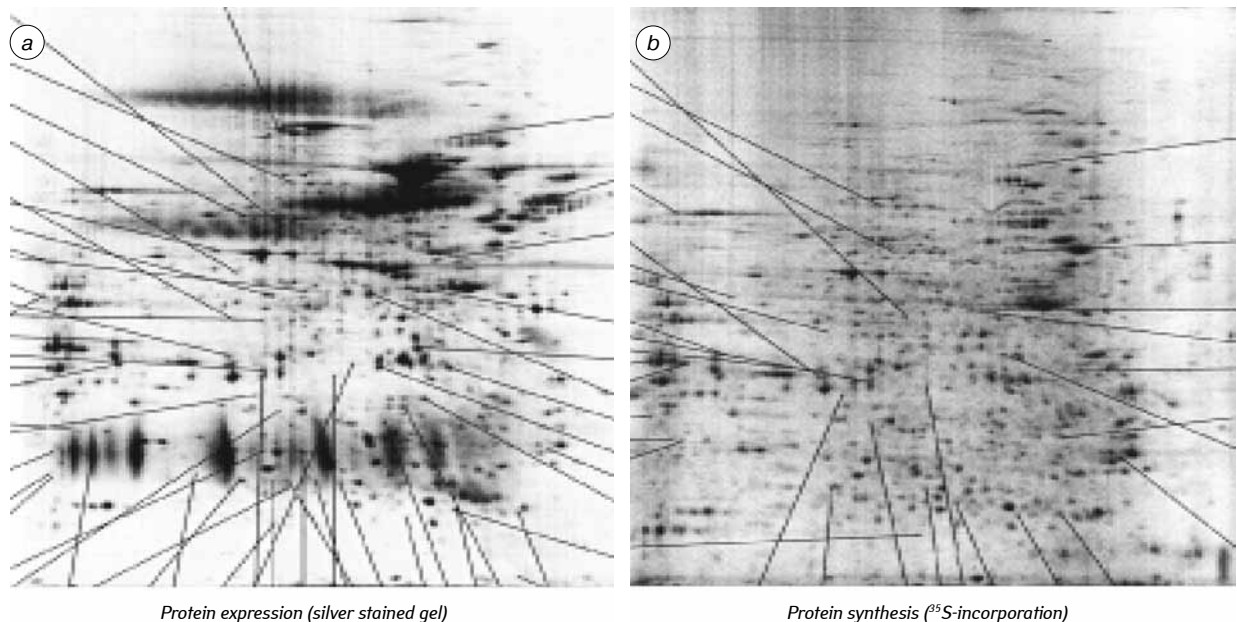


Fig. 2. Representative 2D gels of proteins from cells treated or not with TGF β 1. *a* — representative 2D gel stained with silver is shown. Annotation of proteins is shown in Supplement Table 1 and quantification data in Supplement Table 2; *b* — image of representative ^{35}S -labeled gel obtained after exposure in a phosphorimager. ^{35}S -labeled proteins identified as regulated by TGF β 1, are annotated in Supplement Table 3 and quantification data in Supplement Table 4. Migration positions of identified proteins are shown by lines in all panels

on the 8th hour and the 24th hour (see Fig. 1, *b*). Thus, 184A1 cells were responsive to TGF β 1, although the inhibition of cell proliferation was less pronounced, as compared to tumorigenic breast epithelial cells, e.g. MCF7 or MCF10A cells [9, 14].

We generated two-dimensional gels of proteins extracted from 184A1 cells treated with 10 ng/ml of TGF β 1 for 2 h, 8 h, and 24 h. Three 2D gels per each experimental condition were generated. To evaluate protein expression, we stained proteins in 2D gels with silver (Fig. 2, *a*). To evaluate protein synthesis, we labeled 184A1 cells with [^{35}S]methionine and [^{35}S]cysteine for the last 2 hours of incubation with TGF β 1 (Fig. 2, *b*). In average, we observed 1600 protein spots in silver-stained and in ^{35}S -labeled gels. Variations in the total number of protein spots in gels representing all experimental conditions were less than 10%. Using gel image analysis, we identified protein spots, which changed their expression levels for more than 50% between at least 2 experimental conditions. Only spots with statistically significant differences in expression were considered for identification ($p < 0.05$, Student's t-test, embedded in the Image Master Platinum image analysis software). MALDI TOF mass spectrometry was used to identify proteins in these spots. Hundred unique proteins regulated on the level of expression were identified in 132 protein spots, and 47 unique proteins were identified in 64 ^{35}S -labeled spots (Supplement* Tables 1–4).

To validate proteomics results, we performed immunoblotting of selected proteins. Validation of changes in expression of DNA polymerase κ , cullin-5 and replication protein A confirmed proteomics results (Fig. 3).

Systemic analysis of proteins regulated by TGF β 1. Functional clustering of identified proteins showed that regulation of the cell cycle, cell movement, morphology, antigen presentation, and metabolic processes were among the most affected functional domains. The number and functional roles of identified proteins indicated that the depth of this study was reaching the level of low abundance signaling proteins.

For an overview of signaling pathways and various cellular processes affected by TGF β 1, we explored relations between identified proteins by generating two networks. The first network was based on proteins changing expression (silver stained), and the second was based on proteins changing their ^{35}S -incorporation levels (Fig. 4, *a*, *b*). The general topology of the networks showed a number of nodes with high interconnections, but also a number of nodes with low connections, up to single pairs. It has to be noted that we applied strict expansion limitations, and only closely connected nodes and edges were considered. We observed that the network formed by the proteins changing their expression (silver stained proteins) has more regulatory clusters as compared to the network build with the ^{35}S -labeled proteins (see Fig. 4, *a*, *b*).

To extract information suitable for experimental interrogation, we analyzed nodes related to the regulation of proliferation. As TGF β 1 regulates cell proliferation, the proliferation-related network was analyzed (Fig. 4, *c*). We focused on casein kinase-2, as it phosphorylates p53 tumor suppressor. Among the network components, p53, CK2 α and SSRP1 proteins were found to be connected, with p53 being predicted as regulated by CK2 α and SSRP1 (see Fig. 4, *c*). These network connections are based on database deposited experimental evidence reported by other researchers [13]. p53 was found mutated or inactivated

*Tables as supplementary materials are posted at https://www.researchgate.net/publication/337242574_Supplementary_material_Woksepp_et_al_Experimental_Oncology_2019.

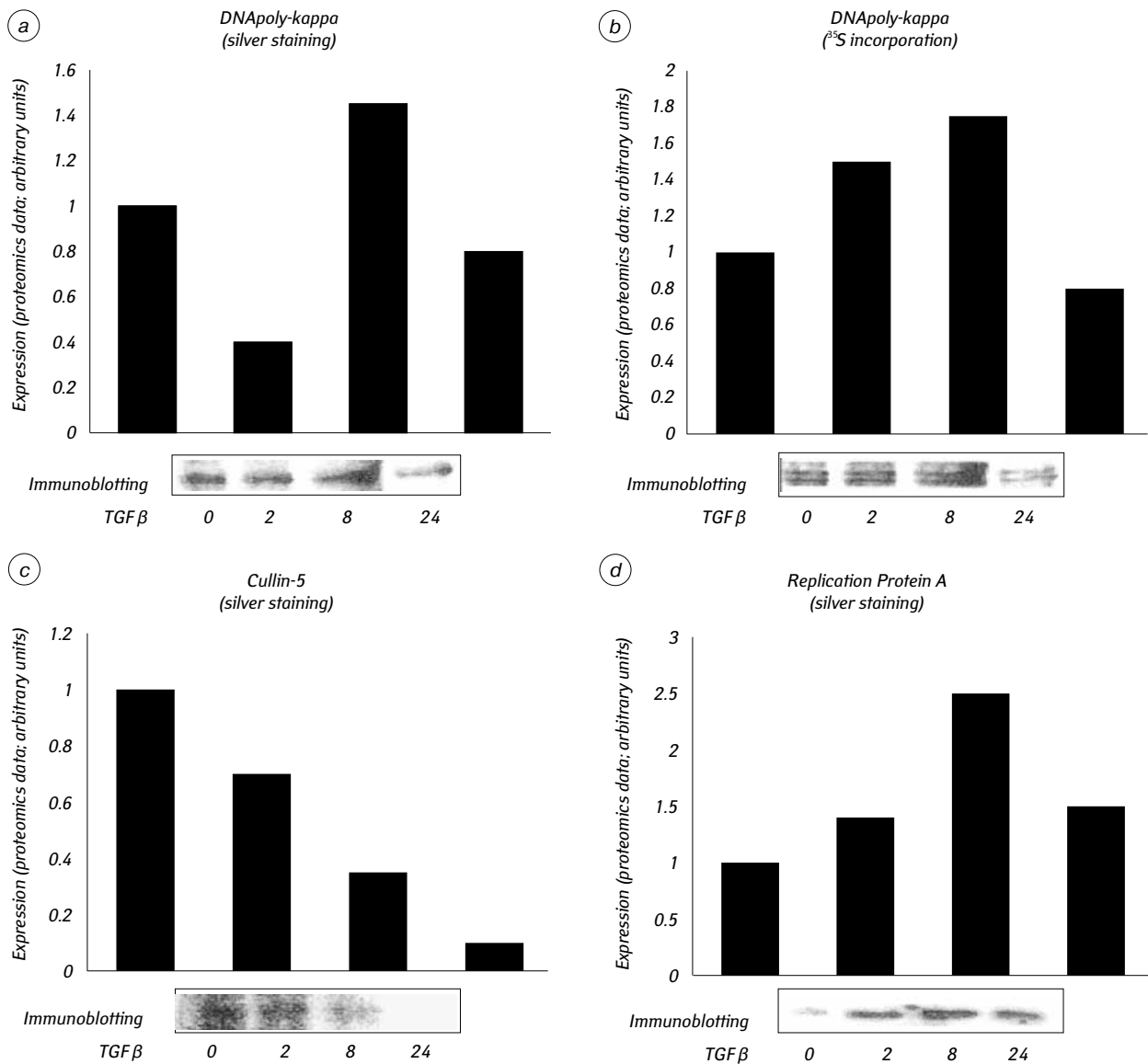


Fig. 3. Validation of proteomics data. Expression of DNA polymerase κ (a, b), cullin-5 (c) and replication protein A (d) were validated by immunoblotting of cell extracts with corresponding specific antibodies, as indicated. Migration positions of the proteins are indicated by arrows. Upper parts of panels show changes observed in 2D gels, and lower panels show immunoblotting images. Time-treatments with TGF β 1 are annotated

in approximately 50% of cancers, and is known to regulate cell proliferation and apoptosis. Therefore, we focused further experimental interrogation on CK2 α and SSRP1, as potential regulators of p53 phosphorylation upon TGF β 1 treatment (see Fig. 4, c).

CK2 α is involved in TGF β 1-dependent phosphorylation of p53 at Ser392 and regulation of cell proliferation. For interrogation of the sub-network shown in Fig. 4, c, we down-regulated CK2 α and SSRP1 or overexpressed CK2 α , alone or in combinations. Transfection of specific siRNAs was used for down-regulation, and a CK2 α expression vector for enhanced expression. Phosphorylation of p53 at serine residue 392, expression of p53, activating phosphorylation of Erk1/2, and cell proliferation test were used to monitor the responsiveness of cells to TGF β 1 (Fig. 5). As expected from proteomics data, TGF β 1 enhanced expression of CK2 α (Fig. 5, a). We also observed

stimulation of p53 phosphorylated at Ser392, and TGF β 1-dependent inhibition of phosphorylation of Erk1/2 (see Fig. 5, a). Total protein levels and the level of p53 were not changed upon treatment with TGF β 1 (Fig. 5, b, control immunoblotting for actin and p53). Thus, we confirmed that TGF β 1 may affect not only CK2 α , as was observed in the proteomics, but also phosphorylation of p53.

The network analysis suggested that the observed changes in expression of CK2 α and phosphorylation of p53 may be coordinated. Therefore, we performed an interrogation of the suggested network by down- or up-regulation of selected components. p53 phosphorylation was up-regulated by the overexpression of CK2 α , and down-regulated by the CK2 α -specific siRNA (see Fig. 5, b). The siRNAs and the construct were tested for their efficacy by the suppliers. The results shown in Fig. 5b confirm that up-regulation of CK2 α induces phosphorylation of p53 at Ser392.

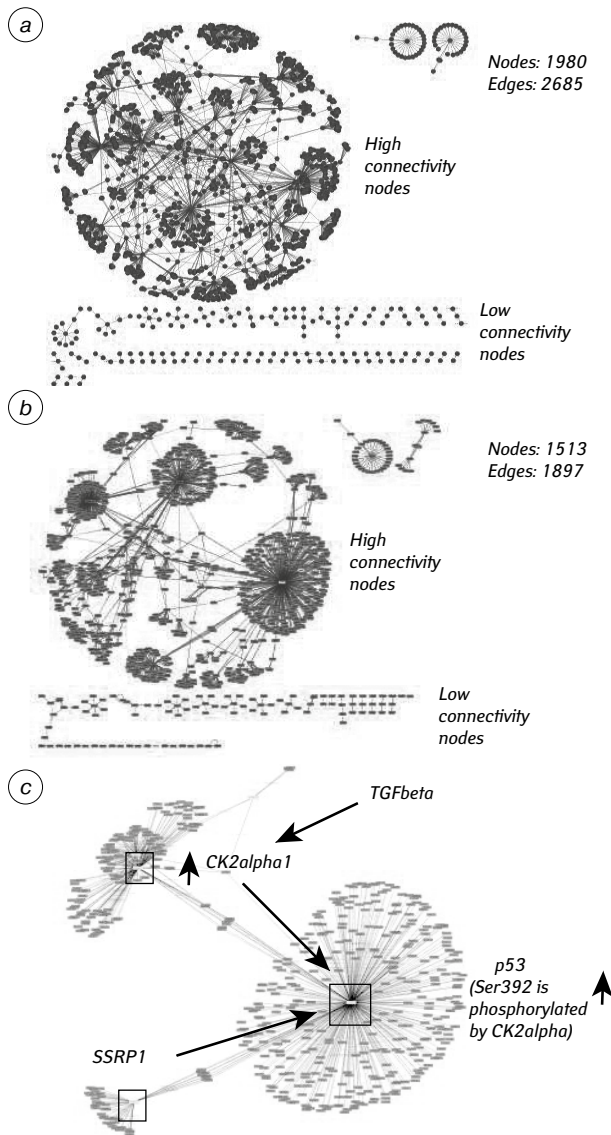


Fig. 4. Networks formed by proteins regulated by TGF β 1 in 184A1 cells. Complete networks formed by proteins which changed expression (a; silver stained proteins) and ^{35}S -label incorporation (b) are shown. The shape of the networks indicates clusters of closely interconnected nodes. (c) A link of CK2 α 1 identified as upregulated by TGF β 1, to p53 and contribution of SSRP1 are shown in the sub-network identified in the network (a)

Down-regulation of SSRP1 led to enhanced phosphorylation of p53. The enhanced phosphorylation was observed also in combinations of siSSRP1 with CK2 α overexpression or down-regulation (see Fig. 5, b). This suggests that SSRP1 has an inhibitory role on the CK2 α -dependent stimulation of p53 Ser392 phosphorylation.

Thus, CK2 α expression is induced by TGF β 1, and may be involved in the stimulation of p53 phosphorylation at Ser392. SSRP1 modulates p53 phosphorylation, as predicted from the network analysis (see Fig. 4 and 5).

We further investigated whether observed changes in p53 phosphorylation upon interrogation of cells with modulated expression of CK2 α and SSRP1 would have an impact on cell proliferation (Fig. 5, c). We observed that the down-regulation of CK2 α slightly enhanced

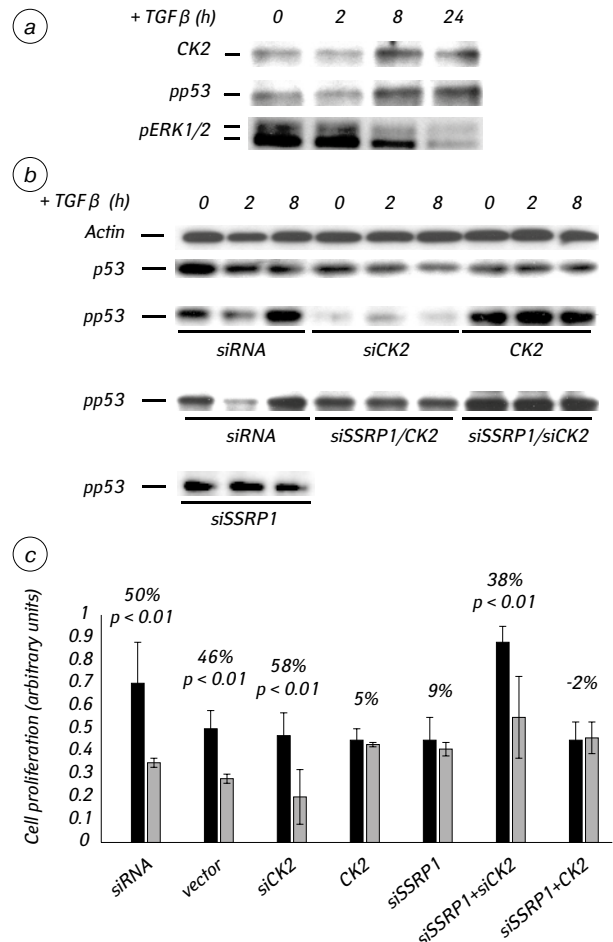


Fig. 5. Modulation of expression of CK2 α regulates p53 phosphorylation at Ser392 in 184A1 cells upon treatment with TGF β 1. 184A1 cells were treated with TGF β 1 (10 ng/ml) for the indicated period of time. a — expression of CK2 α (CK2), phosphorylation of p53 (pp53) and Erk1/2 were monitored by immunoblotting of total cell extracts; b — loading controls (actin) and expression of total p53 (p53) are shown. Phosphorylation of p53 at Ser392 upon transfection of a control scrambled siRNA (siRNA), siRNA to CK2 α 1 (siCK2), the expression vector of CK2 α 1 (CK2), and siRNA to SSRP1 (siSSRP1) is shown. Specific bands in immunoblotting experiments are shown, and proteins of interest are indicated; c — proliferation of cells was measured by an MTT assay in cells subjected to modulation of expression of CK2 α and SSRP1, as indicated. Transfections were performed with siRNA constructs (siCK2 α , siSSRP1), or with an expression vector for CK2 α (CK2 α), alone or in combinations, as indicated. TGF β 1-dependent inhibition of cell proliferation are indicated in % on the top of corresponding conditions. Significance values are annotated as p values of comparisons between TGF β 1-treated and non-treated cells. Representative experiments out of 4 (a, b) and 3 (c) performed are shown

the inhibitory effect of TGF β 1, from 50% to 58% of inhibition. The effect of TGF β 1 was not observed upon expression of CK2 α . This suggests that induction of CK2 α by TGF β 1 may lead to negative feedback for the TGF β 1-dependent inhibition of cell proliferation. Transfection of siRNA SSRP1 resulted in enhanced phosphorylation of p53 (see Fig. 5, b), and subsequently blocking of TGF β 1-dependent inhibition for conditions with transfection of siSSRP1 alone or CK2 α +siSSRP1, or a high total level of proliferation of the cells for the condition with transfection of siCK2 α +siSSRP1 (see Fig. 5, c).

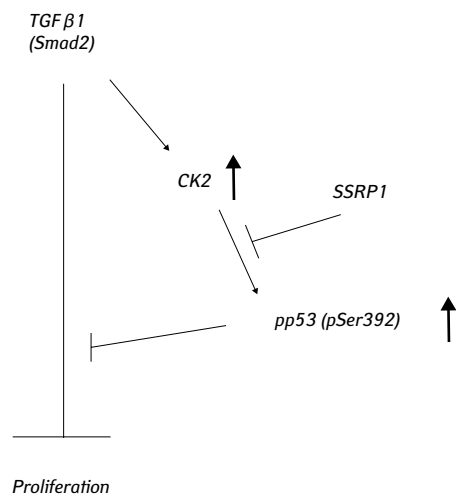


Fig. 6. Schematic model of relations between TGF β 1 upregulation of CK2 α , p53 phosphorylation, modulations by SSRP1, and cell proliferation. The connections reflect observed upregulation of CK2 α , which correlated with phosphorylation of p53, and negative modulation by SSRP1 of CK2 α -dependent phosphorylation of p53. Phosphorylation of p53 may interfere with TGF β 1-dependent inhibition of cell proliferation

DISCUSSION

The multiplicity of TGF β 1 effects on cells is a strong indication of multiple regulatory mechanisms engaged by TGF β 1 [2–4]. The identified proteins (Supplement Tables 1 and 3) represent an insight into TGF β 1 targets in 184A1 human breast epithelial cells, which have a phenotype corresponding to the immortalized cells. Immortalization is one of the first steps in transformation of normal cells into malignant; immortalization is the step crucial for development of cancer. The reported here TGF β 1-regulated proteins reflect regulation of the cell proliferation, death, migration, regulation of transcription and protein expression and activities. These biological processes are engaged in tumorigenesis. Our proteomics and systemic analysis data (see Fig. 2 and 4) are in line with many reports of TGF β 1 engaging hundreds of genes or proteins [2, 3]. The importance of our contribution is that we describe TGF β 1-targets in immortalized but not yet malignant cells, which represent initial steps of tumorigenesis. This would allow a further study of dynamics and specifics of TGF β 1 signaling in cells at different steps of carcinogenic transformation, e.g. from tumorigenic to highly metastatic. For example, it may be by comparing reported here data (see Fig. 2 and 3, Supplement Tables 1–4) with other reports (see examples at www.ebi.ac.uk, search for “TGFbeta” datasets).

Systemic analysis (see Fig. 4) shows connections between the identified proteins and links to potential signaling partners. That allows to transit from a list of affected proteins to a regulatory network. Observation of a denser network built with proteins changing expression (see Fig. 4, a), as compared to ^{35}S -incorporating proteins (see Fig. 4, b), is expected due to a protein synthesis being only one of many mechanisms affecting protein expression. The topology of the networks indicates types of regulatory connections and sub-

sequently systemic features of signaling, e.g. closer connected sub-networks, feedbacks and feed-forward loops [10, 15–17]. The network analysis of TGF β 1 signaling in human breast tumor cells and tumor biopsies showed examples of such topologies [18, 19]. Applied by us limitation of expansion of the reported here networks and incorporation of curated data resulted in the generation of high and low connected nodes. The nodes connectivity distribution indicates priorities of TGF β 1 signaling in the given cells, i.e. 184A1 cells.

One of the priority mechanisms is an observation of upregulation of CK2 α 1 by TGF β 1 (Supplement Tables 1 and 2). Tumor suppressor p53 is phosphorylated by CK2 α at Ser392 [20–23]. This phosphorylation contributes to formation of p53 complexes in p53-dependent regulation of cell death and proliferation [21–23]. SSRP1 is one of such components [22]. p53 phosphorylation at Ser392 is involved in formation of p53 tetramers, and therefore p53 activation [24]. Abrogation of p53 phosphorylation at Ser392 resulted in less active p53 [25]. p53 is one of the key regulators of cell proliferation and apoptosis [6, 20]. The network analysis (see Fig. 4) indicated also that the effect on p53 may be mediated by CK2 α and modulated by SSRP1. SSRP1 was reported to form a complex with CK2 α and CK2 β [22].

Here we report proteins of key importance for TGF β 1 signaling in immortalized breast epithelial cells 184A1, identified by a proteomics approach. Our results show a mechanism that involves TGF β 1, CK2 α and SSRP1, and may have relevance to TGF β 1-dependent inhibition of 184A1 proliferation (Fig. 6). This mechanism involves up-regulation of CK2 α , followed by an increase in p53 phosphorylation at Ser392. siRNA to SSRP1 enhanced p53 phosphorylation, indicating an inhibitory role of SSRP1. The p53 Ser392 phosphorylation correlates with a decrease of inhibitory activity of TGF β 1 (see Fig. 6). This study provides proteome profile and network data of TGF β 1 signaling in cells at the first step to acquiring malignant phenotype, e.g. immortalization. The study of TGF β 1 impact on CK2 α , p53 and SSRP1 confirmed the proteomics and systems biology predicted interactions.

ACKNOWLEDGMENTS

The authors are grateful for the support and encouragement of Oves Minnesfond and the grants from the Qatar National Research Fund (NPRP9-453-3-089) and Qatar University (QUUG-CMED-15/16-1, QUST-CMED-FAU-15/16-1, QUCG-CMED-2018/2019-2) to S.S.

H.W., R.S.K., O.Z. and A.G. performed experiments, N.Z. participated in protein identification by MS, S.S. designed and managed the project, wrote the manuscript.

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