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REVIEW

Characterization of tumor differentiation factor (TDF) and its receptor (TDF-R)

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Abstract Tumor differentiation factor (TDF) is an under-investigated protein produced by the pituitary with no definitive function. TDF is secreted into the bloodstream and targets the breast and prostate, suggesting that it has an endocrine function. Initially, TDF was indirectly discovered based on the differentiation effect of alkaline pituitary extracts of the mammosomatotropic tumor MtTWlO on MTW9/PI rat mammary tumor cells. Years later, the cDNA clone responsible for this differentiation activity was isolated from a human pituitary cDNA library using expression cloning. The cDNA encoded a 108-amino-acid polypeptide that had differentiation activity on MCF7 breast cancer cells and on DU145 prostate cancer cells in vitro and in vivo. Recently, our group focused on identification of the TDF receptor (TDF-R). As potential TDF-R candidates, we identified the members of the Heat Shock 70-kDa family of proteins (HSP70) in both MCF7 and BT-549 human breast cancer cells (HBCC) and PC3, DU145, and LNCaP human prostate cancer cells (HPCC), but not in HeLa cells, NG108 neuroblastoma, or HDF-a and BLK CL.4 cells fibroblasts or fibroblast-like cells. Here we review the current advances on TDF, with particular focus on the structural investigation of its receptor and on its functional effects on breast and prostate cells.

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Abbreviations

TDF	Tumor differentiation factor
TDF-R	TDF-Receptor
HBCC	Human breast cancer cells
HPCC	Human prostate cancer cells
MCF7 cells	Steroid-responsive breast cancer cells
BT-549 cells	Steroid-resistant breast cancer cells
DU145 cells	Steroid-resistant prostate cancer cells
PC3 cells	Steroid-resistant prostate cancer cells
LNCaP	Steroid-responsive prostate cancer cells
HeLa	Cervical cancer cells
NG108 cells	Mouse neuroblastoma x rat glioma cells
BLK CL.4 cells	Embryonic fibroblasts-like cells
HDF-a	Human dermal fibroblasts
ORF	Open reading frame
SDS-PAGE	Sodium dodecyl sulfate–polyacryl amide gel electrophoresis
Ab	Antibodies
AP	Affinity chromatography
IAP	Immunoaffinity purification
MS	Mass spectrometry
ESI-MS	Electrospray ionization mass spectrometry
LC–MS/MS	Liquid chromatography tandem mass spectrometry

TIC	Total ion current
m/z	Mass/charge
CID	Collision-induced dissociation
WB	Western blotting
IF	Immunofluorescence
GRP78 precursor/BiP	Glucose regulated protein (accession # gi6470150/gi386758)
HSP8	Heat shock 70-kDa protein 8 isoform 1 (accession # gi62897129/gi5729877)
HSP70	Heat shock 70-kDa protein (accession # gi386785)
HSP1	Heat shock 70-kDa protein 1 (accession # gi4529893)
HSP90Bb	Heat shock 90Bb protein (accession # gi20149594)
HSP90	Heat shock protein 90 (accession # gi306891)
HSPA9	Heat shock 70-kDa protein 9 (accession # gi12653415)

Introduction

Tumor differentiation factor (TDF) is a recently discovered protein, produced by the pituitary gland and secreted into the blood stream, with an under-investigated mechanism of action. TDF induces morphological and biochemical changes in vitro and in vivo, which suggest that it is involved in the differentiation of HBCC and HPCC [1, 2]. Specifically, it induces markers of differentiation such as the polarization and formation of cell junctions and basement membrane. Furthermore, it promotes milk protein synthesis and the over-expression of E-cadherin [3–10]. However, TDF has no known morphological differentiation effect on fibroblasts, kidney, hepatoma, or leukemic lymphocytic cell lines [1, 2]. The differentiation activity of TDF has not been reproduced by any of the known pituitary hormones or other growth factors produced by the pituitary [1, 2]. TDF is secreted by the pituitary directly into the blood, suggesting that this protein has an endocrine role (Fig. 1). However, it is not yet completely clear where it goes and to what receptor it binds. It is also not clear how TDF protein promotes cell differentiation. Since 1992, when its activity was first discovered and described, there have been only three additional publications, two of which emerged from our group. Here we discuss how TDF was first discovered and then further investigated, with particular focus on the structural investigation of its receptor and its functional effects on breast and prostate cells.

Initial identification of TDF

TDF was indirectly discovered based on its effect on the alkaline pituitary extracts of the mammosomatotropic

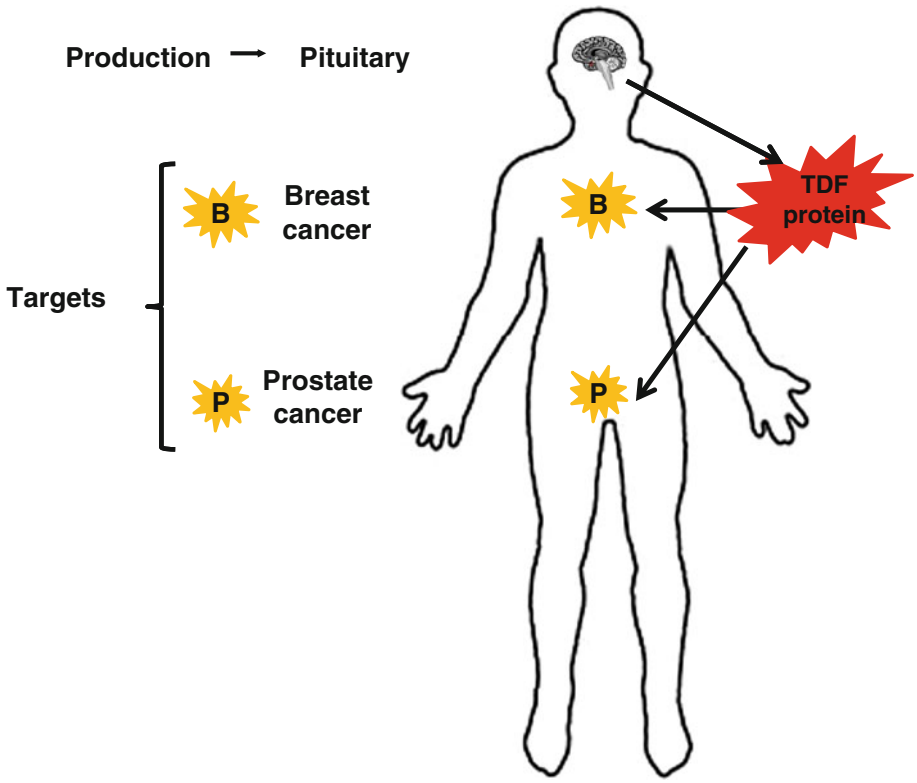

Fig. 1 Schematic of the site of the synthesis of TDF and its target organs in men and women

tumor MtTW10 on MTW9/Pl rat mammary tumor cells. The investigators found that alkaline pituitary extracts of MtTW10 tumors induced aggregation and adhesion of MTW9/Pl cells [1]. They also observed that upon exposure to the pituitary extract, the MTW9/Pl cells started to synthesize lactalbumin and overexpress laminin, as determined by Western blotting (WB). The researchers concluded, using available microscopic and biochemical evidence, that the MTW9/Pl cells aggregate, adhere to each other and differentiate, due to a factor contained within the pituitary extract. Years later, the same group used expression cloning to identify the cDNA responsible for this differentiation activity [2]. They reported the isolation of a cDNA clone of 1.1 kb from a human pituitary cDNA library by expression cloning in *Xenopus* oocytes. The cDNA encodes a 108-aa polypeptide. This protein was named the tumor differentiation factor (TDF). The recombinant TDF protein and a 20-amino-acid peptide, TDF-P1, selected from the open reading frame (ORF) of the gene, induced morphological and biochemical changes similar to the pituitary extract and consistent with differentiation of HBCC and HPCC. Fibroblast, kidney, hepatoma, and leukemic lymphocytic cell lines were unaffected. Breast and prostate cancer cells aggregated in spheroid-like acini after exposure to TDF. This effect was abrogated by anti-TDF-P1 antibodies. E-cadherin expression was increased in a dose-dependent manner by TDF. Moreover, treatment of MCF7 cells with TDF led to production of a lactalbumin-related protein, which did not occur in untreated MCF7 [2].

Characterization of TDF

TDF protein is very small with a predicted molecular mass of 12 kDa. It does not share homology with any protein sequence available in databases (Expasy, NCBI, etc.) and contains a histidine-rich region, two *N*-myristoylation sites (6GTRVGQ11 and 10GQALSF15), and two protein kinase C phosphorylation sites (57SLK59 and 102TFR104) [11, 12]. It also contains additional phosphorylation sites for Casein kinase II and protein kinase A. TDF also contains four cysteine residues that may be disulfide-linked in the secreted TDF isoform. TDF contains no particular motifs for other post-translational modifications such as *N*-glycosylation, or processing/truncation (e.g., a furin-like cleavage site). In addition, although this protein is secreted, it does not have a signal sequence, suggesting that it is secreted through a non-classical secretory pathway [13]. Furthermore, TDF is either glycated or glycosylated at serine and/or threonine residues [2]. The observed molecular mass of TDF protein is much higher than the theoretical one: the glycosylated protein has 45 kDa and the de-glycosylated one is 35 kDa [2], but its theoretical mass is 12 kDa. Currently, the isolation and characterization of this intensely post-translationally modified protein, partially published [14], is underway in our laboratory.

Isolation and identification of the potential TDF receptor candidates

The effect of TDF on HBCC and HPCC can be interpreted through the existence of a receptor: TDF receptor (TDF-R). Therefore, identification and characterization of TDF-R from HBCC and HPCC was always the highest priority in our laboratory. However, when the existence of a receptor is suspected, a strong rationale for its identification must exist, and a good strategy for (1) design of the experiments for isolation and characterization of TDF-R, (2) execution of the experiments, and (3) interpretation of the results is required. A schematic of the design of the experiments is shown in Fig. 2. The outcomes of these experiments were already published by our laboratory [15, 16]. In our

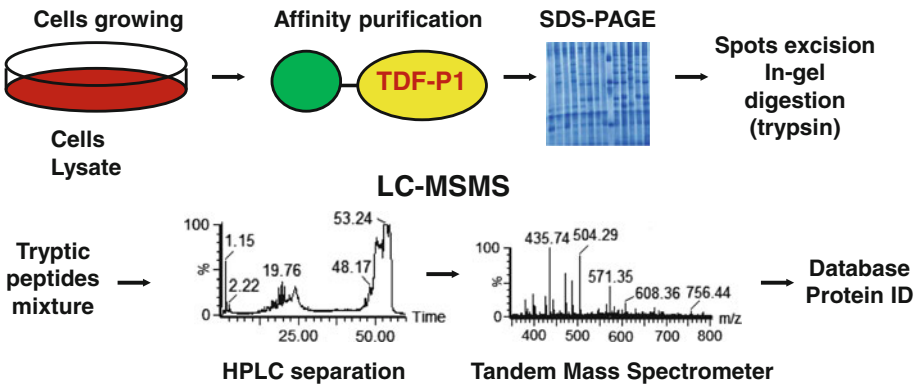

Fig. 2 Strategy for isolation and identification of the TDF-R candidates by AP and LC–MS/MS. The DU145 and MCF7 cell lysates were incubated with TDF-P1 agarose beads and the eluates were separated by SDS-PAGE and stained by Coomassie. The gels were then cut into pieces and digested by trypsin and then the resulting peptide mixtures were analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The raw data were processed by ProteinLynx Global Server (PLGS version 2.4, Waters Corporation) and the pkl files were submitted to Mascot (http://www.matrixscience.com) database search for protein identification

experiments, we used affinity purification (AP) chromatography for purification of the potential TDF-R candidates and liquid chromatography tandem mass spectrometry (LC–MS/MS) for their identification and characterization. Validation and follow-up was performed using additional methods (described later). Initially we grew the cells in vitro, lysed the cells, and then purified the potential TDF-R candidates using the TDF-P1 coupled to agarose beads. We reasoned that if the native TDF, the recombinant TDF (rTDF), and TDF-P1 peptide promote differentiation of HPCC and HBCC, then the potential TDF-R candidates could be isolated by affinity chromatography using TDF-P1 only. Once we purified the potential TDF-R candidates, the eluates were separated by SDS-PAGE and the gel pieces were digested by trypsin and the peptides mixtures were extracted and analyzed by LC–MS/MS and submitted to a Mascot database search for the identification of proteins (Fig. 2).

The work pertaining to the isolation, identification, and characterization of potential TDF-R candidates was performed according to published procedures [17–24] and the outcomes of these studies were recently published [15, 16]. In our experiments, we incubated the TDF-P1 beads with the cell lysates, washed the beads, eluted the TDF receptor candidates, and analyzed them by LC–MS/MS, as described in Fig. 2. We analyzed two cell types that are representative for identification of the potential TDF-R candidates from all cells: steroid-responsive MCF7 HBCC and steroid-resistant DU145 HPCC. Identification of the same potential TDF-R candidates would suggest that the TDF induces differentiation of both HBCC and HPCC, through a steroid-independent pathway. Conversely, identification of the potential TDF-R candidates that are different in HBCC and HPCC would suggest that the (1) TDF-R is different in HBCC compared with HPCC and TDF promotes cell differentiation through a mechanism that is different in breast cells, compared with prostate cells; (2) the TDF-R is the same in both HBCC and HPCC, but specific only to steroid-responsive or steroid-resistant cells. In this case, regardless of whether possibility 1 of 2 is correct, further experiments are required.

Isolation and identification of TDF-R candidates from androgen-resistant DU145 cells by AP and LC–MS/MS

In the AP and LC–MS/MS experiments for identification of TDF receptor candidate using DU145 cells as starting material, we identified with high confidence seven proteins that are potential TDF receptor candidates: GRP78 precursor or BiP (gi6470150), heat shock 70-kDa protein 8 isoform 1 (HSP8, gi62897129), heat shock 70-kDa protein (HSP1, gi386785), Heat shock 90Bb protein (HSP90Bb, gi20149594), heat shock protein 90 (HSP90, gi306891), Sequestosome 1 (gi119574171) and valosin-containing protein (gi11305821). HSP90Bb and HSP90 were not considered since they were identified by only one peptide.

The first hit in our experiments that had the highest probability to be the potential TDF receptor was glucose-regulated protein (GRP78), a 78-kDa protein and a member of the heat shock protein (HSP) family, also named heat shock 70-kDa protein 5 (HSP70 or HSP5) or immunoglobulin heavy chain-binding protein (BiP). Total ion current (TIC), MS, and MS/MS of a peptide that is part of GRP78 are shown in Fig. 3. GRP78 is a member of the heat shock protein (HSP) 70 family of proteins and is involved in the folding and assembly of proteins in the endoplasmic reticulum (ER), but it may also be identified in the cytosol or at the cell membrane [25, 26]. HSPs are highly expressed in cancerous cells [25, 27–34] and are essential to the survival of these cells [35] and therefore HSP inhibitors show promise as anticancer agents [36, 37]. HSPs (HSP70 and HSP90) have been found to be associated with both estrogen and androgen receptors [38–42]. HSPs have a role in cell proliferation and inhibition of one HSP (HSP90) led to dysregulation of a different HSPs (HPS70) and inhibition of cell proliferation [43]. HSPs also have a role in apoptosis and cell differentiation, especially HSP70 and HSP90. These proteins interact with apoptotic proteins and block the apoptotic pathways, thus promoting cell differentiation [43]. HSPs may even determine whether cells should undergo apoptosis or differentiation [44]. Recently, it was demonstrated that GRP78 forms a cell surface complex with Cripto, an oncoprotein that signals via MAPK/ERK, PI3 K/Akt, and Smad2/3 pathways, and mediates signaling in human tumor, mammary epithelial, and embryonic stem cells [25]. Active Cripto from Cripto-GRP78 complex promotes cellular proliferation, decrease of cell adhesion, and down-regulation of E-cadherin. However, Cripto alone is not able to signal and promote the above-mentioned cellular events. Therefore, GRP78, when in complex with Cripto is an oncogene [25], while when it is not complexed with Cripto, it may promote cell differentiation [44]. The two other HSPs, heat shock 70-kDa protein 8 isoform 1 (HSP8, gi62897129), heat shock 70-kDa protein (HSP70, gi386785) are all HSPs from the same family with GRP78 and are currently being investigated.

The second protein that we identified as a potential TDF receptor in our AP experiments using androgen-resistant DU145 cells as a starting material was Sequestosome 1, a 47-kDa cytoplasmatic protein, also named ubiquitin-binding protein p62. Sequestosome 1 has a Phox and Bem1p (PB1) domain, present in many eukaryotic cytoplasmic signaling proteins. The PB1 domain-containing proteins

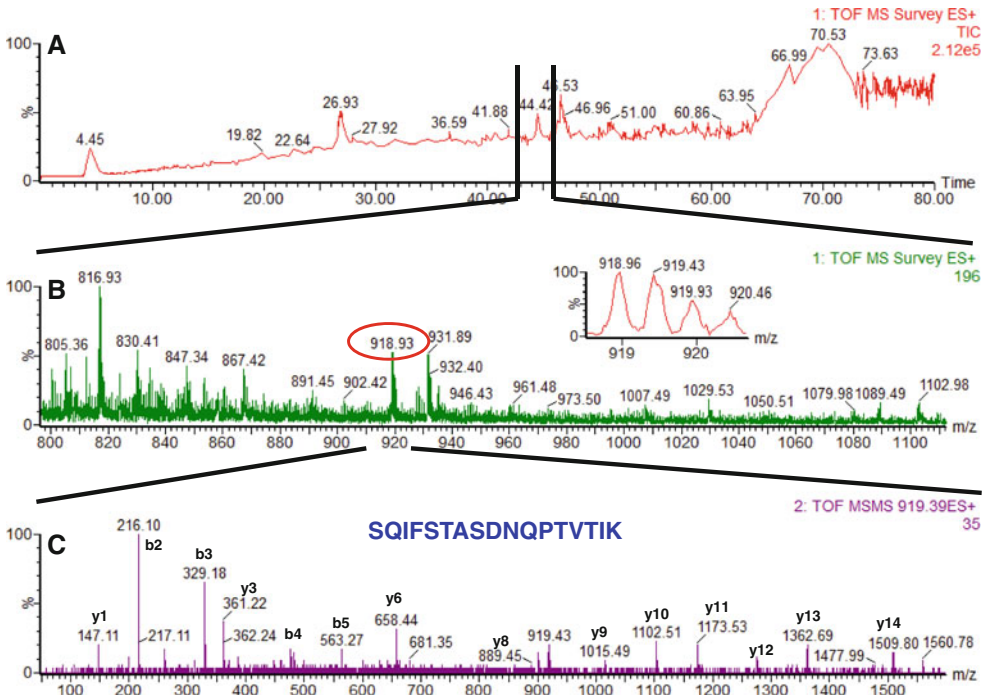

Fig. 3 AP and LC–MS/MS analysis of a peptide mixture for identification of the potential TDF receptor. The TDF-P1 beads were incubated with DU145 cell lysate and the AP protein sample was eluted and then separated on SDS-PAGE. The gel bands were then excised and digested by trypsin and the resulting peptide mixture was separated on a C18 reverse phase column over 75 min of gradient of acetonitrile. **a** TIC of the chromatogram. **b** MS survey mass spectrum, in which one double-charged peak at *m/z* of 918.93 (expanded in the inbox) was fragmented by MS/MS and produced a MS/MS spectrum (**c**). The resulting peaks in the MS/MS spectrum correspond to a series of *b* and *y* ions from a peptide that was part of GRP78. Data analysis of these peaks led to identification of the sequence shown in (**c**) and identification of GRP78 as potential TDF receptor. Figure adapted from reference 15 with permission from the publisher

are usually involved in cell signaling, receptor internalization, protein turnover, and protein–protein interactions. This protein interacts with the proteasome [45], aPKC [46], and MEK5 [47]. However, we did not find for this protein any link to steroid receptors, cell differentiation, or cell proliferation. Since we did not identify this protein in AP experiments using MCF7 cells (see later), it is possible that this protein was a contaminant in our experiments. Nevertheless, we still do not exclude this protein from our list of TDF receptor candidates. The last protein that we identified in our AP and LC–MS/MS experiments with DU145 cells was valosin-containing protein, also named transitional endoplasmic reticulum ATPase, an 89-kDa protein. This protein is usually found as a homohexamer [48] with a predicted and determined molecular mass of 540 kDa [17, 20, 24].

Isolation and identification of TDF receptor candidates from estrogen-responsive MCF7 cells by AP and LC–MS/MS

In our LC–MS/MS experiments performed with the material isolated from estrogen-responsive MCF7 cells, we identified four proteins with high confidence: GRP78 precursor (gi386758); heat shock 70-kDa protein 8 isoform 1 (HSP8, gi5729877 heat shock 70-kDa protein 1 (HSP1, gi4529893), heat shock 70-kDa protein 9 (HSPA9, gi12653415). Additional structural proteins such as actin, keratin, cytokeratin, and tubulin were also identified. So far, the only strong TDF receptor candidates were GRP78 and HSP70. These proteins were identified with high confidence in the experiments using both MCF7 and DU145 steroid-responsive breast and steroid-resistant prostate cancer cells. The two additional proteins, Signalosome 1 and valosin-containing protein, are very unlikely to be TDF-R candidates. The other potential TDF-R candidates (heat shock 70-kDa protein 8 isoform 1, heat shock-induced protein, and heat shock 70-kDa protein 9 isoform) are all HSPs from the same family with GRP78 are also currently being investigated.

Validation of the AP and LC–MS/MS by AP and Western blotting (WB)

Identification of the TDF-R candidates by AP and LC–MS/MS do not hold much value without proper validation. Therefore, validation of the TDF-R was investigated using AP and WB in various cell lines. To validate our

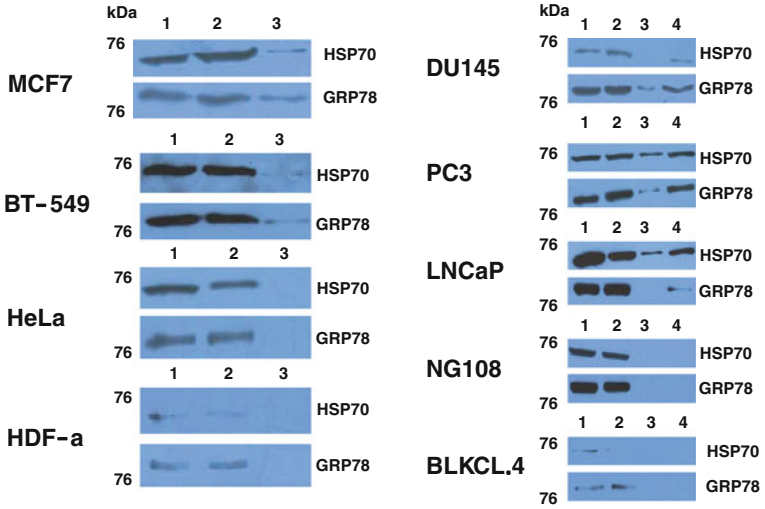

Fig. 4 AP and WB analysis of various cell lines using anti-GRP78 and HSP70 antibodies. The TDF-R candidates were purified from the cell lines indicated by AP using TDF-P1 peptide and then the eluates were investigated by WB. The cell lysates were prepared from HBCC (MCF7 and BT-549), HPCC (DU145, PC3, and LNCaP), cancerous cells (HeLa and NG108 neuroblastoma), and normal cells (HDF-a fibroblasts and BLK CL.4 fibroblast-like cells). In WBs: (*1*) input cell lysate, (*2*) flow through, (*3*) eluate, and (*4*) 5× concentrated eluate. The molecular mass markers are indicated (kDa). Figure adapted from Refs. [15, 16] with permission from the publisher

experiments, we used AP and WB using antibodies (Ab) against GRP78 and against HSP70 protein; anti-GRP78-Ab recognized only human GRP78, while the anti-HSP70-Ab recognized HSP8 and HSP1. We reasoned that GRP78 is the main TDF-R candidate, but HRP70 is also a TDF-R candidate and represents a family of proteins including HSP8 and HSP1 HSP70. We also included the possibility that both GRP78 and HSP70 are TDF-R and form a GRP78-HSP70 core protein complex. The outcome of the AP and WB experiments is shown in Fig. 4.

GRP78 and HSP70 are the potential TDF-R candidates in both steroid-responsive and steroid-resistant HBCC and HPCC, suggesting a common steroid-independent mechanism of TDF-induced cell differentiation in breast and prostate cells

Initially, we tested HBCC to validate the TDF-R candidates. We found the TDF-R candidates (GRP78 and HSP70) by AP and WB in both MCF7 steroid-responsive HBCC and in steroid-resistant BT-549 HBCC (these cells do not express estrogen receptors) [49], suggesting that the TDF-R candidates may activate the TDF pathway through a mechanism independent of and perhaps parallel with the steroid pathway. We also found a similar trend in steroid-responsive LNCaP HPCC and in steroid-resistant DU145 and PC3 HPCC, suggesting that the TDF pathway may be common to breast and prostate cancer cells. However, we did not find the TDF-R candidates in AP and WB experiments in non-breast, non-prostate HeLa cancerous cells, NG108 neuroblastoma × glioma cells, as well as in non-breast, non-prostate, non-cancerous human dermal fibroblasts (HDF-a), or normal embryonal fibroblast-like cells (BLK CL.4). Therefore, TDF-R candidates are restricted to breast and prostate cells, but are not purified from non-breast, non-prostate cancerous cells, or from normal non-breast, non-prostate cells, suggesting that the TDF pathway is restricted to steroid-responsive and steroid-resistant breast and prostate cells.

Investigation of GRP78 and HSP70 as potential TDF-R candidates using immunofluorescence (IF) and confocal microscopy

Additional insights about potential TDF-R candidates came from IF and confocal microscopy studies. In one of the studies, we observed that GRP78 Ab interacts with their antigens outside the plasma membrane, as compared with CM-Dil, a plasma membrane marker [16]. This staining pattern was observed in both steroid-responsive MCF7 and steroid-resistant BT-549 cells, as well as in HeLa cancerous cells, but not in HDF-a cells. In the same study [16], we found that HSP70 antibodies identified their antigens outside of plasma membrane in all cell lines studied (MCF7, BT-549 breast cancer cell lines, HeLa cancer cells, and HDF-a cells), which prompted us to suspect that the HSP70 is not a specific TDF-R, the staining is not specific, or HSP70 indeed exists outside the plasma membrane in all

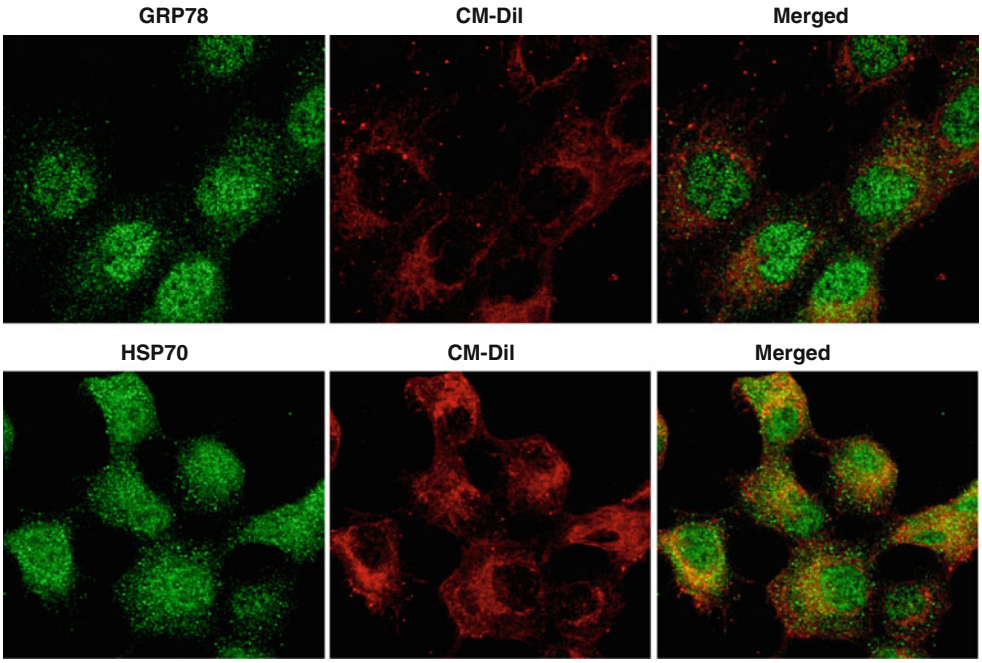

Fig. 5 Immunolocalization of GRP78 and HSP70 proteins in DU145 cells by confocal microscopy. GRP78 and HSP70 proteins were detected by anti-GRP78 and anti-HSP70 antibodies and then visualized with AlexaFluor 488 antibodies (*green*). Plasma membrane was stained with CM-Dil (*red*). The merged images are also shown

cell lines studied, but the TDF-R is a multi-subunit receptor whose specificity and activity depends on the interaction of HSP70 with GRP78 to form a TDF-R complex, composed of at least GRP78 and HSP70. Additional subunits that could fit to our theory are HSP90Bb or HSP90 beta that were identified in our experiments and that are known to interact with HSP70 proteins.

Further evidence about the TDF-R came from a follow-up study in which our lab investigated the HPCC by IF and confocal microscopy [15]. GRP78 protein was detected by their antibodies outside the plasma membrane of steroid-resistant DU145 and PC3 HPCC, steroid-responsive LNCaP HPCC, and in the non-prostate NG108 neuroblastoma cells, but not in the normal fibroblast-like BLK CL.4 cells. For the HSP70 protein, IF experiments showed similar staining in all cell lines investigated (DU145, PC3, LNCaP HPCC, NG108 neuroblastoma, and normal fibroblast-like BLK CL.4 cells. These results demonstrated that while GRP78 is detected outside the cells in HeLa and NG108 cancerous cells, detection of this protein is not sufficient to be named a TDF-R. Therefore, the initial suspicion that TDF-R may contain more than one subunit became stronger. An example of a staining by GRP78, HSP70, and CM-Dil investigated by confocal microscopy is shown in Fig. 5. Therefore, interaction of GRP78 with HSP70 and perhaps with other proteins may indeed lead to a functional TDF-R, which may also be a TDF-induced-formation of TDF-R complex or a constitutive TDF-R complex.

Investigation of GRP78 and HSP70 as potential TDF-R candidates using molecular modeling

Complementary information about GRP78 and HSP70 proteins as potential TDF-R candidates came from molecular modeling of the published work from our laboratory [15, 16], as well as from additional research (Fig. 6). A homology model of protein structure was generated by using the template single-chain PDB structure 2KHO [50], the NMR structure of *E. coli* HSP70 (DnaK). The KHO model is used as TDF-P1 receptor protein, and DnaK is the prokaryotic correspondent of eukaryotic HSP70. The resulting model structure of 2KHO, built using the Swiss-Model automated mode [51–53], has 1.43 Å rmsd when superposed with 2KHO using Cα atoms [54]. A ribbon diagram of TDF-R model protein is depicted in Fig. 6a, where the TDF receptor protein is colored from amino-terminal (blue) to carboxyl-terminal (red). Exposed and buried residues of this model receptor protein are shown in Fig. 6b. Docking experiments were performed using Gramm × docking server [55, 56] and have been described previously [15, 16]. Among the top-ten structures, the TDF-P1 peptide is docked six times onto the TDF receptor model protein as seen in Fig. 7a. Another one of the top-ten solutions led to essentially the same docking position as displayed in Fig. 7d. These binding pockets and the neighboring amino acid residues on the TDF-R candidate are displayed in Fig. 7a–c and d–f. Neighboring amino acid

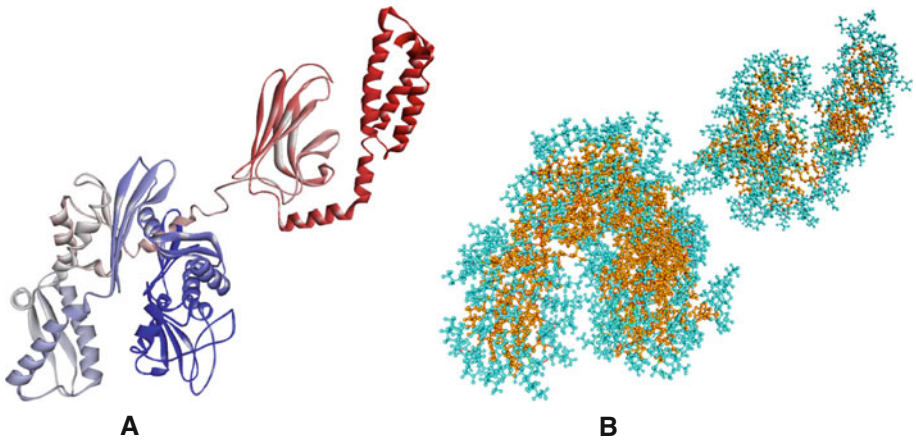

Fig. 6 Structure of the model receptor protein. **a** Ribbon diagram of TDF receptor protein colored from the N-terminal (*blue*) to C-terminal (*red*). **b** Solvent-accessible surface of the model receptor protein. *Cyan* denotes exposed residues with more than 25 % solvent accessible surface (SAS) and *dark brown* residues are buried with 10 % less solvent accessibility. Probe radius is 1.4 Å. Here, the receptor protein is depicted in *ball-and-stick mode*

residues on the model receptor site are depicted in Fig. 7b–c. These key neighboring residues are Met 196, Arg 197, Ile 199, Phe 242, Asp 413, Leu 414, Val 415, Cys 420, Pro 421, Val 443, Val 505, Asn 506, Gly 507, Asn 528, and Ile 538. Here, H bonds are formed between Gln 4 (P1) ··· Phe242 (receptor model) and between Cys 16 (P1) ··· Cys 420 (receptor model). Neighboring amino acid residues on the model receptor as depicted in Fig 7e–f are Val 429, Thr 434, Pro491, Arg492, Gly 493, Lys 556, Asn 563, Glu 566, Ser 567, Gly 577, Asp 578. Here, H bonds are formed between Gly 18 (P1) … Thr434 (receptor model), and Arg 1(P1) ··· Gly 577 (receptor model).

Another set of docking trials were made by using the Patch Dock and refined by Fire Dock servers [57–60]. Three out of the top-ten docking conformations using Fire Dock returned results comparatively similar to the pose shown in Fig. 7a. The remaining five Fire Dock simulations from this group matched the pose in Fig. 7g. The neighbor residues of the receptor in this P1-docked structure (Fig. 7g) are Thr 38, Arg 60, Tyr 65, Lys 81, Phe 93, Asp 94, Phe 114, Gly 227, His252, Leu253, Gly 254-255, Glu 256, Asp 259, Gln 286, Arg 290, Glu 293, Lys294, and Arg 297. These residues are depicted in Fig. 7h–i (red stick). Here, two H bonds are formed between Lys 17(P1) ··· Gly 254 (receptor model) and another one is in between Gln 10 (P1) … Arg 297 (receptor model). The construction as well as the analyses of all these figures were carried out using the Accelrys Discovery Studio 3.1 [61].

Does TDF have any effect on normal breast and prostate cells? Does TDF modulate the puberty or pregnancy?

A malignant phenotype is the result of dysregulation of cell differentiation and proliferation [62–65]. Malignant cells are highly proliferative, but highly undifferentiated [62–65]. An increased rate of cell differentiation is reflected by inhibition of cell growth, manifested by a decreased rate of cell proliferation and cell cycle arrest [44]. TDF promotes cell differentiation in breast and prostate cancer cell lines, but not in fibroblasts or in other cancerous cell lines. However, it is not yet known whether normal breast and prostate cells differentiate when exposed to TDF protein. If TDF promotes the differentiation of normal, non-cancerous breast and prostate cells, this suggests that TDF has additional yet-to-be-investigated roles. For example, if TDF stimulates breast and prostate cancer cell differentiation (but not the differentiation of their normal counterparts for these cells), this could be due to a high level of "un-differentiation" of cancer cells. Since TDF does not have any effect on other non-breast and non-prostate cancer cell lines, this suggests that TDF could promote differentiation of breast and prostate cancer cells because they are both steroid-regulated breast and prostate cells AND un-differentiated cancerous cells. In addition, if TDF promotes cell differentiation only in breast and prostate cancer cell lines, but not in normal breast and prostate cells, then TDF could in theory act specifically on un-differentiated breast and prostate cells as part of a developmental process but not on mature cells, which could explain the differentiation effect of TDF on undifferentiated breast and prostate cancerous cells. Therefore, investigation of TDF-induced cell differentiation through analysis of the cell cycle progression and monitoring of markers specific for cell differentiation (cyclin D1, E-cadherin, beta-catenin, or gamma-catenin) in normal and cancerous cells could lead to elucidation of the molecular mechanisms through which TDF induces cell differentiation. Further questions regarding the function of TDF include: does TDF (1) have a constant concentration in the bloodstream and (2) act on un-differentiated breast and prostate normal cells? Will (3) TDF influence the development and differentiation of the secondary sexual characters during puberty? and (4) Will TDF have the blood levels increased during puberty? This question could extend to pregnancy: (5) will TDF influence the differentiation of

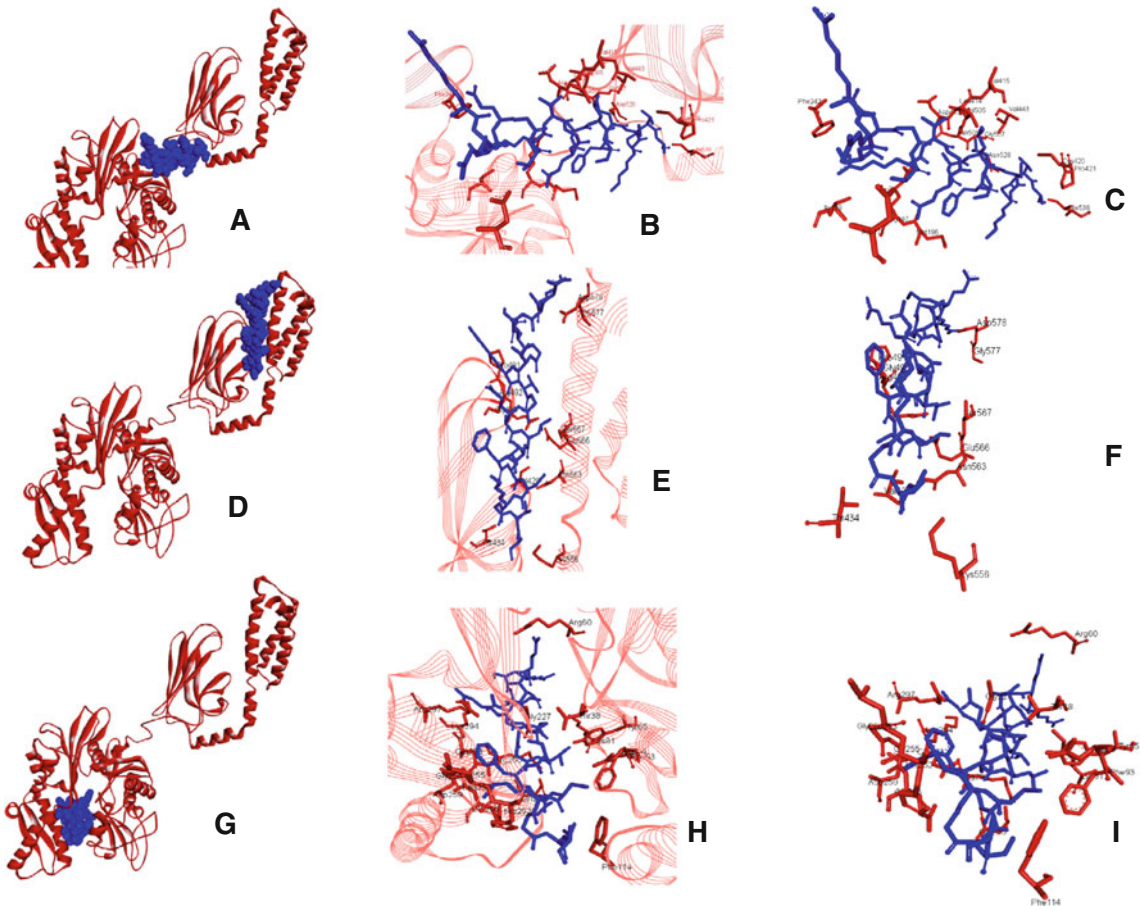

Fig. 7 Prediction and structural analyses of potential peptide binding sites. **a**, **d**, **g** Potential TDF-P1 binding pockets in the model receptor protein. **b**, **c**, **e**–**f**, **h**, **i** Closer look at the TDF-P1 binding site on TDF-R protein. Key residues of the receptor protein in the binding pockets are displayed. TDF-P1 peptide is depicted in *blue* and receptor is depicted in *red*

breast cells and their preparation for lactation? (6) will TDF blood levels increase during pregnancy, due to increased need for differentiation of breast cells? The answer to some of these questions could hopefully reveal important information about TDF, yet to be discovered. In fact, our laboratory has already started to investigate the function of TDF, with particular focus on puberty and pregnancy. In our lab, we compared the levels of TDF in the sera from healthy children and adults, but found no significant difference (data not shown). However, we did not examine the sera from children prior to puberty, during puberty, and after puberty (from the same subject), so it is premature to conclude anything about TDF. We also monitored the TDF levels in the sera of non-pregnant and pregnant women. We found striking differences: the TDF levels in the sera of pregnant women were at least twice as high when compared with the TDF levels in the sera of non-pregnant women (data not shown). However, since the number of samples analyzed was too small, it is too early to conclude anything about a potential role of TDF in pregnancy. However, our experiments will have to be first reproduced with a larger number of samples before we can conclude anything regarding a potential role of TDF in pregnancy. In a last experiment, we also investigated by AP and WB using anti-GRP78 and anti-HSP70 in the cell lysate from MCF10A normal breast cells (data not shown). We found no reaction for any of the two proteins investigated, suggesting that the normal breast cells do not have sufficient GRP78 and perhaps HSP70 at the cell surface to allow us to purify them by AP and identify them by WB.

Based on our recent studies, TDF activates a possible novel pathway through the members of HSP70 family of proteins. We also concluded that TDF may activate a pathway that is specific to breast and prostate cancer cells, but not to other cancer cells (e.g., HeLa and NG108 cells) or normal cells (e.g., HDF-a). In the view of TDF-R, we found that at least two proteins are TDF-R candidates (GRP78 and HSP70) and they are identified by AP and WB in both HBCC and HPCC but not on other cells. Therefore, the actions of TDF could be dependent on the presence of

GRP78 and HSP70 at the right time, in the right place (outside the membrane and close to each other). As such, the actions of TDF on normal undifferentiated breast and prostate cells during puberty and on the breast cells during pregnancy could in theory be dependent on (and perhaps limited by) the availability of the GRP78 and HSP70 at the plasma membrane as a functional TDF-R complex. However, until the experiments confirm our theory, this is just speculation and rational reasoning.

Targeting TDF and potential TDF-R candidates to prevent breast and prostate cancer

Breast cells are responsive to steroid hormones [66–70]. Prostate cells are also responsive to steroid hormones [71–91]. The current cytotoxic and hormonal therapy for breast and prostate cancer has temporary effectiveness, but mainly palliates the patient's symptoms, and rarely cures the disease. The need for new therapeutic strategies to prolong survival or to cure this cancer is obvious [92, 93]. One option is differentiation therapy, which uses agents that induce cell differentiation. Therefore, if TDF induces differentiation of HBCC and HPCC through a novel pathway, then manipulation of the TDF pathway to produce differentiation of breast and prostate cancer cells could provide an additional therapy alternative for the treatment of breast and prostate cancer. If TDF and TDF-P1 can induce cell differentiation, then two types of compounds with better differentiation activity can be created: small molecules, TDF-R agonists that can be obtained after identification of TDF-R and (2) TDF-P1 analogs, which are TDF-P1 derivatives with increased differentiation activity, increased stability, and reduced toxicity. Therefore, designing TDF-P1 analogs using D amino acids or substitutions of glycine with alanine to create a restrain point would be the first step in increasing the biological activity of TDF-P1. There is still one caveat with the TDF-P1 analogs: they are peptides, well known for their short life regarding its stability. In addition, designing and testing TDF-R agonists will also be a new avenue that could be explored for the identification of enhanced differentiation agents. However, it is worth noting and perhaps discussing, what types of TDF agonists should be designed, built, and tested. Should we target GRP78, HSP70, or both? Based on the AP and WB, as well as immuno-AP and WB studies, GRP78 should be targeted. However, based on the structural biology studies, HSP70 proteins also interact with TDF-P1. Therefore, advancement in understanding the function of TDF and TDF-R will not be possible without elucidating whether the TDF-R is a TDF-R complex, formed of at least GRP78 and HSP70, or whether it is just one protein (GRP78 or HSP70 but not both).

Table 1 Summary of the cell lines where GRP78 and HSP70 proteins were identified by AP and LC–MS/MS or by AP and WB

Cell lines	GRP78	HSP70
Breast cancer cell lines		
MCF7	+	+
BT-549	+	+
Prostate cancer cell lines		
DU145	+	+
PC3	+	+
LNCaP	+	+
Other cancer cell lines		
HeLa	–	–
NG108	–	–
Normal cell lines		
HDF-a	–	–
BLK CL.4	–	–

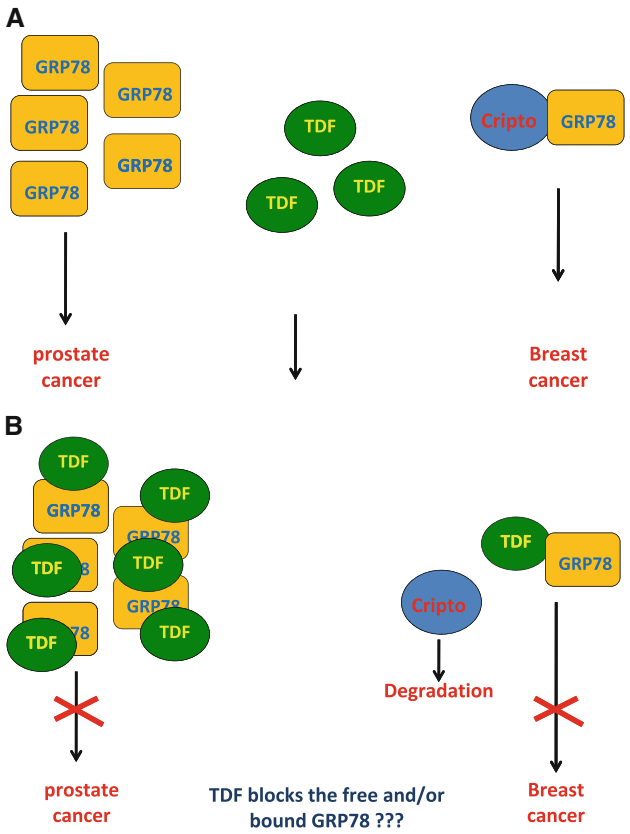

Fig. 8 Hypothetical mechanism of action of TDF that considers only GRP78 as TDF-R. **a** Overexpression of GRP78 at the cell surface leads to prostate cancer; when in complex with Cripto, GRP78 enables Cripto to induce breast cancer. **b** When the blood TDF binds to GRP78, it blocks the free (in prostate cancer) and Cripto-bound GRP78, which blocks the development of the prostate and breast cancers. When Cripto is not bound to GRP78, it is degraded and therefore prevented from binding other GRP78 molecules

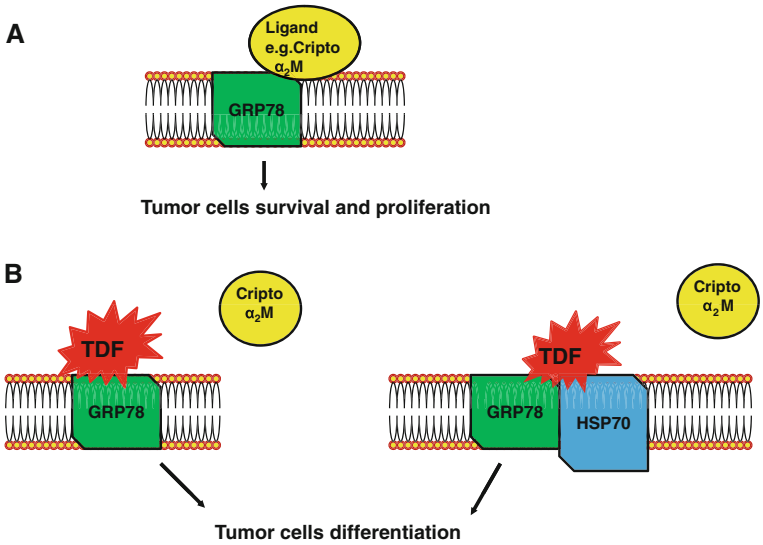

Fig. 9 Hypothetical mechanism of action of TDF that considers TDF-R as a protein complex. **a** This model considers more interaction partners for GRP78 (e.g., Cripto, alpha2 macroglobulin (a2 M). These GRP78 partners, either soluble (a2 M) or membrane-bound (Cripto) are competitively replaced by TDF. **b** TDF-R is considered as one molecule (GRP78) or a protein complex formed from at least GRP78 and HSP70

Conclusions

We reviewed the available literature related to the discovery and the investigation of TDF. We then compared the AP and LC–MS/MS data published by our laboratory that mostly focused on identification of TDF-R candidates from a variety of cell lysates. The most likely TDF-R candidates are GRP78 and HSP70, which are common in both DU145 HPCC and MCF7 HBCC. We also concluded using AP and WB that the TDF-R candidate is specific to HPCC (DU145, PC3, and LNCaP) and in HBCC (MCF7 and BT-549), but not to other cancer (HeLa or NG108) or normal (HDF-a BLK CL.4) fibroblast or fibroblast-like cells investigated (Table 1). Identification of these two proteins was then further compared in HBCC and HPCC and investigated by fluorescence and confocal microscopy and by molecular modeling. The possibility that TDF-R is a multi-subunit protein complex, composed of at least GRP78 and HSP70 is also considered. Using all of these data, two main working models/hypothetical mechanisms of action for TDF were built (Figs. 8, 9), that do not exclude each other. These models are the basis of future investigations.

Perspectives

Many questions regarding the function of TDF and its receptor are still waiting for an answer. Some of the questions will hopefully receive an answer, but most likely many of them will still have to wait for a long time. Among the most pressing questions are: What are the post-translational modifications that account for the differences between the theoretical and experimental molecular mass of TDF? What cells synthesize TDF? Is TDF restricted to one organ or tissue? Is TDF specific to mammals? Do other mammals and non-mammals (e.g., fish) have a TDF homologue? Do TDF levels from the blood increase in pregnancy? If yes, is TDF a pregnancy-protective? Do TDF levels in the blood decrease in cancer? If yes, can TDF be used as a biomarker for prediction of breast and prostate cancer?

Future directions

In addition to the questions described in the perspective section and that are waiting for an answer, one additional question that requires an answer should be considered: could TDF be considered a biomarker? Could overexpression of GRP78 and/or other HSP70 members be good indicators of the onset and perhaps treatment of breast and prostate cancer, simply by investigating the clinical samples (normal versus cancerous)? If this is the case, targeting TDF-R with TDF and TDF-P1 agonists, as well as with TDF-R agonists could help us not only in the treatment of breast and prostate cancer but most importantly in its prevention.

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References

1. Platica M, Chen HZ, Ciurea D, Gil J, Mandeli J, Hollander VP (1992) Pituitary extract causes aggregation and differentiation of rat mammary tumor MTW9/Pl cells. Endocrinology 131(6): 2573–2580
2. Platica M, Ivan E, Holland JF, Ionescu A, Chen S, Mandeli J, Unger PD, Platica O (2004) A pituitary gene encodes a protein that produces differentiation of breast and prostate cancer cells. Proc Natl Acad Sci USA 101(6):1560–1565
3. Brabant G, Hoang-Vu C, Cetin Y, Dralle H, Scheumann G, Molne J, Hansson G, Jansson S, Ericson LE, Nilsson M (1993) E-cadherin: a differentiation marker in thyroid malignancies. Cancer Res 53(20):4987–4993
4. De Leeuw WJ, Berx G, Vos CB, Peterse JL, Van de Vijver MJ, Litvinov S, Van Roy F, Cornelisse CJ, Cleton-Jansen AM (1997) Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma in situ. J Pathol 183(4): 404–411
5. Edelman GM, Crossin KL (1991) Cell adhesion molecules: implications for a molecular histology. Annu Rev Biochem 60:155–190
6. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeier W (1991) E-cadherin-mediated cell–cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 113(1):173–185
7. Ray DB, Horst IA, Jansen RW, Kowal J (1981) Normal mammary cells in long-term culture. I. development of hormone-dependent functional monolayer cultures and assay of alpha-lactalbumin production. Endocrinology 108(2):573–583
8. Sommers CL, Thompson EW, Torri JA, Kemler R, Gelmann EP, Byers SW (1991) Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. Cell Growth Differ 2(8):365–372
9. Thean ET, Toh BH (1990) Serum human alpha-lactalbumin as a marker for breast cancer. Br J Cancer 61(5):773–775
10. Vleminckx K, Vakaet L Jr, Mareel M, Fiers W, van Roy F (1991) Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 66(1): 107–119
11. Karlin S, Bucher P, Brendel V, Altschul SF (1991) Statistical methods and insights for protein and DNA sequences. Annu Rev Biophys Biophys Chem 20:175–203
12. Wootton JC, Federhen S (1996) Analysis of compositionally biased regions in sequence databases. Methods Enzymol 266: 554–571
13. Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S (2004) Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng Des Sel 17(4):349–356
14. Roy U, Sokolowska I, Woods AG, Darie CC (2012) Structural investigation of tumor differentiation factor (TDF). Biotechnol Appl Biochem 0 (0):xxx. In Press
15. Sokolowska I, Woods AG, Gawinowicz MA, Roy U, Darie CC (2012) Identification of a potential tumor differentiation factor receptor candidate in prostate cancer cells. FEBS J 279(14): 2579–2594. doi:10.1111/j.1742-4658.2012.08641.x
16. Sokolowska I, Woods AG, Gawinowicz MA, Roy U, Darie CC (2012) Identification of potential tumor differentiation factor (TDF) receptor from steroid-responsive and steroid-resistant breast cancer cells. J Biol Chem 287(3):1719–1733. doi:10.1074/jbc.M111.284091
17. Darie CC, Shetty V, Spellman DS, Zhang G, Xu C, Cardasis HL, Blais S, Fenyo D, Neubert TA (2008) Blue Native PAGE and mass spectrometry analysis of the ephrin stimulation-dependent protein–protein interactions in NG108-EphB2 cells. Applications of Mass Spectrometry in life safety, NATO Science for Peace and Security Series. Springer, Berlin Heidelberg New York
18. Darie CC, Biniossek ML, Gawinowicz MA, Milgrom Y, Thumfart JO, Jovine L, Litscher ES, Wassarman PM (2005) Mass spectrometric evidence that proteolytic processing of rainbow trout egg vitelline envelope proteins takes place on the egg. J Biol Chem 280(45):37585–37598. doi:10.1074/jbc.M506709200
19. Darie CC, Biniossek ML, Jovine L, Litscher ES, Wassarman PM (2004) Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. Biochemistry 43(23):7459–7478. doi:10.1021/bi0495937
20. Darie CC, Deinhardt K, Zhang G, Cardasis HS, Chao MV, Neubert TA (2011) Identifying transient protein–protein interactions in EphB2 signaling by blue native PAGE and mass spectrometry. Proteomics 11(23):4514–4528. doi:10.1002/pmic.201000819
21. Sokolowska I, Dorobantu C, Woods AG, Macovei A, Branza-Nichita N, Darie CC (2012) Proteomic analysis of plasma membranes isolated from undifferentiated and differentiated HepaRG cells. Proteome Sci. 10(1):47. doi:10.1186/1477-5956-10-47
22. Sokolowska I, Gawinowicz MA, Ngounou Wetie AG, Darie CC (2012) Disulfide proteomics for identification of extracellular or secreted proteins. Electrophoresis (In Press). doi:10.1002/elps.201200182
23. Woods AG, Sokolowska I, Darie CC (2012) Identification of consistent alkylation of cysteine-less peptides in a proteomics experiment. Biochem Biophys Res Commun 419(2):305–308. doi:10.1016/j.bbrc.2012.02.016
24. Woods AG, Sokolowska I, Yakubu R, Butkiewicz M, LaFleur M, Talbot C, Darie CC (2011) Blue Native PAGE and mass spectrometry as an approach for the investigation of stable and transient protein–protein interactions. In: Oxidative stress: diagnostics and therapy. ACS Books. doi:10.1021/bk-2011-1083.ch012
25. Kelber JA, Panopoulos AD, Shani G, Booker EC, Belmonte JC, Vale WW, Gray PC (2009) Blockade of Cripto binding to cell surface GRP78 inhibits oncogenic Cripto signaling via MAPK/PI3 K and Smad2/3 pathways. Oncogene 28(24):2324–2336
26. Ni M, Zhou H, Wey S, Baumeister P, Lee AS (2009) Regulation of PERK signaling and leukemic cell survival by a novel cytosolic isoform of the UPR regulator GRP78/BiP. PLoS ONE 4(8):e6868
27. Graner MW, Raynes DA, Bigner DD, Guerriero V (2009) Heat shock protein 70-binding protein 1 is highly expressed in high-grade gliomas, interacts with multiple heat shock protein 70 family members, and specifically binds brain tumor cell surfaces. Cancer Sci 100(10):1870–1879. doi:10.1111/j.1349-7006.2009.01269.x
28. Wu B, Wilmouth RC (2008) Proteomics analysis of immunoprecipitated proteins associated with the oncogenic kinase cot. Mol Cells 25(1):43–49
29. Fu Y, Lee AS (2006) Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. Cancer Biol Ther 5(7):741–744
30. Kuwabara H, Yoneda M, Hayasaki H, Nakamura T, Mori H (2006) Glucose-regulated proteins 78 and 75 bind to the receptor for hyaluronan mediated motility in interphase microtubules. Biochem Biophys Res Commun 339(3):971–976

31. Lim SO, Park SG, Yoo JH, Park YM, Kim HJ, Jang KT, Cho JW, Yoo BC, Jung GH, Park CK (2005) Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. World J Gastroenterol 11(14):2072–2079
32. Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA, Patierno SR (2000) Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. Breast Cancer Res Treat 59(1):15–26
33. Menoret A, Bell G (2000) Purification of multiple heat shock proteins from a single tumor sample. J Immunol Methods 237(1–2):119–130
34. Stoeckle MY, Sugano S, Hampe A, Vashistha A, Pellman D, Hanafusa H (1988) 78-kilodalton glucose-regulated protein is induced in Rous sarcoma virus-transformed cells independently of glucose deprivation. Mol Cell Biol 8(7):2675–2680
35. Daneshmand S, Quek ML, Lin E, Lee C, Cote RJ, Hawes D, Cai J, Groshen S, Lieskovsky G, Skinner DG, Lee AS, Pinski J (2007) Glucose-regulated protein GRP78 is up-regulated in prostate cancer and correlates with recurrence and survival. Hum Pathol 38(10):1547–1552
36. Didelot C, Lanneau D, Brunet M, Joly AL, De Thonel A, Chiosis G, Garrido C (2007) Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. Curr Med Chem 14(27):2839–2847
37. Solit DB, Rosen N (2006) Hsp90: a novel target for cancer therapy. Curr Top Med Chem 6(11):1205–1214
38. Nair SC, Toran EJ, Rimerman RA, Hjermstad S, Smithgall TE, Smith DF (1996) A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor. Cell Stress Chaperones 1(4):237–250
39. Nemoto T, Ohara-Nemoto Y, Ota M (1992) Association of the 90-kDa heat shock protein does not affect the ligand-binding ability of androgen receptor. J Steroid Biochem Mol Biol 42(8):803–812
40. Sanchez ER, Faber LE, Henzel WJ, Pratt WB (1990) The 56–59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. Biochemistry 29(21):5145–5152
41. Veldscholte J, Berrevoets CA, Brinkmann AO, Grootegoed JA, Mulder E (1992) Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. Biochemistry 31(8):2393–2399
42. Veldscholte J, Berrevoets CA, Zegers ND, van der Kwast TH, Grootegoed JA, Mulder E (1992) Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from non-transformed receptors. Biochemistry 31(32):7422–7430
43. Jensen MR, Schoepfer J, Radimerski T, Massey A, Guy CT, Brueggen J, Quadt C, Buckler A, Cozens R, Drysdale MJ, Garcia-Echeverria C, Chene P (2008) NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. Breast Cancer Res 10(2):R33
44. Lanneau D, de Thonel A, Maurel S, Didelot C, Garrido C (2007) Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. Prion 1(1):53–60
45. Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24(18):8055–8068
46. Moscat J, Diaz-Meco MT (2000) The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. EMBO Rep 1(5):399–403
47. Diaz-Meco MT, Moscat J (2001) MEK5, a new target of the atypical protein kinase C isoforms in mitogenic signaling. Mol Cell Biol 21(4):1218–1227
48. DeLaBarre B, Brunger AT (2003) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. Nat Struct Biol 10(10):856–863
49. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R (2000) Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep 7(1):157–167
50. Bertelsen EB, Chang L, Gestwicki JE, Zuiderweg ER (2009) Solution conformation of wild-type *E. coli* Hsp70 (DnaK) chaperone complexed with ADP and substrate. Proc Natl Acad Sci U S A 106(21):8471–8476. doi:10.1073/pnas.0903503106
51. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22(2):195–201. doi:10.1093/bioinformatics/bti770
52. Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18(15):2714–2723. doi:10.1002/elps.1150181505
53. Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31(13):3381–3385
54. Maiti R, Van Domselaar GH, Zhang H, Wishart DS (2004) SuperPose: a simple server for sophisticated structural superposition. Nucleic Acids Res 32 (Web Server issue):W590-594. doi:10.1093/nar/gkh477
55. Tovchigrechko A, Vakser IA (2005) Development and testing of an automated approach to protein docking. Proteins 60(2):296–301. doi:10.1002/prot.20573
56. Tovchigrechko A, Vakser IA (2006) GRAMM-X public web server for protein–protein docking. Nucleic Acids Res 34 (Web Server issue):W310–W314. doi:10.1093/nar/gkl206
57. Andrusier N, Nussinov R, Wolfson HJ (2007) FireDock: fast interaction refinement in molecular docking. Proteins 69(1):139–159. doi:10.1002/prot.21495
58. Duhovny D, Nussinov R, Wolfson HJ (2002) Efficient unbound docking of rigid molecules. In: Gussfield et al. (ed) Proceedings of the 2nd Workshop on Algorithms in Bioinformatics (WABI) Lecture Notes in Computer Science Rome, Italy, vol 2452. Springer, pp 185–200
59. Mashiach E, Schneidman-Duhovny D, Andrusier N, Nussinov R, Wolfson HJ (2008) FireDock: a web server for fast interaction refinement in molecular docking. Nucleic Acids Res 36 (Web Server issue):W229–W232. doi:10.1093/nar/gkn186
60. Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ (2005) PatchDock and SymmDock: servers for rigid and symmetric docking. Nucleic Acids Res 33 (Web Server issue):W363–W367. doi:10.1093/nar/gki481
61. Accelrys_Software Inc (2012) Discovery studio modeling environment, Release 3.1. San Diego. Accelrys Software Inc 1:1–5
62. Ashkenas J, Muschler J, Bissell MJ (1996) The extracellular matrix in epithelial biology: shared molecules and common themes in distant phyla. Dev Biol 180(2):433–444
63. Fagotto F, Gumbiner BM (1996) Cell contact-dependent signaling. Dev Biol 180(2):445–454
64. Koornstra JJ, de Jong S, Hollema H, de Vries EG, Kleibeuker JH (2003) Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. Crit Rev Oncol Hematol 45(1):37–53
65. Pusztai L, Lewis CE, Lorenzen J, McGee JO (1993) Growth factors: regulation of normal and neoplastic growth. J Pathol 169(2):191–201
66. Chen GG, Zeng Q, Tse GM (2008) Estrogen and its receptors in cancer. Med Res Rev 28(6):954–974

67. Enmark E, Gustafsson JA (1999) Oestrogen receptors—an overview. J Intern Med 246(2):133–138
68. Gustafsson JA (1999) Estrogen receptor beta—a new dimension in estrogen mechanism of action. J Endocrinol 163(3):379–383
69. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA (2001) Mechanisms of estrogen action. Physiol Rev 81(4): 1535–1565
70. Zeng Q, Chen G, Vlantis A, Tse G, van Hasselt C (2008) The contributions of oestrogen receptor isoforms to the development of papillary and anaplastic thyroid carcinomas. J Pathol 214(4): 425–433
71. Kobayashi T, Shimizu Y, Terada N, Yamasaki T, Nakamura E, Toda Y, Nishiyama H, Kamoto T, Ogawa O, Inoue T (2010) Regulation of androgen receptor transactivity and mTOR-S6 kinase pathway by Rheb in prostate cancer cell proliferation. Prostate 70(8):866–874. doi:10.1002/pros.21120
72. Yuan X, Balk SP (2009) Mechanisms mediating androgen receptor reactivation after castration. Urol Oncol 27(1):36–41. doi:10.1016/j.urolonc.2008.03.021
73. Urbanucci A, Waltering KK, Suikki HE, Helenius MA, Visakorpi T (2008) Androgen regulation of the androgen receptor coregulators. BMC Cancer 8:219. doi:10.1186/1471-2407-8-219
74. Zhu ML, Kyprianou N (2008) Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocr Relat Cancer 15(4):841–849. doi:10.1677/ERC-08-0084
75. Rinnab L, Hessenauer A, Schutz SV, Schmid E, Kufer R, Finter F, Hautmann RE, Spindler KD, Cronauer MV (2008) Role of androgen receptors in hormone-refractory prostate cancer: molecular basics and experimental therapy approaches. Urologe A 47(3):314–325. doi:10.1007/s00120-008-1637-1
76. Shimada K, Nakamura M, Ishida E, Higuchi T, Yamamoto H, Tsujikawa K, Konishi N (2008) Prostate cancer antigen-1 contributes to cell survival and invasion though discoidin receptor 1 in human prostate cancer. Cancer Sci 99(1):39–45. doi:10.1111/j.1349-7006.2007.00655.x
77. Tararova ND, Narizhneva N, Krivokrisenko V, Gudkov AV, Gurova KV (2007) Prostate cancer cells tolerate a narrow range of androgen receptor expression and activity. Prostate 67(16): 1801–1815. doi:10.1002/pros.20662
78. Margiotti K, Wafa LA, Cheng H, Novelli G, Nelson CC, Rennie PS (2007) Androgen-regulated genes differentially modulated by the androgen receptor coactivator L-dopa decarboxylase in human prostate cancer cells. Mol Cancer 6:38. doi:10.1186/1476-4598-6-38
79. Richter E, Srivastava S, Dobi A (2007) Androgen receptor and prostate cancer. Prostate Cancer Prostatic Dis 10(2):114–118. doi:10.1038/sj.pcan.4500936
80. Reddy GP, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH, Sheng S (2006) Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. J Cell Biochem 98(6):1408–1423. doi:10.1002/jcb.20927
81. Dehm SM, Tindall DJ (2006) Molecular regulation of androgen action in prostate cancer. J Cell Biochem 99(2):333–344. doi:10.1002/jcb.20794
82. Hobisch A, Fritzer A, Comuzzi B, Fiechtl M, Malinowska K, Steiner H, Bartsch G, Culig Z (2006) The androgen receptor pathway is by-passed in prostate cancer cells generated after prolonged treatment with bicalutamide. Prostate 66(4):413–420. doi:10.1002/pros.20365
83. Dong Y, Zhang H, Gao AC, Marshall JR, Ip C (2005) Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. Mol Cancer Ther 4(7):1047–1055. doi:10.1158/1535-7163.MCT-05-0124
84. Linja MJ, Visakorpi T (2004) Alterations of androgen receptor in prostate cancer. J Steroid Biochem Mol Biol 92(4):255–264
85. Marandola P, Bonghi A, Jallous H, Bombardelli E, Morazzoni P, Gerardini M, Tiscione D, Albergati F (2004) Molecular biology and the staging of prostate cancer. Ann N Y Acad Sci 1028: 294–312. doi:1028/1/29410.1196/annals.1322.034
86. Sommer A, Haendler B (2003) Androgen receptor and prostate cancer: molecular aspects and gene expression profiling. Curr Opin Drug Discov Dev 6(5):702–711
87. Suzuki H, Ueda T, Ichikawa T, Ito H (2003) Androgen receptor involvement in the progression of prostate cancer. Endocr Relat Cancer 10(2):209–216
88. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlage RE, Shabanowitz J, Hunt DF, Weber MJ (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem 277(32):29304–29314. doi:10.1074/jbc.M204131200
89. Haendler B (2002) Androgen-selective gene regulation in the prostate. Biomed Pharmacother 56(2):78–83
90. Sadar MD, Hussain M, Bruchovsky N (1999) Prostate cancer: molecular biology of early progression to androgen independence. Endocr Relat Cancer 6(4):487–502
91. Griffiths K, Morton MS, Nicholson RI (1997) Androgens, androgen receptors, antiandrogens and the treatment of prostate cancer. Eur Urol 32(Suppl 3):24–40
92. Corona G, Baldi E, Maggi M (2011) Androgen regulation of prostate cancer: where are we now? J Endocrinol Invest 34(3): 232–243
93. Loda M, Kaelin WG Jr (2010) Prostate cancer: beta control your hormones. Cancer Cell 17(4):311–312