Table 2
Proteins with altered expression during the differentiation from monkey ES cells to NS cells and neurons by the NSS method. Molecular functions and possible roles in neurogenesis are shown for the proteins identified in this study. Molecular functions were obtained from the Gene Ontology Consortium (http://www.geneontology.org/). Listed proteins were classified into eight groups: D, cytoskeleton; C, heat shock/stress proteins; S, signal transduction; PM, protein metabolism; LM, lipid metabolism; EM, energy metabolism; RM, RNA metabolism; E, cell cycle.

Protein name	Class	Function	Neurogenesis	Ref.
Elongation factor 1 beta	PM	Catalytic subunit of the guanine nucleotide exchange factor of the eukaryotic elongation factor 1 complex	Not known	–
14-3-3 Protein zeta/delta	S	Adaptor protein implicated in the regulation of signaling pathway	Activates the tyrosine hydroxylase catalyzing rate-limiting step of dopamine synthesis	[20]
Reticulocalbin 1	S	Contains four functional EF-hand domains and regulates calcium-dependent activities in endoplasmic reticulum	Not known	–
Vimentin	D	Class-III intermediate filaments interspecies interaction between organisms	Increased in NS cells	[10]
Hetrogeneous nuclear ribonucleoprotein K	RM	Plays a role in the nuclear metabolism of hnRNAs with cytidine-rich sequences, such as mRNA splicing	Decreases during neuronal differentiation from the human NS cell line	[10]
Rho GDP dissociation inhibitor 1	S	Regulation of GDP/GTP exchange reaction of Rho to maintain Rho proteins in an inactive state	Present in human NS cell lines	[6,10]
Annexin A5	S	Calcium-dependent binding to phospholipid membranes, interacts selectively with a receptor that possesses protein tyrosine kinase activity	Not known	–
Heat shock protein 90, beta	C	Processes and transports secreted proteins	Present in human NS cells	[6,10]
Actin cytoplasmic 1	D	ATP-binding structural constituent of cytoskeleton	Increases during neuronal differentiation from the human NS cell line	[10]
Fatty acid binding protein 7	LM	Fatty acid-binding activity involved in transport or propagation of processes extending from the cell	Maintenance of neuroepithelial cells of rat cortex. Functional links to glutamate receptor	[27–31]
Lamin B1	D	Components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane	Decreases during neuronal differentiation from the human NS cell line	[10]
Heat shock cognate 71-kDa protein	C	Molecular chaperone	Decreases during neuronal differentiation from the human NS cell line	[10]
Cellular retinoic acid binding protein 1	LM	Binds retinoic acid and regulates the access of retinoic acid to nuclear retinoic acid receptors	Increases in human ES cell-derived neuroectodermal spheres	[11]
Prohibitin	E	Inhibition of DNA synthesis to regulate proliferation	Increases during neural differentiation from mouse ES cells	[8,34]
Peroxiredoxin-2	C	Redox regulation of the cell	Increases during retinoic acid-induced neural differentiation from mouse ES cells	[9]
Lamin B2	D	Components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane	Not known	–
Creatine kinase B type	EM	Catalysis of transfer of phosphate between ATP and various phosphogens	Increases during neuronal differentiation from the human NS cell line	[8,10]
Collapsin response mediator protein 2	S	Plays a role in axon guidance, neuronal growth cone collapse, and cell migration	Present in rat and human NS cells	[6,10]
Glutathione S-transferase P	C	Removes wound substances by conjugation of reduced glutathione	Decreases during neuronal differentiation from mouse ES cells	[7]

and Ser^{522}, respectively, and suggested that CRMP2-4 was triphosphorylated on Thr^{514}, Ser^{522} and X (undetermined acid residue). These results were consistent with the report of Patrakitkomjorn et al. [21].

Comparisons of protein and mRNA expression levels suggested that the upregulation of CRABP1 protein in NS cells and the upregulation of CRMP2-2, CRMP2-3, and CRMP2-4 in NS cells and neurons also occurred at the post-transcriptional level.

4. Discussion

In this study, we examined differentially regulated proteins in monkey NS cells and neurons derived from ES cells. Using highly homogeneous cells differentiated from ES cells via the formation of NSS, we identified 17 proteins with increased expression and seven with decreased expression from ES cells to NS cells, and three proteins with increased expression and 10 with decreased expression in going from NS cells to neurons.

Among the proteins identified, some have been reported to be differentially regulated in mammalian neurogenesis from ES cells to neural cells. For example, the expression of creatine kinase B type is increased in neural cells differentiated from the human NS cell line ReNcell VM [10]. Glutathione S-transferase P is downregulated from mouse ES cells to neurons [7]. FABP7, which is expressed in neuroepithelial cells, including NS cells and differentiating neurons downstream of the transcription factor Pax6, is essential for their maintenance during early embryonic development of the rat cortex [27]. FABP7 is expressed in mouse radial glia, which serve as neuroprogenitors in the adult mouse brain [28]. Moreover, FABP7 is strongly expressed in radial glia and immature astrocytes in pre- and perinatal brain, but its expression is remarkably attenuated in the astrocytes of adult rat brain [29]. Fabp7 has functional links to the glutamate receptor, *N*-methyl-D-aspartic acid receptor, in mice [30] and has been reported to be associated with human schizophrenia [31]. These observations indicate that the same proteins are involved in mammalian neurogenesis, from ES cells to neural cells, supporting the reliability of the data obtained by proteomic analysis.

In contrast, observations that differ from our data have been reported. For example, proliferating cell nuclear antigen, heterogeneous nuclear ribonucleoprotein K, and peroxiredoxin 4 have been reported to decrease from proliferating human neuronal stem cells (ReNcell VM) to differentiating neuronal stem cells [10]; α-3/α-7 tubulin has been reported to decrease from mouse ES cells to neurons [9]; and translationally controlled tumor protein decreases, but tubulinα-6 and actin-related protein 3 increase, from mouse ES cells to neurons [7]. In addition, although Hsp84 has been reported to increase from adult rat hippocampus NS cells to neurons [6], heat shock protein 90 beta/Hsp84 decreased from NS cells to neurons in monkeys but decreased from ES cells to NS cells in mice [8]. These discrepancies may be due to differences in the origin of the cell lines, cell homogeneity, culture conditions used, and developmental stages of differentiation from ES cells to neurons. Moreover, the competency of NS cells has been reported to change over time during development [35–38].

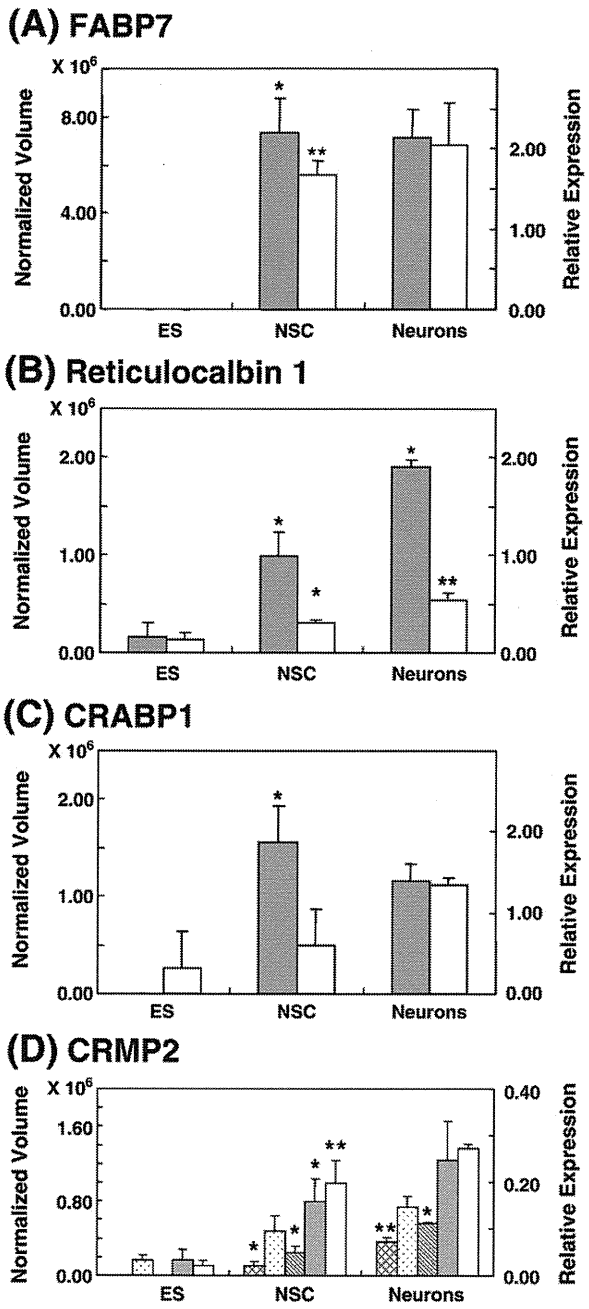

Fig. 3. Protein and RNA expression of five identified genes. RNA expression assayed by real-time RT-PCR and protein expression assayed by 2-DE are plotted for each stage of differentiation (ES cells, NS cells, and neurons). (A–C) White bars indicate RNA expression, whereas gray bars indicate protein expression. (D) White bars refer to RNA expression; gray, slanted stripes, dotted, and checked bars refer to the expression of the proteins CRMP2-1, CRMP2-2, CRMP2-3, and CRMP2-4, respectively. The vertical axis on the left shows the normalized volume of each protein spot on 2-DE, and that on the right shows the relative amounts of RNA normalized with respect to signals from ubiquitously expressed GAPDH mRNA. ES, ES cells; NSC, NS cells. "*" and "**" indicate the *P*-values of 0.05 and 0.01, respectively, for comparisons of the corresponding expression at two stages.

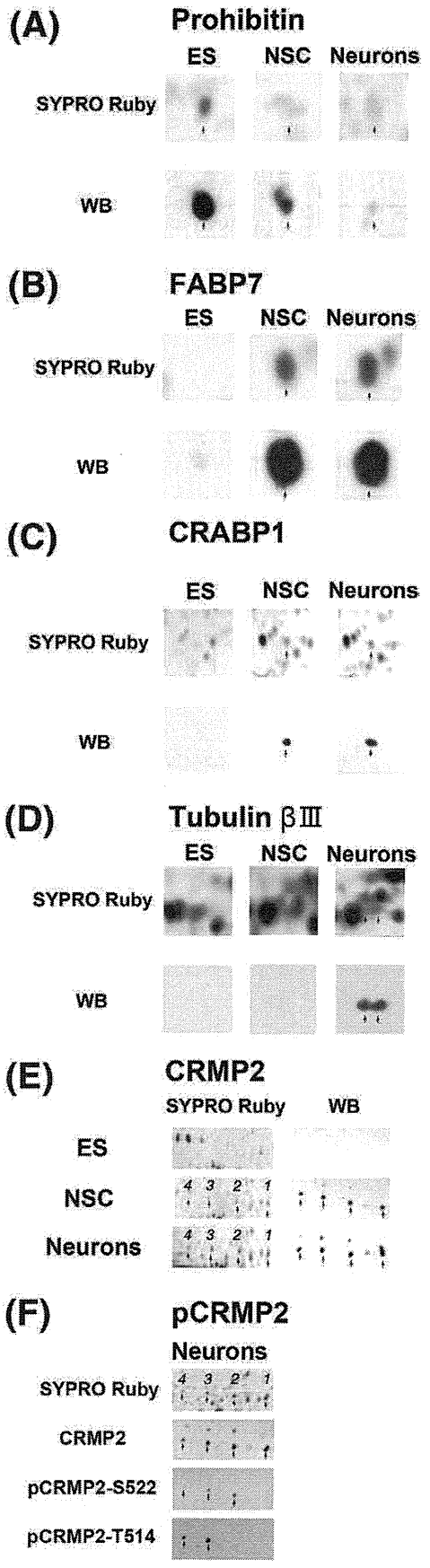

Fig. 4. Two-dimensional Western blotting analysis of a typical enlarged portion from two-dimensional gel electrophoretic gel from ES cells, NS cells, and Neurons. Proteins were visualized by staining with SYPRO Ruby, and by Western blotting analysis with the following antibodies: (A) anti-prohibitin, (B) anti-FABP7, (C) anti-CRABP1, (D) anti-tubulin beta III, (E) anti-CRMP2, and (F) anti-CRMP2 (CRMP2), anti-CRMP2 phosphorylated on Ser-522 (pCRMP2-S522), anti-CRMP2 phosphorylated on Thr-514 (pCRMP2-T514), to detect phosphorylated CRMP2 (pCRMP2). Western blotting analysis of 2-DE gels was performed as described in Materials and methods. ES, ES cells; NSC, NS cells.

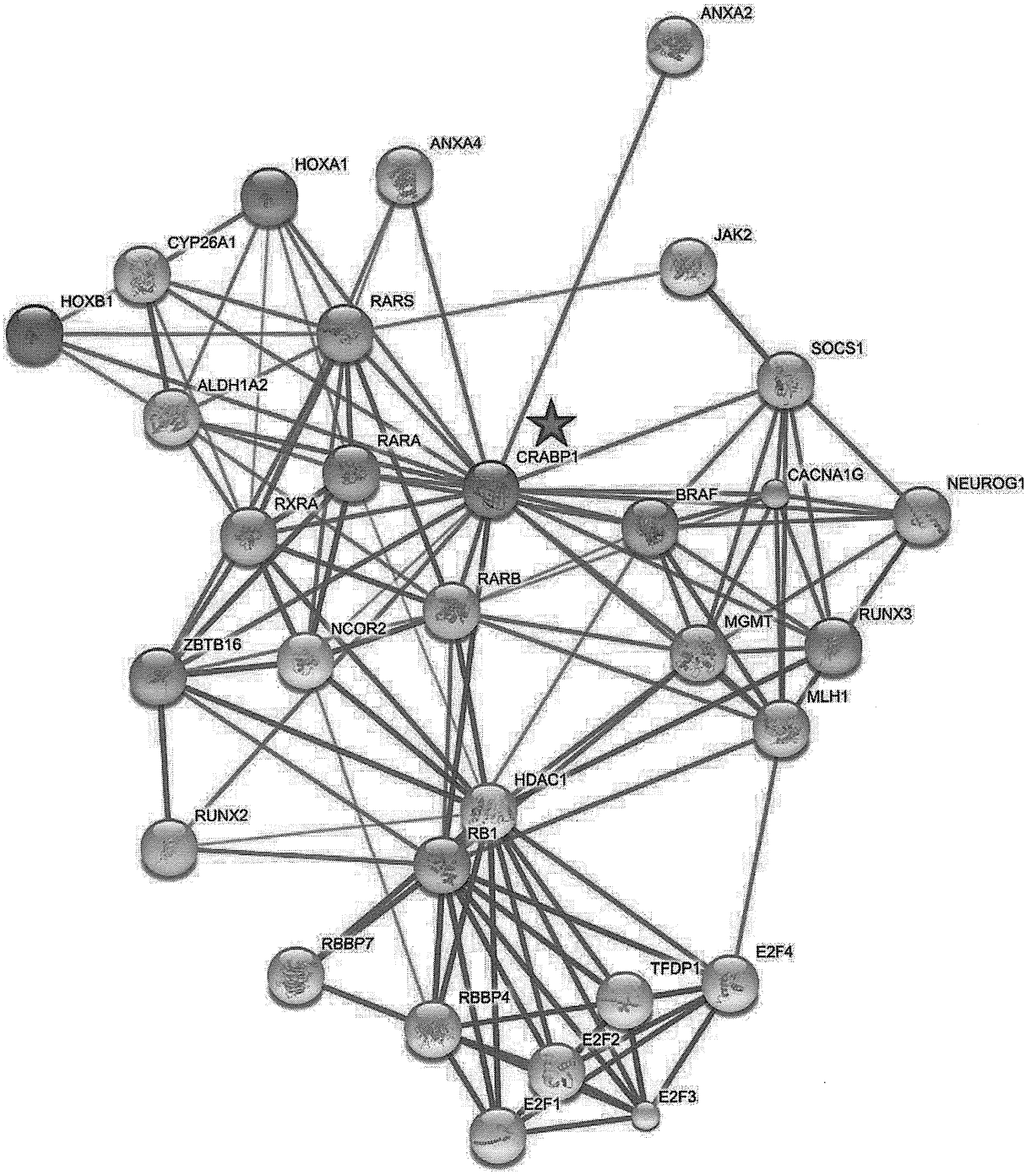

Fig. 5. The protein–protein interaction network of CRABP1. The interaction network was extracted using search tool STRING (http://string.embl.de/).

We found that the expression of galectin 1 and laminin receptor was not altered from monkey ES cells to neural cells, whereas galectin 1 has been found to be increased in mouse NS cells, while both galectin 1and laminin receptor have been reported to decrease in mouse neurons [8]. Prohibitin decreased in monkey NS cells and neurons, in contrast to its upregulation during mouse neural differentiation from ES cells to NS cells [8,34]. The protein–protein interaction network of prohibitin, which was extracted using search tool STRING (http://string.embl.de/) based on reported protein interactions, suggested that mouse and human prohibitins differed in their protein–protein interactions (Supplementary Fig. 1, A and B). Accordingly, the discrepancy between prohibitin expression during neural differentiation from ES cells of mouse and monkey origin may be due to differences in the mechanisms to regulate prohibitin expression via the protein–protein interactions.

Among the newly identified proteins showing increased expression from ES cells to NS cells and neurons were peroxiredoxin 2, CRMP2, and CRABP1, all of which are related to nervous system disorders. For example, the level of the oxidized form of peroxiredoxin 2 has been found to increase in individuals with Alzheimer's disease [39]. It has been reported that CRMP2 is a risk factor of schizophrenia [40], and its hyperphosphorylation is an early event in Alzheimer's disease progression [41].

CRMP2 has been found to bind directly to kinesin light chain and tubulin, resulting in a trimeric complex that stimulates tubulin transport to the distal part of the growing axon [42]. The interaction

between CRMP2 and tubulin has been reported to be altered by CRMP2 phosphorylation, by Cdk5 at Ser^{522} and by GSK-3β at Thr^{514}, inhibiting axon outgrowth [43]. Cdk5 has been reported to be activated in neurons [44] and may play important roles in neurite outgrowth [45] and neuron migration [46]. GSK-3β has been reported to be activated via the activation of Cdk5 as a priming kinase [47]. These findings supported our observation that CRMP2-1 (non-phosphorylated CRMP2) was upregulated in NS cells, and that CRMP2-2 (CRMP2 mono-phosphorylated on Ser522), CRMP2-3 (CRMP2 di-phosphorylated on Ser522 and Thr514), and CRMP2-4 (CRMP2 probably tri-phosphorylated on Ser522, Thr514, and X) were upregulated in NS cells and neurons.

All-trans retinoic acid is used extensively to promote neurogenesis in vitro [7,11,48]. CRABP1 is thought to facilitate catabolism and/or sequestering of retinoic acid, rendering it unavailable to nuclear receptors [32]. Moreover, CRABP2 has been reported to facilitate the transfer of all-trans retinoic acid to the nuclear envelope, where it can interact with its specific receptors. These receptors undergo conformational changes upon ligand binding, allowing the subsequent binding of retinoic acid response elements in certain target genes and their activation [48]. We found that protein expression of CRABP1 was upregulated in NS cells and neurons although its mRNA expression level was unchanged. In NS cells and neurons, however, the level of CRABP2 protein was not altered, although its mRNA expression was downregulated (Supplementary Table 2). The recent finding that CRABP1 is upregulated in human ES cell-derived neuroectodermal spheres bearing neuroprogenitors [11] supports our observations. Taken together, our results suggest that the retinoic acid concentrations and gene activation are tightly regulated during neural differentiation from ES cells. This is interesting, because the practicality of NS cells derived from ES cells may be limited by the teratogenic potential caused by retinoic acid. Retinoic acid concentrations have been reported to be regulated differently under various culture conditions, including in NS cells prepared via ectodermal cells in embryoid bodies [49,50], in the absence of embryoid bodies, by culturing ES cells on mouse-cultured stroma cells [51], or in chemically defined low-density cultures.

Protein interactions of the differentially expressed proteins were explored using STRING (http://string.embl.de/) based on reported protein interactions. As shown in Fig. 5, the interaction map suggested CRABP1 as a date hub, which connects modules as regulators, mediators, or adaptors [52], as well as FABP7, peroxiredoxin 2, and CRMP2 (Supplementary Fig. 1C–E). And the interaction maps suggested Rho GDP dissociation inhibitor 1 and 14-3-3 protein zeta/delta as party hubs, which are involved in signal transduction (Supplementary Fig. 1F and G). Additional studies are needed to elucidate the importance of the specific date hub proteins suggested here.

These results clearly showed that the proteomic approach used here is useful for gaining insight into the molecular mechanism of differentiation and induction from monkey ES cells to NS cells via NSS, and from NS cells to neurons. Together with our previous findings [8], the results presented here suggest that neural differentiation in monkeys is regulated in a manner similar to, but somewhat different from, that in mice, and that higher neural differentiation in monkeys is regulated by more proteins than in mice. In addition, our results provide further evidence that the NSS method we have for the differentiation and induction from monkey ES cells to NS cells and neurons in vitro is useful in providing an experimental model of primate neurogenesis from ES cells to neural cells. Furthermore, our findings indicate that NS cells and neurons differentiated from ES cells by the NSS method may also be useful for fast and effective high throughput drug screening for primate neural diseases.

Further application of proteomics to the detailed stages of differentiation may provide a more complete picture of early primate neurogenesis and enable us to elucidate the differences in characteristics of primate fetal and adult NS cells and neurons.

Conclusion

Using highly homogeneous monkey cells differentiated from ES cells via the formation of NSS, we identified 17 proteins with increased expression and seven with decreased expression from ES cells to NS cells, and three proteins with increased expression and 10 with decreased expression in going from NS cells to neurons. Particularly, FABP7 and CRABP1 were upregulated in NS cells, and CRMP2 was upregulated in NS cells and neurons. FABP7 and CRMP2 mRNAs were also upregulated in NS cells, while CRABP1 mRNA was unchanged. These results provide insight into the molecular basis of monkey neural differentiation.

Supplementary materials related to this article can be found online at doi: 10.1016/j.bbapap.2010.10.009.

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〔特別企画II：臨床検査領域におけるプロテオミクスの現状と未来像〕

卵巣明細胞腺がん関連タンパク質の発現調節

荒川憲昭[1,*]・増石有佑[1]・平野　久[1,2]

[1]横浜市立大学大学院生命ナノシステム科学研究科生体超分子科学専攻
[2]横浜市立大学先端医科学研究センター

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SUMMARY

Proteomics is a useful aid to discover new diagnosis markers or drug-targets for various diseases. To identify proteins expressed specifically in ovarian clear cell adenocarcinoma (CCA), a malignant strain of ovarian cancer subtype, we compared the proteomic patterns of cell lines derived from CCA and non-CCA using Two-Dimensional Differential Gel Electrophoresis (2D-DIGE) analysis. We identified several proteins, including annexin IV, which were increased in several CCA cell lines. These proteins were verified to be upregulated in several CCA cell lines by Western blot and quantitative RT-PCR analyses. Promoter analysis of annexin IV gene revealed that the p53-binding motif was involved in CCA-specific expression. Mutations in the p53 gene were absent in several CCA-derived cell lines, and gene silencing of p53 by RNA interference decreased the ANX4 expression in the CCA cell lines. Thus, the annexin IV gene is regulated by p53 in the CCA cells. Elevations of several p53-induced proteins were also observed in CCA cells. Therefore, we concluded that p53 functional status is involved in forming the unique features of the proteome in CCA cell lines.

Key words: disease proteomics, 2D-DIGE, ovarian clear cell adenocarcinoma, annexin IV, p53

緒　言

疾患プロテオーム研究は，疾患の診断に用いるバイオマーカー，創薬の標的分子の探索や病気の原因になるタンパク質の解明などを目的として行われている．この研究ではまず，蛍光標識二次元ディファレンスゲル電気泳動（2D-DIGE），ナノ液体クロマトグラフィー（nanoLC）質量分析装置（MS/MS）など，高感度，高精度，ハイスループットな分析技術を用いて生体組織や血液などに存在するバイオマーカーや創薬ターゲット候補タンパク質が検出・同定される．同定された疾患関連タンパク質の特徴や，機能，疾患との関連がウエスタンブロット法，MS/MS，RNA干渉法，ノックアウトマウスなど様々な方法を用いて明らかにされる．また，多数の検体を対象にしてELISAやMS/MS法などを用いて疾患関連タンパク質が疾患特異的に発現しているのか検証される．

著者らは，抗癌剤に抵抗性であり早期発見されても多数の予後不良例が存在する悪性度の高い卵巣癌組織型，卵巣明細胞腺癌（CCA）のプロテームを解析し，CCA細胞が特徴的に産生するアネキシンIVを含む様々なタンパク質を検出・同定した．同定したタンパク質のCCA特異的な発現には，何らかの共通したメカニズムが関係しているという仮説のもとにまずアネキシンIV遺伝子の発現調節機構を解析した．その結果，本遺伝子は転写因子p53により発現制御を受けていることが明らかになった．さらにCCA細胞ではp53標的遺伝子群が発現上昇しているという，CCA関連タンパク質群の一つの共通性が浮き彫りになった．本論文ではこの事例について報告する．

1. CCA細胞のプロテオーム解析

上皮性卵巣癌は組織型に富む癌であり，大きく分けて漿液性，粘液性，類内膜，CCAの4種類に分類される．この

Regulation of protein expression in relation with ovarian clear cell adenocarcinoma

Noriaki Arakawa[1], Yusuke Masuishi[1], Hisashi Hirano[1,2]
[1]Yokohama City University, Graduate School of Nanobioscience; and [2]Yokohama City University, Advanced Medical Research Center
* Corresponding author: Noriaki Arakawa; Yokohama City University, Graduate School of Nanobioscience, Suehiro 1-7-29, Tsurumi, Yokohama 230-0045, Japan
Fax: 045-508-7667
E-mail: arakawa@tsurumi.yokohama-cu.ac.jp

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中で特にCCAは悪性度の高い組織型であるとされている．この組織型は抗癌剤に抵抗性を示し，早期発見されても多くは予後不良となる．

全卵巣癌組織型の中でCCAが占める割合は，欧米諸国では5～6%であるのに対し，日本では22%を越えている[1]．我が国におけるCCAの発生頻度は極めて高い．したがって，日本では，CCAを他の卵巣癌組織型とは一線を画する疾患ととらえており，CCAに対する新規抗悪性腫瘍薬の開発ならびにCCAの診断マーカーの確立が喫緊の課題となっている．そこで，著者らは，CCA細胞が特徴的に産生するタンパク質を探索するために，CCA由来の培養細胞株のプロテオームを2D-DIGE法にて解析した[2]．

まず，CCA由来細胞株OVTOKOおよびOVISE，比較対照としてnon-CCA細胞株MCASをそれぞれ7M尿素，2Mチオ尿素，4%CHAPSを含む緩衝液にて溶解し，タンパク質を抽出した．各々のタンパク質抽出液を様々な組み合わせで蛍光標識（Cy2，Cy3，Cy5）した．標識産物を混合して二次元電気泳動後，Typhoon蛍光スキャナー装置にて各泳動像を取得し，画像解析を行うことで比較定量解析を行った．

その結果，2000個以上のタンパク質が検出され，その中で，non-CCA細胞株であるMCASと比較してCCAを由来とするOVTOKOとOVISEの両細胞において発現量が2倍以上高いタンパク質を10種類以上同定することができた[2]．

これらのタンパク質が他のCCA細胞株でも発現量が高いのかどうかを調べるために，non-CCA細胞群，CCA細胞群合わせて11種類の卵巣癌細胞株を用いて，当該タンパク質群の発現量を定量的リアルタイムPCR法，およびウエスタンブロット法により比較した．その結果，アネキシンIVの発現は，mRNAおよびタンパク質ともにCCA細胞群で特徴的に高いことが確認された（Fig. 1）．

2. ANX4遺伝子のプロモーター解析

細胞中でタンパク質が量的に増加する原因として最初に考えられることは，遺伝子発現を引き起こす転写因子の活性化である．癌遺伝子の中で，JunやSTAT，Myc，Etsなどの転写因子は正常細胞においては厳密に制御されているが，様々な癌腫では調節から逸脱し恒常的に活性化している．そこでCCA細胞にもこのような癌遺伝子に分類される転写因子が活性化している可能性が考えられた．

ANX4がCCA細胞において特徴的に発現していることは，著者らが行ったプロテオーム解析[2]やDNAマイクロアレイ解析[3,4]により以前から指摘されている．さらに最近，100症例以上の卵巣癌組織を免疫組織染色により解析することによってもANX4とCCAの関連性が明確にされている[5]．しかし，その転写調節機構については，CCA細胞に限らず他の組織細胞においても解析はなされていない．

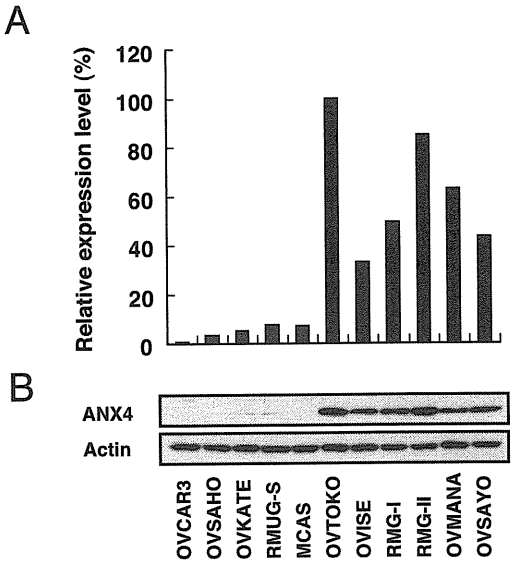

Fig. 1.　mRNA and protein levels of ANX4 in various ovarian cancer cell lines.

Total RNA and protein were extracted from five non-CCA (OVCAR-3, OVKATE, OVSAHO, RMUG-S and MCAS) cell lines and six CCA (OVTOKO, OVISE, RMG-I, RMG-II, OVMANA and OVSAYO) cell lines. (A) Real time RT-PCR analysis of ANX4 mRNA. The values were normalized to the level of 18S ribosomal RNA expression in each sample. (B) Western blot analysis of ANX4 protein. Actin was included as a loading control.

そこでCCA細胞に特異的なANX4遺伝子の発現に関わる転写因子を同定し，それがCCA関連タンパク質に共通しているものであるのかどうか検討した．

プロモーター解析を行うため，ANX4遺伝子の転写開始点上流−1534から下流+1010塩基までの領域をpGL3-Basicルシフェラーゼベクター（プロメガ社）に挿入した．この挿入配列をもとに様々な変異体を作製し，OVISE，OVTOKOおよびMCAS細胞に導入し，転写活性を測定した．

その結果，挿入したANX4遺伝子は，CCA細胞であるOVTOKOおよびOVISE細胞で極めて高い転写活性を示したが（Fig. 2A），転写開始点下流に存在するp53結合配列を欠損もしくは変異導入すると，MCAS細胞では変化は見られなかったのに対し，2種のCCA細胞における転写活性は著しく減少した（Fig. 2B）．このANX4遺伝子上のp53結合配列にp53が結合するのかどうか，ゲルシフトアッセイにより検証した．CCA細胞株から核タンパク質を抽出し，^{32}P標識した本領域の二本鎖DNAプローブと反応させた．その結果，検出されたDNA-タンパク質複合体のバンドは100倍量の未標識プローブを添加することで消失し，抗p53抗体を添加するとさらに高分子側にシフトするバンドが認められた（Fig. 3）．したがって，CCA細胞におけるp53は本領域に結合することがわかった．さらにCCA細胞中のp53をRNA干渉法により発現抑制したり，マイトマイシンCの投与により活性化すると，ANX4の発現の変動が認め

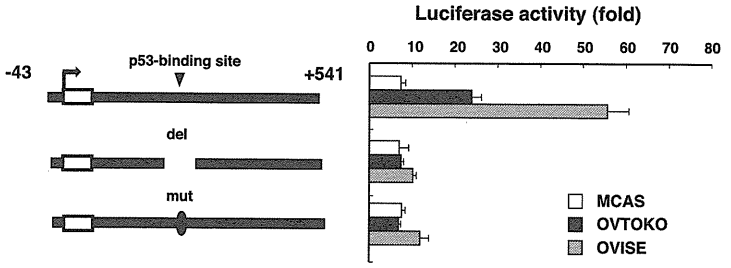

Fig. 2. CCA-specific transcriptional activity of *ANX4* depends on the p53-binding site.

The luciferase vector containing the flanking region of the *ANX4* transcriptional start site were introduced into OVTOKO, OVISE, and MCAS cells, and the transcriptional activities were measured. The luciferase activities of the –43/+541 luciferase vector and the mutants with p53-binding motif deletion or mutation were compared. Luciferase activity is expressed as the fold-change relative to pGL3-basic vector activity in each cell. The b-galactosidase control vector was co-transfected as an internal control. Schematic diagrams of the *ANX4* promoter-luciferase plasmids are shown on the left, where the location of the 5'- and 3'-ends are indicated relative to the transcription start site.

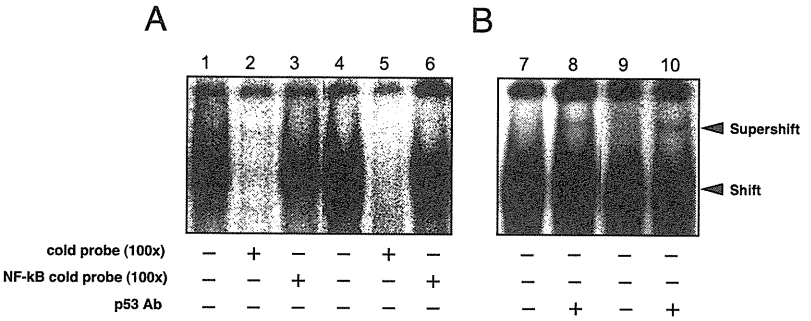

Fig. 3. p53 protein binds to the +180 region. Gel shift assay was carried out using a radiolabeled double-stranded oligonucleotide probe recognizing the +180 region sequence.

Nuclear extracts were isolated from OVTOKO and OVISE. *A.* Specific bands showing a protein-DNA complex were detected in the cells (lanes 1 and 4). Binding specificity was evaluated with the addition a 100-fold excess of an unlabeled +180 probe (lanes 2 and 5) or the NF-kB-binding probe (lanes 3 and 6). *B.* Supershifted bands were demonstrated in the presence of the anti p53 IgG. Nuclear extracts were preincubated in the presence (lanes 8 and 10) or absence (lanes 7 and 9) of anti p53 IgG, PAb-421.

られたことから，CCA 細胞特異的な ANX4 発現には p53 が寄与していることが示唆された[6]．

3. CCA 細胞のプロテオームの特徴

50%以上の癌患者の腫瘍細胞では，p53 遺伝子に変異が起きる．しかし，CCA では p53 変異は稀であることが知られており，本研究で用いた CCA 細胞株も例に漏れず，解析に用いた全ての CCA 細胞株からは p53 変異は検出されなかった．しかし，ANX4 発現が微弱な non-CCA 細胞株では全てにおいて変異が認められた．CCA 細胞における p53 標的遺伝子 p21 や MDM2 の発現量は，non-CCA 細胞と比較して，タンパク質と mRNA の両レベルともに高かったことから，CCA では p53 が機能的状態にあることが示唆される．実際に，多くの CCA 患者組織でも p21 が検出される[7]．

筆者らは CCA 細胞株のプロテオミクス解析を，先に述べた 2D-DIGE 法の他にも，iTRAQ 法を含めたショットガン解析を用いて進めており，ANX4 以外にも様々な CCA 関連タンパク質を同定しているが（荒川，増石ら未発表），それぞれの生理的役割や分子機能，あるいは関与する情報伝達経路などを考えると，これらのタンパク質には一見共通性がないように見える．しかし，タンパク質をコードする遺伝子群が p53 の標的となるのかどうか文献情報[8]により調べたところ，p53 により直接的に発現誘導を受けるタンパク質が種々存在する．ANX4 も含めて，このような p53 標的遺伝子群の翻訳産物が増加していることは，non-CCA 細胞のプロテオームとは区別すべき CCA 細胞のプロテオームの一つの特徴であると言えるだろう．CCA 細胞は p53 をある程度活性化しているというユニークな分子生物学的性質

がプロテオームの特徴に寄与している可能性が考えられる．

おわりに

プロテオミクス手法を用いて同定されたCCA関連タンパク質の変動を血液中でも検出することができれば，これらのタンパク質の診断マーカーとしての利用価値は高い．このような観点から，組織検体や培養細胞で検出されたCCAマーカー候補タンパク質を血液中で検出できないか調べた．中空糸膜や抗体カラムを用いてアルブミンなどの血液中に含まれる高濃度タンパク質を除去し，LC/nanoLC-MS/MSにより約3,000近いタンパク質を解析した．しかし，この方法でもCCA関連タンパク質の変動を検出することはできなかった．血液中でCCAマーカー候補タンパク質を検出するためには，新しい検出技術の開発が必要である．

プロテオーム研究ではしばしば，同定されるタンパク質群のジーンオントロジー情報から，例えば転写関連因子が多く変動している，あるいはアポトーシス抑制因子群が多く変動している，といった共通した“偏り”に着目することで，プロテオームデータから細胞動態を予測することが試みられる．このような情報は病態を理解する上で非常に重要であると考えられる．しかしながら実際は，同定されたタンパク質の性質だけでは共通点はなかなか見えない．著者らが同定しているCCA関連タンパク質においても一見共通性はない．しかし，遺伝子の発現調節メカニズムという観点からタンパク質群を眺めると，p53により発現誘導されるタンパク質が多く存在するというCCAの特徴を見いだすことができる．MS/MSにより同定されるタンパク質の性質だけでなく，その発現に関わる転写因子，あるいは相互作用ネットワークなども含めて考慮することで，細胞内で起きている特異現象が見えてくる可能性が高くなると思われる．たとえ注目したタンパク質一種類ではマーカーとして有効でなくても，共通した病態特異的現象に関わる周辺のタンパク質群，CCAを例に挙げるとp53標的遺伝子を数種類組み合わせることで，より効率的な病態診断が可能になるのではないだろうか．

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Identification of Phosphorylated Proteins Involved in the Oncogenesis of Prostate Cancer Via Pin1-Proteomic Analysis

Kanji Endoh,[1,2] Mayuko Nishi,[2] Hitoshi Ishiguro,[3,4] Hiroji Uemura,[3] Yohei Miyagi,[5] Ichiro Aoki,[6] Hisashi Hirano,[7] Yoshinobu Kubota,[3] and Akihide Ryo[2]*

[1]Drug Discovery Research Center, Taiho Pharmaceutical Co., Ltd, Tsukuba, Japan
[2]Department of Microbiology, Yokohama City University School of Medicine, Yokohama, Japan
[3]Department of Urology, Yokohama City University School of Medicine, Yokohama, Japan
[4]Photocatalyst Group, Kanagawa Academy of Science and Technology, Takatsu-ku, Kawasaki, Kanagawa, Japan
[5]Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research institute, Yokohama, Japan
[6]Department of Molecular Pathology, Yokohama City University School of Medicine, Yokohama, Japan
[7]Department of Nanobioscience, Yokohama City University, Yokohama, Japan

BACKGROUND. The peptidyl-prolyl isomerase Pin1 regulates a subset of phosphorylated proteins by catalyzing the *cis-trans* isomerization of their specific phosphorylated Ser/Thr-Pro motifs. Although Pin1 has been shown to be involved in cell transformation and the maintenance of the malignant phenotype in prostate cancer, its specific substrates during these processes have not yet been determined.

METHODS. Cancer-specific phosphorylated proteins were isolated from two human prostate cancer cell lines (PC-3, LNCaP) and the Dunning rat prostate cancer cell lines by GST-pull down analysis with recombinant GST-Pin1 protein. These proteins were then identified by the LC-MS/MS analysis using a Q-Tof micro mass spectrometer and processed for further functional analysis.

RESULTS. We newly identified five prostate cancer-specific Pin1 binding proteins (PINBPs) in this screen. Among these, TRK-fused gene (TFG) was found to be preferentially up-regulated in prostate cancer cell lines and tissues. The targeted inhibition of TFG by specific siRNA resulted in the reduced cell proliferation and the induction of premature senescence in PC3 prostate cancer cells. We further found that TFG can facilitate the cell signaling mediated by NF-kappaB and androgen receptor (AR). Tissue micro-dissection based quantitative RT-PCR analysis of prostate cancer tissues following radical prostatectomy further revealed that TFG expression is closely associated with both a higher probability and shorter period of tumor recurrence following surgery.

CONCLUSIONS. Pin1-based proteomics analysis is a useful tool for the identification of prostate cancer-specific phosphorylated proteins. TFG could be a potential diagnostic and/or prognostic marker and therapeutic target in prostate cancer. *Prostate 72:626–637, 2012.* © 2011 Wiley Periodicals, Inc.

KEY WORDS: **prostate cancer; tumor recurrence; androgen receptor; prognostic marker**

INTRODUCTION

Prostate cancer is one of the most common tumors to arise in males and its incidence is increasing steadily worldwide [1]. Despite the possibility of an earlier diagnosis using serum prostate specific antigen (PSA), prostate cancer is still the leading cause of male cancer death in many countries [2]. Radiation and a radical prostatectomy are definitive forms of therapy for clinically localized prostate cancers, but a

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*Correspondence to: Prof. Akihide Ryo, PhD, MD, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. E-mail: aryo@yokohama-cu.ac.jp

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substantial number of cases have demonstrated tumor recurrence, even when tumors have been localized pathologically to the prostate at the time of surgery. Recurrent cancers after radiation and radical prostatectomy are often refractory to treatments with chemical reagents and radiation [3,4]. Although the Gleason grade is an excellent diagnostic indicator of prostate cancer based on histopathological features, it is currently difficult to predict whether a clinically localized prostate cancer will remain latent or progress to aggressive or metastatic disease [5]. Novel prostate cancer biomarkers based on the molecular cell biology of prostate tumorigenesis are thus desirable.

Recent advances in mass spectrometry (MS) have enabled the analysis of not only overall protein expression profiling in certain cancer cells or tissues [6–8], but also a substantial number of post-translational modifications (PTMs), such as protein phosphorylation, from small cancer tissue specimens in a short timeframe. Whilst these technically advanced MS methods can detect a large number of candidate proteins [9,10], it remains difficult to select functionally significant oncoproteins and the corresponding PTMs that contribute to the progression of cellular oncogenesis. It is thus desirable to develop a new strategy for selectively capturing phosphorylated proteins that are functionally important in cell transformation and oncogenesis.

Pin1 is an enzyme that specifically binds phosphorylated serine or threonine, immediately preceding proline (pSer/Thr-Pro) in a subset of proteins [11]. Pin1 regulates the function of its substrate proteins by promoting *cis/trans* isomerization of the peptide bond between Ser/Thr and Pro [12,13]. We previously demonstrated that the peptidyl prolyl-isomerase Pin1 plays an important role in the maintenance of the tumorigenic properties of prostate cancer [14]. Targeted depletion of Pin1 results in the stable suppression of both cell growth and tumorigenicity in prostate cancer cells [14]. Furthermore, Pin1 inhibition significantly suppresses several malignant phenotypes such as cell proliferation, invasion, and angiogenesis. These results indicate that Pin1 plays crucial role in a range of tumorigenic properties in prostate cancer cells. However, specific Pin1 substrates during these processes have not so far been determined.

In our present study, we utilized Pin1 as a molecular probe in combination with MS analysis to capture and identify prostate cancer-specific phosphorylated proteins. We thereby identified TRK-fused gene (TFG) as a new Pin1-interacting oncogenic protein and provide evidence that it could be a potent therapeutic target for prostate cancer.

MATERIALS AND METHODS

Cell Lines

DU145, PC3, and LNCaP cells (human prostate cancer cell lines) were obtained from the American Type Culture Collection (Rockville, MD). Prostatic epithelial cells (hPrEC) were purchased from Takara Bio (Shiga, Japan). PrEC were cultured in prostate epithelial basal medium (PrEBM, Takara Bio) supplemented with human epidermal growth factor, triiodothyronine, transferrin, epinephrine, gentamicin sulfate, amphotericin B, bovine pituitary extract, bovine insulin, hydrocortisone, and retinoic acid additives provided by the manufacturer. Prior to the experiments, DU145, LNCaP, and PC3 cells were cultured in RPMI1640 (Wako, Osaka, Japan) supplemented with 10% fetal calf serum (FCS), and incubated under 5% CO_2. In the experiments, these cells were cultured in phenol red-free RPMI plus 0.1% bovine serum albumin (BSA) or F-12 medium supplemented with charcoal-stripped 10% FCS, and stimulated with reagents.

Pin1 Proteomic Analysis

GST-fused Pin1 was purified using a conventional strategy in *E. Coli* and used in pull down analysis with cell lysates. Prostate cancer or normal prostate epithelial cell lysates were processed for pull down with GST or GST-Pin1 at 4°C for 3 hr. Pin1-binding proteins were thereby recovered and collected by thrombin cleavage which digests at a site between GST and Pin1 followed by SDS–PAGE, as described previously [15]. Gels were subjected to silver staining and specific bands were systematically excised. The gel pieces were then reduced, alkylated, and trypsinized. Peptides were analyzed by liquid chromatography-tandem MS (LC-MS/MS) analysis with a Q-Tof micro Mass Spectrometer (Micromass). Protein identification was performed using a Mascot search (Matrix Science).

RNA Extraction, cDNA Preparation, Real-Time RT-PCR, and siRNA

Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Quantitative reverse transcription (qRT)-PCR was performed with an ABI 7700 sequence detector system (Applied Biosystems) as described previously. Primers for TFG (5′-GGAACACAAAAGACCAAAATGG-3′; 5′-AGGGCTCTACTTTAGTACATC-3) were designed using Primer Express (Applied Biosystems). Reagents from the One-Step Cyber Green RT-PCR Master Mix Reagents Kit (TaKaRa) were used in accordance with the

manufacturer's protocol. PCR reactions were performed in a total volume of 25 μl using ABI Prism770 (Applied Biosystems). A standard curve method was used to determine the expression levels and all measurements were found to be within the linear range of the standard curve. β-actin was used as an internal control and to normalize Pin1 expression levels. TFG-targeted siRNA sequences were as follows: (TFG1: 5′-GUCUGCUUCUGAUUCUUCU-3′; TFG2: 5′- GGUCAGAUGUACCAACAGU -3′; control: 5′- UCGTAUGUUGUGUGGAAUU -3′).

Cell Culture

Human prostate cancer cell lines PC3, LNCaP, and DU145 were cultured in RPMI1640 or DMEM, supplemented with 10% FCS and penicillin/streptomycin. Dunning rat tumor sub lines of variable cell growth (doubling time, days) and variable degrees of metastatic potential; AT-1 (2.5 ± 0.2 days, 0–5%), AT-2 (2.5 ± 0.2 days, 0–20%), AT-3 (1.8 ± 0.2 days, 75–100%), and MAT-LyLu (1.7 ± 0.3 days, 75–100%) were grown in RPMI 1,640/10% fetal bovine serum (FBS) supplemented with 0.1% dexamethasone and 1% L-glutamine [16].

Antibodies

An anti-TFG polyclonal antibody was generated in rabbits against a synthetic peptide corresponding to the region 320–336 of TFG conjugated to keyhole limpet hemocyanin, and purified on a peptide affinity column. TFG phospho-specific polyclonal antibodies (anti-TFGpThr206 and anti-TFGpThr372) were generated in rabbits against synthetic peptides "CEDRSGpTPDSIX" and "CXXGSTMpTPPPSX" (where X donates any one of the 20 amino acids) respectively, as described previously [17]. Antibodies were affinity-purified on sepharose resin to which the appropriate phosphorylated peptide had been covalently coupled and passed through a column containing the unphosphorylated peptides to remove any antibodies that did not recognize the phosphorylated epitope. Phosphospecificity was established by ELISA and western blot assays.

Gene Reporter Assay

Cells were transfected with plasmid vectors encoding either NF-kB-Luc or PSA-Luc reporter construct and co-transfected with pRL-TK or pRL-SV40 using Effectene transfection reagent (Qiagen). One day after transfection, the cells were resuspended in passive lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Patients and Samples

Paired human untreated primary prostate cancer tissues and normal (or benign prostatic hypertrophy (BPH) (n = 29)) tissues from same patients were obtained during radical prostatectomy at Yokohama City University Hospital and its affiliates. The clinicopathological data for these samples are summarized in Table I. The postoperative serum PSA levels were assayed every 2–3 months. The occurrence of these consecutive elevations of the PSA level by more than 0.20 ng/ml was defined as biochemical recurrence [18]. The pathological stage was determined according to the International Union against Cancer (UICC) [19]. The sampling and usage of all prostate tissues in this study were approved by the ethical committee of Yokohama City University Graduate School of Medicine and performed only after obtaining informed consent from each patient.

Statistics

All statistical analyses were performed using a software for statistic analysis PASW statistics 18 (SPSS, Chicago, IL). TFG expressions and clinicopathological characteristics were compared using chi-square test or the Fisher's exact test. For analysis of the correlation between TFG expression, clinicopathological characteristics, and biochemical recurrence, Kaplan–Meier and log rank tests were utilized. Each parameter and biochemical recurrence was further analyzed using a Cox promotional hazard regression model in univariate and multivariate analyses. The hazard ratio and 95% confidence interval (95%) are also shown in the results. $P \leq 0.05$ was considered to be statistically significant.

TABLE I. Clinicopathological Characteristics of Examined Prostate Cancer Samples

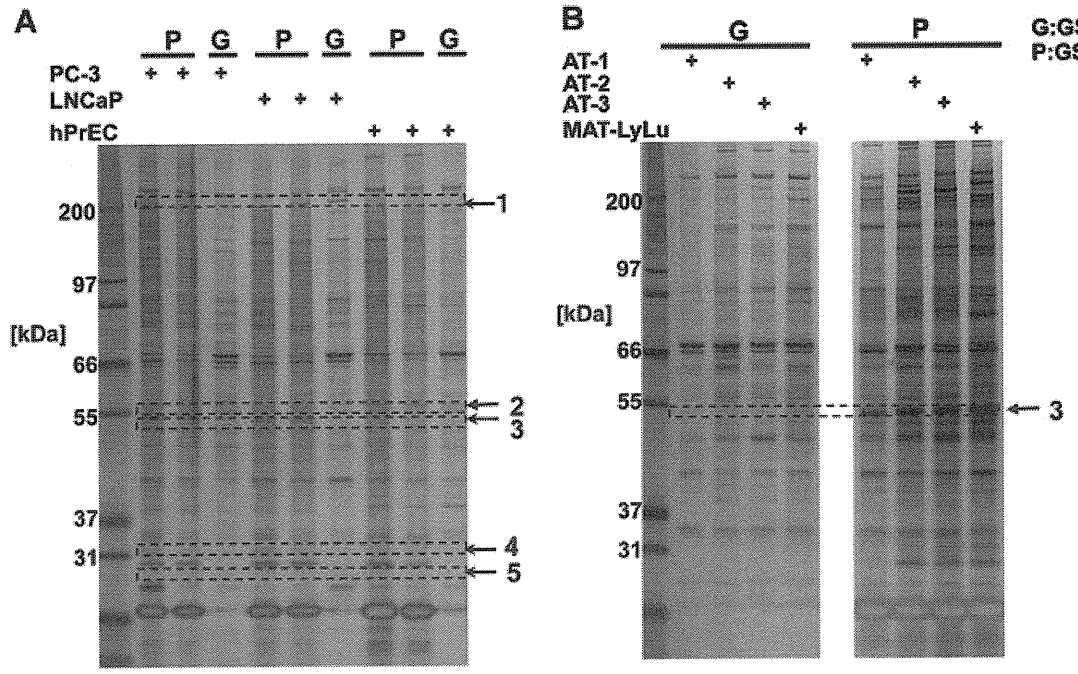
		n	(%)
Age (years)	≦68	15	51.7
	>68	14	48.3
Median(Range)	68(52–75)		
Preoperative PSA(ng/ml)	≦10.0	15	51.7
	>10.0	14	48.3
Median(Range)	8.8(4.1–35.7)		
UICC Stage	pT2a-b	16	55.2
	pT3a-b	13	44.8
Gleason score	≦7	20	69.0
	>8	9	31.0

RESULTS

Isolation of Prostate Cancer-Specific Phosphorylated Proteins using Pin1 Proteomics

Previous reports of Pin1-prostate cancer interaction have demonstrated that Pin1 contributes to the transformation and tumorigenicity of prostate epithelial cells by modulating the function of its substrate proteins [20]. To identify prostate cancer-specific Pin1 substrates, we performed proteomics analysis in which Pin1 was used as a molecular probe to detect phosphorylated targets. We detected multiple bands in then prostate cancer cell lines that were not present in normal prostate epithelial cells. These bands were excised from the gel and subjected to in-gel digestion followed by LC-MS/MS analysis with a Micromass Q-Tof micro Mass Spectrometer. We identified five Pin1 binding proteins (PINBP 1-5) specific to prostate cancer cells (Fig. 1, left panel).

We next performed parallel experiments using the Dunning rat tumor model developed by Issacs et al. [16]. This is a well-characterized animal model that has been used to study prostate cancer pathogenesis. These cell lines have also been shown to exhibit many characteristics including the androgen responsiveness and differential metastatic ability observed during the progression of human prostate cancer. We investigated Pin1-binding proteins in several Dunning tumor sublines (AT1, AT-2, AT-3, and MAT-LyLu), the spectra of which are illustrated in Figure 1 (right panel). When we evaluated the differences in the PINBP profiles for each subline, a 55 kDa band was found to be prominent. This band intensity was significantly increased in malignant Dunning tumor cells. Subsequent LS-MS/MS analysis revealed that the corresponding protein is derived from rat TFG. Since TFG was also identified as a Pin1-interacting protein in human prostate cancer cell lines, we focused on this protein for further analysis.



No	protein	accession No	AA
1	Microtuble-associated protein 1A	NP 002364.5	2803
2	Non-POU protein	AAH69636	471
3	TFG protein	NP_001007566	400
4	necleophosmin	AAA36380.1	294
5	Complement component1	NPQ07021	282

Fig. 1. Identification of TFG as a Pin1 binding protein in prostate cancer cells. **A**: Cell lysates of PC-3, LNCaP, or normal prostatic epithelial cells (hPrEC) were subjected to pull down analysis with either GST or GST-Pin1. Proteins bound to glutathione sepharose beads were isolated and detected by SDS–PAGE and silver staining. Arrows point to the bands containing TFG protein. **B**: Cell lysates from each Dunning subline were processed for GST-pull down analysis as in (A). Bound proteins were separated by SDS–PAGE and detected by sliver staining. Arrow indicates the bands containing TFG protein.

TFG is Overexpressed in Prostate Cancer Cells and Tissues

Our initial analysis indicated that TFG is a Pin1-binding protein in prostate cancer cells and we thus examined its expression in these cells. Both quantitative RT-PCR analysis and immunoblotting with TFG antibodies revealed that TFG expression is preferentially up-regulated in prostate cancer cell lines as compared with normal prostate epithelial cells (Fig. 2A and B). Immunohistochemical analysis of representative prostate cancer tissues with TFG antibodies further revealed that TFG is expressed in glandular cancer cells but not in adjacent non-cancerous epithelial cells (benign prostatic hyperplasia: BPH) or interstitial cells (Fig. 2C). Since prostate cancer cells often infiltrate non-cancerous glands and the intervening fibro-muscular stroma, it was necessary to precisely quantitate the TFG expression levels in cancerous glandular cells. We utilized tissue microdissection based quantitative RT-PCR analysis for this purpose. Twenty nine paired human prostate cancer and adjacent non-cancerous prostate tissues from the same individual were thereby examined. The results of this analysis showed that the TFG expression levels in prostate cancer tissues are higher than in non-cancerous tissues in 63.9% ($P = 0.031$) of the cases (Fig. 2D). These results together demonstrate that TFG is overexpressed in prostate cancer cells.

TFG Interacts With Pin1 on its Thr206-Pro and Thr372-Pro Motifs

Previous reports have indicated that Pin1 can bind only phosphorylated Ser/Thr-Pro motifs [21] of which only two (Thr206-Pro and Thr372-Pro) are present in the TFG protein (Fig. 3A, upper). Interestingly, this motif is conserved between various species including human, rhesus monkey, mouse, and xenopus (Fig. 3A, lower). We thus generated TFG site-directed mutants at these sites by substituting threonine with alanine (T206A, T372A). GST-pull down

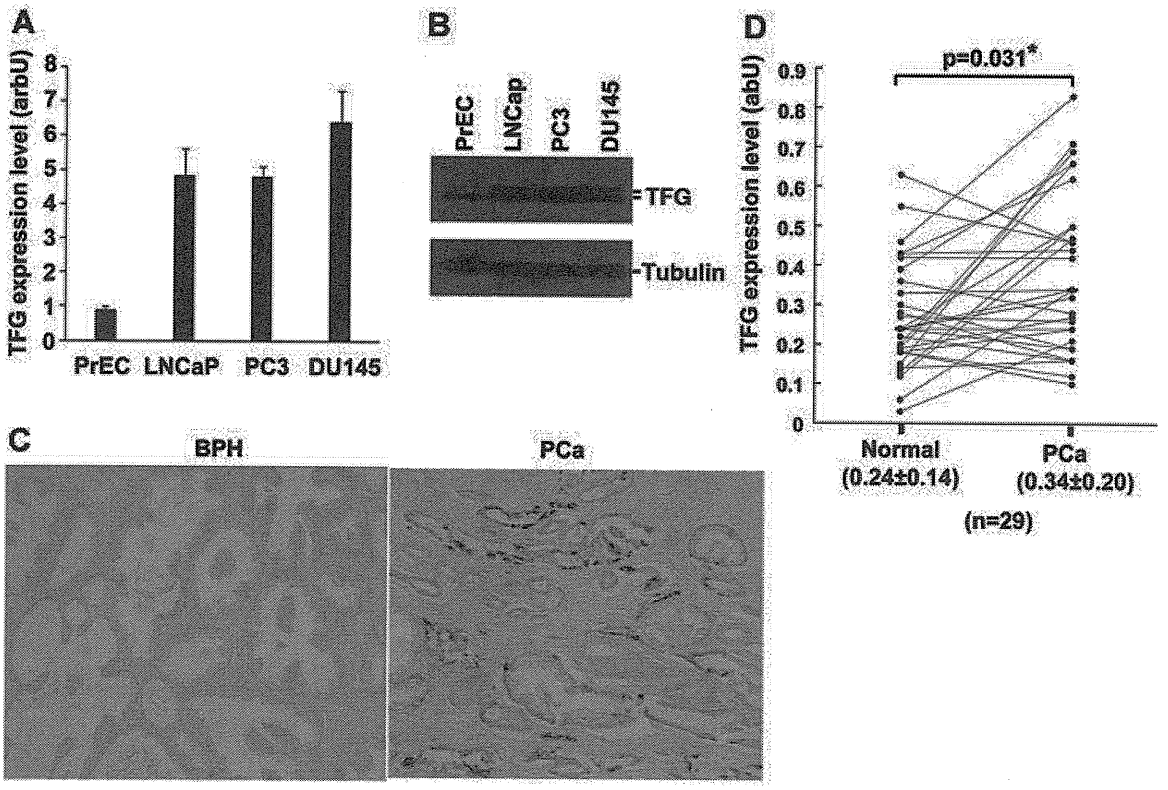


Fig. 2. TFG is overexpressed in prostate cancer cells and tissues. **A, B**: TFG mRNA expression levels were monitored by quantitative RT-PCR analysis of normal prostatic epithelial cells (PrEC) or three different prostate cancer cell lines (A). Cell lysates from the indicated cells were subjected to immunoblot analysis with either anti-TFG or anti-tubulin antibody. Tubulin was used as a loading control. **C**: Immunostaining of TFG in human prostate cancer. Sections from paraffin-embedded tissues were subjected to antigen retrieval, followed by immunostaining with anti-TFG antibody. Non-cancerous benign epithelial hyperplastic tissues (left) show no visually detectable TFG staining, while invasive prostate cancer cells (right) show focally, but intense TFG staining. **D**: TFG mRNA expression levels in prostate cancer tissues and adjacent normal tissues ($n = 29$). TFG expression in prostate cancer was significantly higher than that in normal prostate tissue (Mann–Whitney U-test, $P = 0.031$, *: $P < 0.05$).

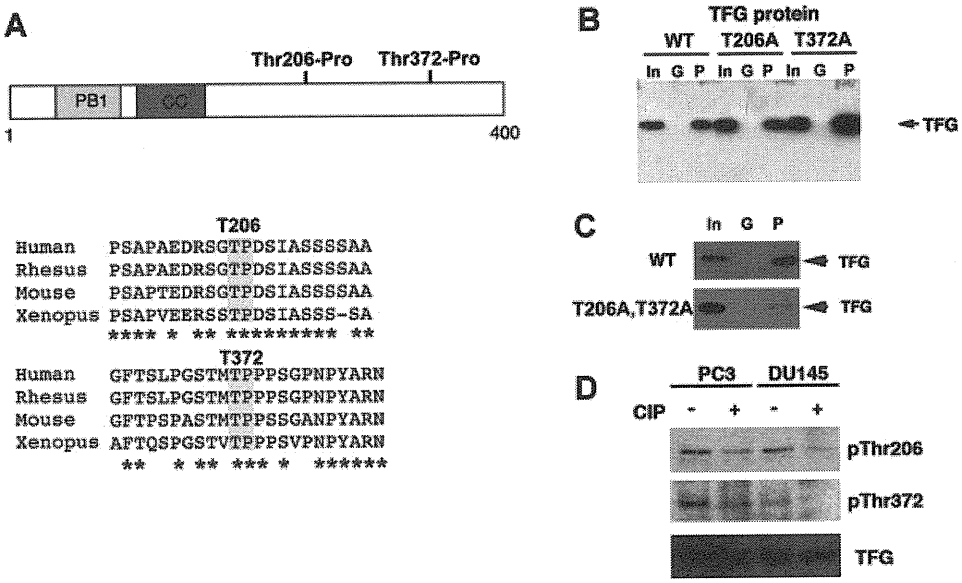


Fig. 3. TFG interacts with Pin1 via its Thr206-Pro and Thr372-Pro motifs. **A**: Schematic representation of human TFG protein (upper). Amino acid sequence alignment of the human, rhesus, mouse, and xenopus TFG proteins (lower). The conserved Thr206-Pro and Thr372 motifs are boxed. **B**: PC3 cells were transfected with the FLAG-tagged TFG (wild-type, WT) or its site-directed mutants, TFG-T206A, or TFG-T372A. At 24 hr following transfection, cell lysates were subjected to GST pull-down analysis followed by immunoblotting with anti-FLAG antibody. In, Input, G, GST; P, GST-Pin1. **C**: PC3 cells were transfected with either FLAG-tagged TFG (wild-type, WT) or its site-directed mutant on both Thr206 and Thr372 sites (T206, 372A). At 24 hr following transfection, cell lysates were subjected to GST pull-down analysis followed by immunoblotting with anti-FLAG antibody. **D**: Lysates from PC3 or DU145 cells were treated or untreated with calf-intestine alkaline phosphatase (CIP) followed by immunoblotting analysis with an anti-TFG antibody or phospho-specific antibodies targeting p-Thr206 or p-Thr372, respectively.

analysis subsequently revealed that Pin1 binds wild-type TFG and its single site-directed mutants (T206A, T372A) (Fig. 3B), but fails to interact with the corresponding TFG double mutant (2A: T206A, T372A) (Fig. 3C). These results indicate that Pin1 binds TFG on both the Thr206-Pro and Thr372-Pro motifs.

We next addressed whether Pin1 binding sites in TFG were indeed phosphorylated in prostate cancer cells. Cell lysates from PC3 and DU145 cells were treated or untreated with calf-intestine alkaline phosphatase and then subjected to immunoblotting analysis with Thr206 or Thr372 phospho-specific antibodies. We found that these two sites are indeed phosphorylated in the two prostate cancer cell lines as the signals were abolished by the pre-treatment with alkaline phosphatase (Fig. 3D). These results together indicate that Pin1 interacts with the phosphorylated Thr206 and Thr372 sites on TFG.

TFG Activates NF-kappaB in Cooperation With Pin1

A previous report has indicated that TFG binds NEMO and TANK proteins that modulate the NF-kappaB pathway and enhance NF-kappaB activity [21]. We thus speculated as to whether Pin1 enhances the function of TFG during NF-kappaB signaling. A luciferase reporter assay using the 3X kappaB-luciferase reporter construct was performed in LNCaP cells co-transfected with TFG or Pin1. Whilst the sole expression of either TFG or Pin1 had only minor effects, the co-expression of TFG and Pin1, but not its catalytic domain mutant (K63A), produced a significant increase in reporter activity (Fig. 4A). However, this is not the case in the TFG mutant lacking two Pin1 binding sites (T206A, T372A; Fig. 4B). This indicated that Pin1 promotes TFG-mediated NF-kappaB activation via its interaction with the Thr206-Pro and Thr372-Pro motifs on TFG.

TFG Promotes the Transcriptional Activity of AR

We next tested whether Pin1 enhances the transcriptional activity of androgen receptor (AR). A luciferase reporter assay using a PSA promoter containing androgen responsive elements (AREs) was performed in either LNCaP or 293T cells co-transfected with AR or TFG. Whilst the sole expression of TFG had no significant effects, the co-expression of TFG and AR produced a significant increase in the reporter activity of the PSA promoter with and without

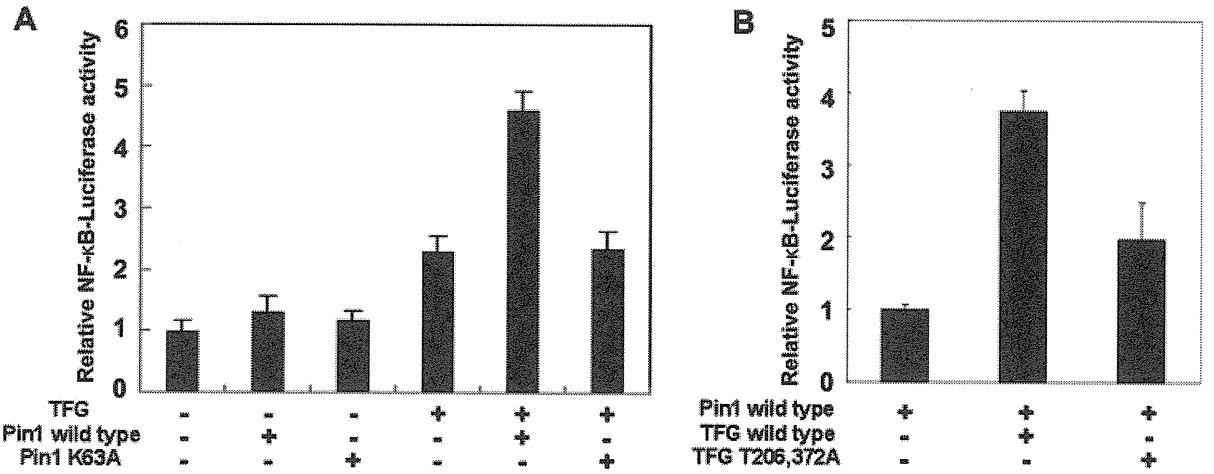


Fig. 4. Pin1 enhances TFG-mediated NF-kappaB activation. **A, B**: LNCaP cells were transiently transfected with the indicated plasmid vectors and co-transfected with an NF-kappaB reporter gene and pRL-TK. At 24 hr post-transfection, the cells were collected and subjected to a gene reporter assay. Immunoblots for TFG-FLAG and HA-Pin1 are shown in the upper left box. Pin1 K63A is a Pin1 mutant lacking its catalytic activity.

an AR ligand dihydrotestosterone (DHT) (Fig. 5A). Immunoprecipitation analysis revealed that TFG can specifically bind to AR (Fig. 5B). Immunofluorescent analysis further revealed that the subcellular localization of AR shifts from being cytoplasmic only to a nuclear and cytoplasmic pattern when TFG is overexpressed (Fig. 5C). These results together indicate that TFG associates with AR and thereby enhances the transcriptional activity of AR.

The Suppression of TFG Affects Cell Growth and Morphology

The above results indicated that TFG is a putative Pin1 substrate and plays an important role in oncogenicity in prostate cancer. We next addressed whether the specific depletion of TFG in prostate cancer cells has any effect on cell proliferation and tumorigenicity. For this purpose, we constructed two different siRNAs that target TFG and used the prostate cancer cell lines PC3, DU145, and LNCaP in which TFG has been shown to be highly overexpressed (Fig. 2A and B). As shown in Figure 6A, TFG protein levels were significantly reduced in cells treated with TFG-siRNAs when compared with control siRNA treated cells.

We next evaluated the effects of TFG depletion on in vitro cell growth and morphology. The suppression of TFG resulted in a substantially decreased cell growth (Fig. 6B). Morphological analysis also revealed that cells expressing TFG-siRNAs were relatively larger and contained vacuolated nuclei and a granular cytoplasm, thus showing specific features of senescent cells. Senescence-associated beta galactosidase staining (SABG) of these cells further revealed significant increases in the number of SABG-positive cells in the TFG-siRNA population, but not in the control siRNA cells (Fig. 6C and D). These results indicate that the loss of TFG expression affects both cell proliferation and morphology by partly inducing cellular senescence in prostate cancer cells.

The Association Between TFG Expression and Prostate Cancer Recurrence

We next investigated the relationship between TFG expression levels and cancer recurrence. The Kaplan–Meier Method and log rank test were used to compare the significance of the recurrence free curves between two groups among 29 prostate cancer samples taken from patients after a radical prostatectomy. The results indicated a strong association between TFG expression and tumor recurrence (Fig. 7A). However, there were no such association between cancer recurrence and age, pre-operative PSA, Gleason score, or UICC stage (Fig. 7B–E). These relationships were also confirmed using the multi-variate analysis between the TFG expression levels and recurrence-free survival times (Table II).

We also investigated the relationship between TFG expression level and Age, Pre-operative PSA, Gleason score, or UICC stage among 29 prostate cancer samples taken from patients after prostatectomy (Fig. 8). There was no significant relevance between TFG expression levels and these parameters. These results demonstrate that TFG expression in prostate cancer tissues may predict a high risk of recurrence after a prostatectomy.

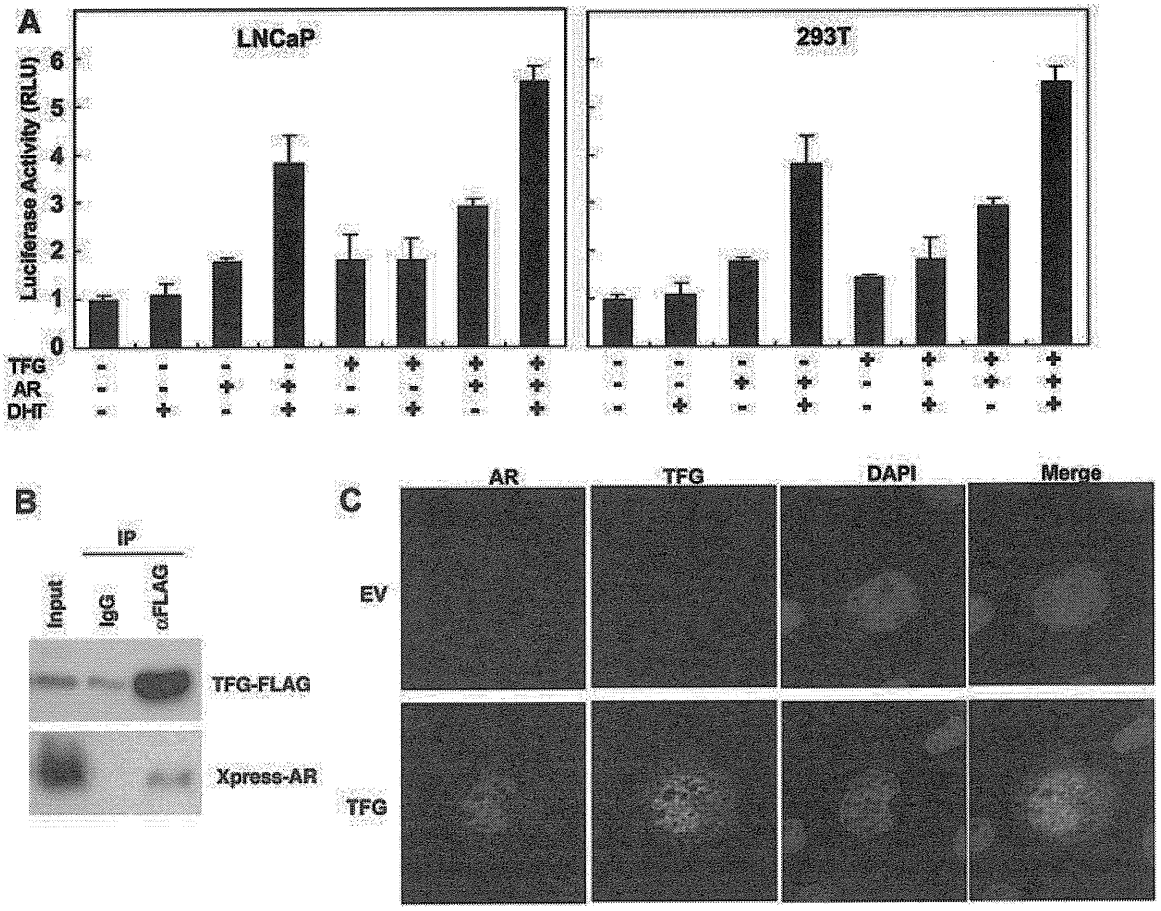


Fig. 5. Effects of TFG on prostate specific antigen (PSA) promoter activity. **A**: A luciferase reporter assay using a PSA promoter containing androgen responsive elements (AREs) was performed in LNCaP and 293T cells co-transfected with AR or TFG. Total DNA content was kept constant in all wells. Cells were treated or untreated with 10 nM dihydro-testosterone (DHT). **B**: 293T cells were co-transfected with TFG-FLAG and Xpress-AR. After 24 hr, cells were lysed and subjected to immunoprecipitation analysis with either rabbit anti-FLAG polyclonal antibody or non-immunized rabbit IgG (IgG). Immunoprecipitates were then processed for immunoblotting analysis with either anti-FLAG or anti-Xpress monoclonal antibodies. **C**: COS7 cells were transfected with Xpress-tagged AR and co-transfected with either TFG-FLAG or empty vector (EV). After 24 hr, cells were fixed and immunostained with anti-Xpress (Red) and anti-FLAG antibodies (Green) followed by confocal microscopy. Cell nuclei were counterstained with DAPI (blue).

DISCUSSION

In our present study, we demonstrate that TFG is a novel Pin1-binding phosphorylated protein in prostate cancer. TFG was found to be highly expressed in prostate cancers and its expression level was closely associated with a high tumor recurrence. Furthermore, TFG activates NF-kappaB and AR signaling and a targeted TFG depletion by specific siRNA prevents cell proliferation and induces cellular senescence in prostate cancer cells. Our current Pin1-based proteomics analysis has thus revealed that TFG could be a new potential diagnostic/prognostic marker and anti-cancer target.

Ayala et al. [22] have reported that Pin1 expression is highly correlated with a higher probability of recurrence in prostate cancer after a prostatectomy. Another report has shown that a Pin1 scoring system which sums both the nuclear and cytoplasmic grade can predict the prognosis of prostate cancer patients after a prostatectomy [23]. We have previously shown in our laboratory that Pin1 plays an important role not only in tumorigenesis but also in the maintenance of the transformed phenotype in prostate cancer cells [20]. However, Pin1 target proteins in prostate cancer have not been reported till date, and the underlying molecular mechanism has remained elusive. To address these questions, we performed Pin1-mediated proteome analyses and identified TFG as a novel Pin1 binding protein in prostate cancer cells. TFG binds Pin1 specifically in prostate cancer cells, and this interaction increases according to the degree of malignancy in Dunning rat sublines. We further found that TFG is overexpressed in prostate cancer cells or

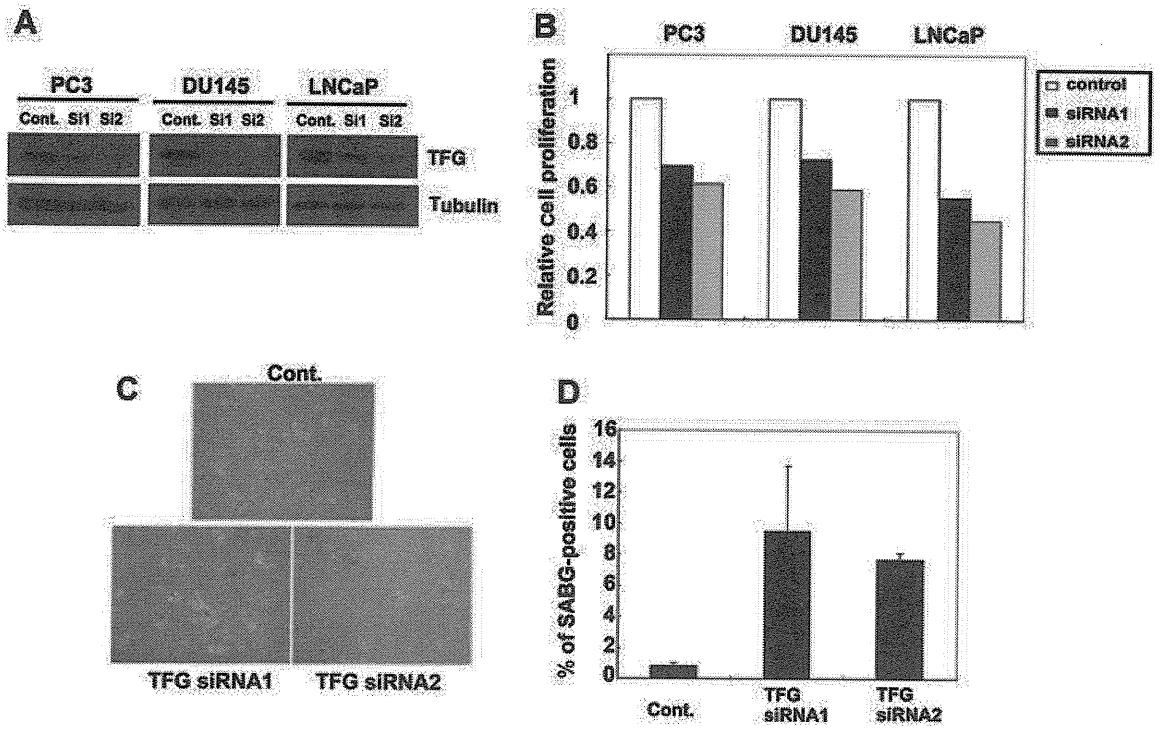


Fig. 6. TFG depletion inhibits cell growth in prostate cancer cells and induces cellular senescence. **A**, **B**: PC3, DUI45, and LNCaP cells were transduced with control-siRNA (Cont) or two different TFG siRNAs (siRNAI, siRNA2) for 96 hr. Cell lysates were extracted and then subjected to immunoblot analysis with indicated antibodies (A). Cell proliferation was assessed by the MTT assay 96 hr after siRNA transfection and the figure represents the mean of three independent experiments (B). **C**: PC3 cells transduced with the indicated siRNA were subjected to senescence-associated β-galactosidase (SABG) staining (blue). The average number of SABG–positive cells is depicted in (D).

tissues and that its inhibition results in the suppression of cell proliferation and the induction of premature senescence in these cells. In addition, Pin1 promotes TFG-mediated NF-kappaB activation by interacting with TFG on Thr206-Pro and Thr372-Pro motifs. These results indicate that Pin1 is a positive regulator of prostate cancer malignancy through TFG-mediated NF-kappaB activation.

A previous report indicates that TFG is an uncharacterized protein that associates with NEMO and TANK to enhance NF-kappaB activity [21]. TFG was shown also here to activate AR transcriptional activity suggesting that it may engage in the hormone dependency of recurrent prostate cancer. Consistent with this hypothesis, we found in our current experiments that TFG expression positively correlates with a higher rate of tumor recurrence after radical prostatectomy. These data suggest that TFG plays an important role in prostate cancer tumorigenesis by facilitating the intracellular signaling mediated by NF-kappaB and AR. Further analysis will be required to more fully characterize the collaborative actions of Pin1 and TFG during oncogenesis. Cell growth is reduced by extracellular signals including cytokines, apoptosis induction caused by DNA damage, and cell cycle arrest via cellular senescence [24,25]. We have reported previously that the suppression of Pin1 results in a substantial decrease of cell growth and the onset of specific features of senescence in prostate cancer cells [20]. In our present study, we demonstrate that the suppression of TFG results in a phenotype that is similar to when Pin1 is suppressed in prostate cancer cells.

To predict clinical outcomes in prostate cancer patients, the Gleason grade and/or PSA test are commonly used. These are standard clinicopathological parameters that can be useful in defining the clinical stage of prostate cancer. However, these parameters cannot predict the clinical outcome of prostate cancer patients with a moderate Gleason score (GS6 or GS7) [5] and a more precise predictive marker is thus desirable. Our current data suggest that TFG could be a potent and independent biomarker for recurrent prostate cancers and might therefore be a potential future therapeutic target.

CONCLUSIONS

This study reveals the potential utility of Pin1 as a molecular probe that can be used to screen prostate cancer specific phosphorylated proteins. This approach has identified TFG as a new Pin1 binding

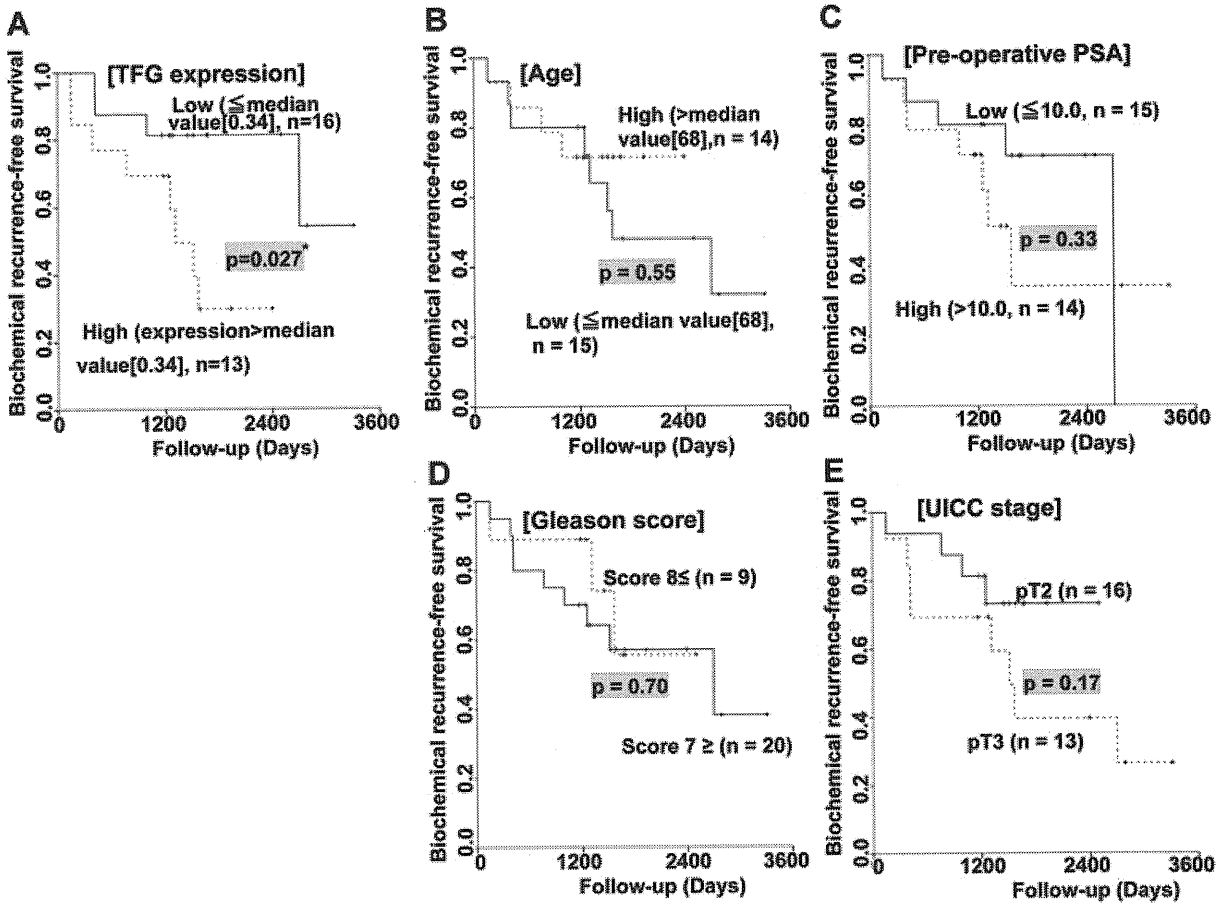


Fig. 7. Association between TFG expression and prostate cancer recurrence. **A–E**: Association of TFG expression levels (A), Age (B), Pre-operative PSA (C), Gleason score (D), or UICC stage (E) with recurrence-free survival. The Kaplan–Meier method and Log rank test were performed in prostate cancers (n = 29) with each parameter. $P \leq 0.05$ indicates statistical significance. (*: $P < 0.05$).

TABLE II. Relative Hazard of Recurrence Free Survival in Multivariate Analysis

		HR	95% CI	*P*
TFG expression				
Low expression (≦0.34)	(n = 16)			
High expression (0.34<)	(n = 13)	4.35	1.08–17.58	0.04*
Pre-operative PSA (ng/ml)				
≦10.0	(n = 15)			
10.0<	(n = 14)	1.8	0.45–7.12	0.41
Gleason score				
≦7	(n = 20)			
8≦	(n = 9)	0.53	0.11–2.56	0.43
UICC stage				
pT2	(n = 16)			
pT3	(n = 13)	1.86	0.48–7.20	0.37
Age (years)				
≦68	(n = 15)			
68<	(n = 14)	0.79	0.18–3.52	0.75

HR, hazard ratio; 95% CI, 95% confidence interval.
*: $P < 0.05$.

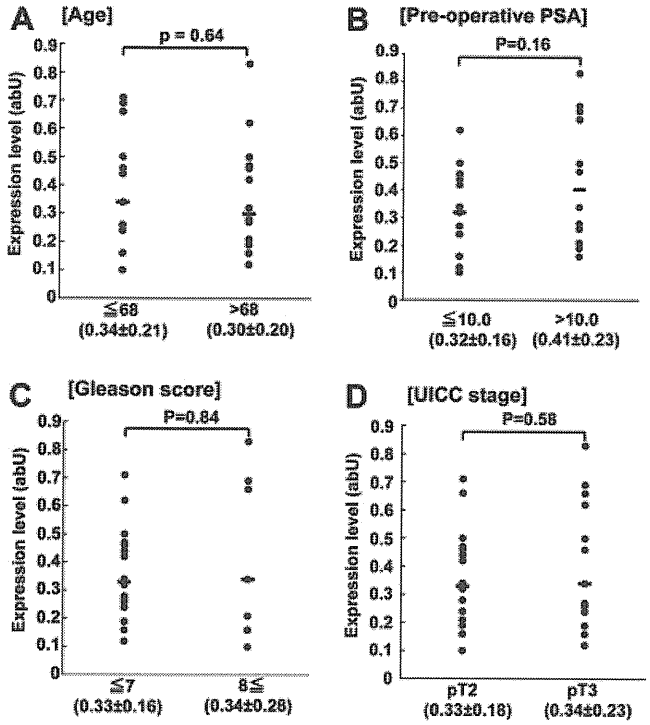


Fig. 8. Association between TFG expression and clinical parameters. **A–D**: Association of the TFG expression levels with Age (A), Pre-operative PSA (B), Gleason score (C), or UICC stage (D). Real-time quantitative PCR was carried out as described in Material and Methods section. Numerical values below columns indicate mean ± standard deviation. *P*-values were calculated by Mann–Whitney U-test. $P \leq 0.05$ indicates statistical significance.

protein in prostate cancer cell lines. TFG was shown to enhance the activity of both NF-kappaB and AR, presumably thereby contributing to prostatic oncogenesis. TFG expression shows a strong association with prostate cancer recurrence after prostatectomy. Our current data therefore indicate that TFG is a potential therapeutic target for prostate cancer and a biomarker for tumor recurrence.

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