Characterization of Gene Expression Induced by RET with *MEN2A* or *MEN2B* Mutation

Tsuyoshi Watanabe,^{*,†} Masatoshi Ichihara,^{*}
Mizuo Hashimoto,^{*} Keiko Shimono,[†]
Yoshie Shimoyama,^{*} Tetsuro Nagasaka,[‡]
Yoshiki Murakumo,^{*} Hideki Murakami,^{*}
Hideshi Sugiura,[†] Hisashi Iwata,[†] Naoki Ishiguro,[†]
and Masahide Takahashi^{*}

From the Departments of Pathology,^{*} Orthopedic Surgery,[†] and
Laboratory Medicine,[‡] Nagoya University Graduate School of
Medicine, Nagoya, Japan

Germ-line point mutations of the *RET* gene are responsible for multiple endocrine neoplasia (MEN) type 2A and 2B that develop medullary thyroid carcinoma and pheochromocytoma. We performed a differential display analysis of gene expression using NIH 3T3 cells expressing the RET-MEN2A or RET-MEN2B mutant proteins. As a consequence, we identified 10 genes induced by both mutant proteins and eight genes repressed by them. The inducible genes include cyclin D1, cathepsins B and L, and cofilin genes that are known to be involved in cell growth, tumor progression, and invasion. In contrast, the repressed genes include type I collagen, lysyl oxidase, annexin I, and tissue inhibitor of matrix metalloproteinase 3 (*TIMP3*) genes that have been implicated in tumor suppression. In addition, six RET-MEN2A- and five RET-MEN2B-inducible genes were identified. Among 21 genes induced by RET-MEN2A and/or RET-MEN2B, six genes including cyclin D1, cathepsin B, cofilin, ring finger protein 11 (*RNF11*), integrin- $\alpha 6$, and stanniocalcin 1 (*STC1*) genes were also induced in TGW human neuroblastoma cells in response to glial cell line-derived neurotrophic factor stimulation. Because the *STC1* gene was found to be highly induced by both RET-MEN2B and glial cell line-derived neurotrophic factor stimulation, and the expression of its product was detected in medullary thyroid carcinoma with the *MEN2B* mutation by immunohistochemistry, this may suggest a possible role for *STC1* in the development of MEN 2B phenotype. (Am J Pathol 2002; 161:249–256)

The *RET* proto-oncogene encodes a receptor tyrosine kinase with a cadherin-related motif and a cysteine-rich domain in the extracellular domain and is located on chromosome 10q11.2.^{1,2} It has been demonstrated that RET is a functional receptor for four related neurotrophic factors including glial cell line-derived neurotrophic fac-

tor (GDNF), neurturin, artemin, and persephin. These factors are known to require glycosylphosphatidylinositol-anchored co-receptors, GFR- α s, as ligand-binding components and to promote the survival of various central and peripheral neurons in culture.^{1,2} In addition, gene knockout studies revealed that the GDNF/RET signaling plays a crucial role in the development of the enteric nervous system and the kidney.^{3–6}

Germline mutations of the *RET* gene cause dominant inherited cancer syndromes; multiple endocrine neoplasia (MEN) type 2A and 2B.^{7–10} MEN 2A is characterized by the development of medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid hyperplasia. MEN 2B shows a more complex phenotype with association of MTC, pheochromocytoma, and developmental abnormalities such as mucosal neuroma, hyperganglioneosis of the intestinal tract, and marfanoid skeletal changes. The *MEN2A* mutations were identified in cysteine residues of the RET extracellular domain, leading to ligand-independent RET dimerization.^{11,12} The *MEN2B* mutations were detected in methionine at codon 918 or in alanine at codon 883 in the tyrosine kinase domain and appear to activate RET without dimerization.^{12,13}

A variety of signaling molecules were shown to be activated by GDNF or RET with *MEN2* mutations.^{1,2} These include extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), AKT, c-Jun amino-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK), and phospholipase-C γ (PLC- γ). Intriguingly, it turned out that several major intracellular signaling pathways such as RAS/ERK, PI3-K/AKT, JNK, p38MAPK, and ERK5 pathways are activated mainly through phosphorylated tyrosine 1062 present in the carboxy-terminal region of RET.^{14–17} Consistent with this finding, we showed that the transforming activity of all MEN 2 mutant forms of RET was markedly impaired by a mutation at tyrosine 1062, indicating the importance of tyrosine 1062 on signal transduction for oncogenesis.^{18,19}

To further elucidate the mechanisms of development of MEN 2A or MEN 2B phenotype, it is important to know which genes are induced by RET-MEN2A or RET-MEN2B mutant proteins. We performed a screening analysis of

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Address reprint requests to Masahide Takahashi, Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: mtakaha@med.nagoya-u.ac.jp.

differential gene expression using a defined *in vitro* model of NIH 3T3 cells expressing RET-MEN2A and RET-MEN2B. In this study, we identified a number of genes induced downstream of RET signals and suggest that the stanniocalcin1 (*STC1*) gene expression may play a role in the MEN 2B phenotype.

Materials and Methods

Cell Culture

NIH 3T3 cells and transfectants expressing RET with *MEN2A* mutation (cysteine 634 → arginine) or RET with *MEN2B* mutation (methionine 918 → threonine) were maintained in Dulbecco's modified Eagle's medium supplemented with 8% calf serum (Hyclone, Logan, UT).

Differential Display Analysis

Total RNAs were isolated from NIH 3T3 cells and transfectants expressing RET-MEN2A or RET-MEN2B mutant proteins using Trizol reagent (Gibco, Tokyo, Japan). After treating with RNase-free DNase I to eliminate contaminated chromosomal DNA, differential display-polymerase chain reaction (PCR) was performed using the TaKaRa rhodamine fluorescence differential display system (TaKaRa, Kyoto, Japan). The fluorescence products were resolved by electrophoresis on denaturing urea-4% polyacrylamide gels. Differentially expressed bands were identified using FM-BIO II (TaKaRa).

Northern Blot Analysis

Total RNA (10 μ g) was separated on 1% agarose-formamide gels with formaldehyde and transferred onto Hybond-XL nylon membranes (Amersham Biosciences, Uppsala, Sweden). DNA fragments identified by the differential display method were labeled with [α - 32 P] dCTP (3000 Ci/mmol, Amersham Biosciences) using the High Prime DNA-labeling system (Roche Diagnostics, Mannheim, Germany) and used as probes for Northern hybridization at 68°C for 3 hours in QuikHyb Solution (Stratagene, Austin, TX). Signals were detected on X-ray films (RX-U) after exposure for appropriate time. To confirm equal loading of RNA, the membranes were also hybridized with [α - 32 P] dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA. Densitometric analysis was performed by scanning the imaging plate with the BAS-2000 system (Fujifilm, Tokyo, Japan).

SYBR Green-Based Real-Time Quantitative Reverse Transcriptase-PCR

Total RNAs were isolated from TGW human neuroblastoma cells stimulated with GDNF (100 ng/ml) for 0.5, 1, 2, 4, 8, 16, 24, and 48 hours, and used for real-time quantitative reverse transcriptase-PCR studies. cDNAs produced from total RNAs were added to 12.5 μ l of PCR reaction mixture plus 12.5 μ l of 2 $\times$ SYBR Green master

mix (Applied Biosystems, Foster City, CA) with 900 nmol/L of gene-specific primers and assays were performed according to the manufacturer's instructions. All data were analyzed with the Applied Biosystems model 7700 and were normalized by the expression level of GAPDH mRNA as an internal control.

Western Blot Analysis

Anti-STC1 rabbit polyclonal antibody was generated against the carboxy-terminal 19 amino acids of mouse STC1. Cells were lysed in sodium dodecyl sulfate sample buffer (50 mmol/L Tris-HCl, pH 6.8, 5 mmol/L ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate, 10% sucrose, 20 μ g/ml bromophenol blue) containing 80 mmol/L of dithiothreitol and protein concentrations in the lysates were determined by using the DC Protein Assay kit (Bio-Rad, Hercules, CA). Twenty μ g of the proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were blocked with 3% ovalbumin in TPBS (Tris-buffered saline with 0.5% Tween 20), and probed with the antibody. The blots were washed and probed with the swine anti-rabbit horseradish peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) before being visualized with the standard chemiluminescent technique (ECL, Amersham Biosciences).

Tet-Off System

MEF3T3 Tet-Off cells (Clontech, Palo Alto, CA) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% Tet-Off system-approved fetal bovine serum (Clontech) containing 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mmol/L L-glutamine, and 100 μ g/ml G418 (Roche Diagnostics), and maintained at 37°C under a 5% CO₂ atmosphere. The MEF3T3 Tet-Off cells grown on 60-minute tissue culture dishes were co-transfected with 10 μ g of pTRE/*RET-MEN2A* or pTRE/*RET-MEN2B* plasmid and 0.5 μ g of pTK-Hygr plasmid (Clontech) by Lipofectamine 2000 (Invitrogen, Tokyo, Japan). The cells were detached with trypsin 3 days after transfection and plated in the presence of doxycycline hydrochloride (0.01 to 1 μ g/ml) (Sigma-Aldrich Japan K.K., Tokyo, Japan) and hygromycin B (200 μ g/ml; Roche Diagnostics). The resulting hygromycin-resistant colonies were expanded and maintained in media supplemented with 100 μ g/ml of G418, 100 μ g/ml of hygromycin, and 200 ng/ml of doxycycline. To induce the expression of RET-MEN2A and RET-MEN2B mutant proteins, the cells were cultured in the absence of doxycycline for 72 hours.

Immunohistochemical Analysis

Human MTC tissues were obtained from eight patients who underwent operations at Nagoya University Hospital. Sections were deparaffinized with xylene, rehydrated, and preincubated with blocking buffer [10% normal goat serum in phosphate-buffered saline (PBS)] for 15 minutes

at room temperature. Then they were reacted with the anti-RET or anti-STC1 antibody overnight at 4°C. After washing with PBS, they were treated with the secondary dextran peroxidase-conjugated goat anti-rabbit antibody (DAKO Envision system) and diaminobenzidine was used to visualize immune complexes.

Results

Gene Expression Induced or Suppressed by RET with MEN2 Mutations

To identify differentially expressed genes in NIH 3T3 cells and NIH 3T3 cells expressing RET-MEN2A or RET-MEN2B mutant proteins [designated NIH-RET(MEN2A) or NIH-RET(MEN2B) cells], we performed a differential display analysis using their RNAs. We detected a total of 336 cDNA bands whose intensities showed clear differences (more than three times) among the samples from these RNAs (data not shown). After further separated by hemagglutinin-yellow and HA-red splitting techniques (TaKaRa), the cDNAs derived from the isolated bands were inserted into the TA cloning vector and sequenced. As a result, 130 known genes and 13 previously unidentified sequences were found (data not shown).

We analyzed the differential expression of 130 known genes in NIH 3T3, NIH-RET(MEN2A), and NIH-RET(MEN2B) cells by Northern blotting. Twenty-nine genes were confirmed to be differentially expressed in these cells (Figure 1). Based on their expression patterns, they were classified into four types: 1) 10 genes induced by both RET-MEN2A and RET-MEN2B mutant proteins (type I); 2) six genes induced predominantly by RET-MEN2A (type II); 3) five genes induced predominantly by RET-MEN2B (type III); and 4) eight genes repressed by RET-MEN2A and RET-MEN2B (type IV) (Figure 1). Type I includes cyclin D1, cathepsin B, cofilin, and cathepsin L and B genes that are known to be involved in cell growth, tumor progression, and invasion whereas type IV includes type I collagen, lysyl oxidase, annexin I, and tissue inhibitor of matrix metalloproteinase 3 (TIMP3) genes that have been implicated in tumor suppression. Type II and type III include various genes associated with different physiological functions.

Identification of GDNF-Inducible Genes

We next investigated whether RET-MEN2A- and/or RET-MEN2B-inducible genes were induced by GDNF stimulation. TGW human neuroblastoma cells that endogenously express RET and GFR- α 1 were treated with GDNF for 0.5, 1, 2, 6, 12, 24, and 48 hours, and their total RNAs were extracted for Northern blotting. As shown in Figure 2A, six of 21 differentially expressed genes were also induced by GDNF, although the time course of their induction was different depending on the genes. They included cyclin D1, cathepsin B, cofilin, ring finger protein 11 (*RNF11*), integrin- α 6 (*ITGA6*), and stanniocalcin 1 (*STC1*) genes. Cyclin D1, cathepsin B, cofilin, and *RNF11* belonged to the type I gene group, and *ITGA6* and *STC1*

Figure 1. Characterization of differentially expressed genes by Northern blot analysis. A portion (10 μ g) of total RNA extracted from NIH 3T3, NIH-RET(MEN2A), or NIH-RET(MEN2B) cells were analyzed by Northern blotting with cDNA probes of the designated mouse genes. Type I, the genes induced by both RET-MEN2A and RET-MEN2B mutant proteins; type II, the genes induced predominantly by RET-MEN2A; type III, the genes induced predominantly by RET-MEN2B; type IV, the genes repressed by both RET-MEN2A and RET-MEN2B. *RNF11*, ring finger protein 11; *IFN- β* , interferon- β ; *TB-2-like 1*, *TB-2-like 1* protein; *HSC73*, heat shock protein 73; *EMK2*, ELK1 motif kinase 2; *ITGA6*, integrin- α 6; *PKA-RI*, protein kinase A regulatory subunit I; *TACC3*, transforming acidic coiled coil-containing gene family 3; *PRNPA*, prion-related protein A; *MPT*, mitochondrial phosphate transporter; *STC1*, stanniocalcin 1; *PheX*, phosphate-regulating gene with homology to endopeptidases on the X chromosome; *EIF4G3*, eukaryotic translation initiation factor 4G3; *neuropsin*, serine protease 19; *PLOD2*, procollagen-lysine,2-oxyglutarate,5-dioxygenase 2; *COLIA1*, type I collagen α 1 chain; *COLIA2*, type I collagen α 2 chain; *TIMP3*, tissue inhibitor of metalloproteinases 3; *SDF1 α* , stromal cell-derived factor-1 α ; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

belonged to type II and type III gene groups, respectively. These results were confirmed by real-time quantitative reverse transcriptase-PCR using RNAs from GDNF-treated TGW cells that were separately extracted (Figure 2B).

STC1 Expression in Cells Expressing RET-MEN2B Mutant Proteins

Because only *STC1* was found to be induced by both RET-MEN2B and GDNF, and was suggestive of a role in early skeletal development²⁰ that is affected in MEN 2B, we further investigated the expression of the *STC1* protein in the transfectants.

We developed a rabbit polyclonal antibody against the carboxy-terminal 19 amino acids of mouse *STC1*. To show the specificity of this antibody, we transiently trans-

Figure 2. Identification of GDNF-inducible genes. **A:** Total RNAs isolated from TGW neuroblastoma cells treated with GDNF (100 ng/ml) for 0, 0.5, 1, 2, 6, 12, 24, or 48 hours were analyzed by Northern blotting with cDNA probes of the designated human genes. The membranes were also probed with *GAPDH* cDNA as a control. **B:** Total RNAs separately isolated from TGW cells treated with GDNF were analyzed by real-time quantitative reverse transcriptase-PCR as described in Materials and Methods. The results show averages from at least three independent experiments. The bars indicate standard errors.

fecting Flag-tagged mouse *STC1* cDNA into COS7 cells and their cell lysates were analyzed by Western blotting with anti-Flag and anti-STC1 antibodies. Both antibodies specifically detected a 32-kDa band that is consistent with the predicted molecular mass of STC1 (Figure 3A). As expected from the result of Northern blot analysis (Figure 1), the 32-kDa band was detected more strongly in the lysate from NIH-RET(MEN2B) cells than in the lysate from NIH-RET(MEN2A) cells (Figure 3B).

To confirm that the STC1 expression is highly induced by the RET-MEN2B mutant protein, we used MEF 3T3 Tet-Off cells in which the gene expression was repressed by addition of tetracycline (doxycycline). The cells were co-transfected with the pTRE (tetracycline-responsive element)-2 vector containing *RET-MEN2A* or *RET-MEN2B* cDNA and the pTK-Hyg selection vector. When tetracycline was removed from media in the transfectants, the expression of RET-MEN2A or RET-MEN2B mutant proteins were clearly induced, accompanying the increase of ERK1/2 phosphorylation (Figure 3C). Under the same condition, the STC1 expression was induced more strongly in the cells expressing RET-MEN2B than in the cells expressing RET-MEN2A (Figure 3C).

Figure 3. Detection of STC1 protein by Western blot analysis. **A:** COS-7 cells were transfected with the expression vector carrying Flag-tagged full-length mouse *STC1* cDNA. The cell lysates from original COS-7 cells (lanes 2, 4, and 6) or transfectant (lanes 1, 3, and 5) were analyzed by Western blotting with normal rabbit IgG (lanes 1 and 2), anti-STC1 antibody (lanes 3 and 4), or anti-Flag antibody (lanes 5 and 6). A 32-kDa STC1 band is indicated. **B:** Total cell lysates from NIH 3T3, NIH-RET(MEN2A), or NIH-RET(MEN2B) cells were analyzed by Western blotting with anti-RET, anti-phosphotyrosine (pY), anti-ERK2, and anti-STC1 antibodies. **C:** MEF3T3 Tet-Off cells were transfected with pTRE expression vector (Clontech) carrying *RET-MEN2A* or *RET-MEN2B* cDNA. Cell lysates from NIH-RET(MEN2B) or MEF3T3 Tet-Off transfectants incubated with or without doxycycline (100 ng/ml) were analyzed by Western blotting with anti-RET, anti-pY, anti-ERK2, anti-phosphoERK1/2, and anti-STC1 antibodies. Tet, tetracycline (doxycycline).

Figure 4. Expression of STC1 in human MTC carrying the *MEN2B* mutation. Bright-field micrographs of histological paraffin sections from sporadic MTC (**A** and **D**), MEN2A-MTC (**B** and **E**), and MEN2B-MTC (**C** and **F**) were stained with anti-RET (**A–C**) or anti-STC1 (**D–F**) antibody. Diaminobenzidine was used to visualize immune complexes. They were counterstained by hematoxylin. When immunohistochemical analyses were performed without the first antibody as a negative control, no staining was observed in these cases. Original magnifications, $\times 200$.

STC1 Expression in Medullary Thyroid Carcinoma

Finally, we examined the STC1 expression in specimens of human MTC by immunohistochemistry. We stained two sections of sporadic MTCs, four sections of MEN2A-MTCs, and two sections of MEN2B-MTCs. No *RET* mutations were detected in two sporadic MTCs. Interestingly, two specimens of MEN2B-MTC were strongly stained with anti-STC1 antibody (Figure 4F). Two MEN2A-MTCs were weakly stained with the antibody (Figure 4E), and two sporadic MTCs and two MEN2A-MTCs were almost unstained (Figure 4D and data not shown). RET expression was detected in all specimens of both MEN2A MTCs and MEN2B MTCs to variable degrees (Figure 4, B and C).

Discussion

MEN 2A and MEN 2B are dominantly inherited cancer syndromes characterized by the development of MTC and pheochromocytoma. In addition to these tumors, 10 to 30% patients of MEN 2A develop parathyroid hyperplasia whereas MEN 2B is characterized by a variety of developmental anomalies including mucosal neuroma, intestinal ganglioneuroma, marfanoid skeletal changes, and a male reproductive defect. The *MEN2A* mutations were detected mainly in one of six cysteine residues of the RET extracellular domain, leading to disulfide-linked RET dimerization.^{11,12} The *MEN2B* mutations were found at methionine 918 or alanine 883 in the kinase domain and activated RET without dimerization.^{12,13} Although we and other investigators reported the differences in signal transduction activated by RET-MEN2A or RET-MEN2B mutant proteins,^{21–24} which signalings are critical for the

development of disease phenotypes still remains unknown. To elucidate further the mechanisms of development of MEN 2A and MEN 2B phenotypes, it is interesting to characterize the differential gene expression induced by RET-MEN2A and RET-MEN2B.

In the current study, we performed a differential display analysis of gene expression using RNAs from NIH 3T3, NIH-RET(MEN2A), and NIH-RET(MEN2B) cells. We identified a total of 29 genes whose differential expression was confirmed by Northern blotting. Although we identified 101 other genes by differential display, the differences in the levels of their mRNA expression were less than two times among three cell lines in Northern blot analyses (data not shown). Thus, the interpretation of the results obtained by sensitive techniques such as differential display and DNA microarray should be cautious without confirmation by different techniques.

Some of RET-MEN2A and RET-MEN2B-inducible genes (type I genes) including cyclin D1, cofilin, and cathepsin B and L genes were reported to be involved in the cell growth, cell motility, and cancer invasion,^{25–28} suggesting that the increased expression of these genes contributes to malignant properties of RET-MEN2A- or RET-MEN2B-expressing cells. However, the roles of other genes such as decorin and interferon- β are controversial because they were reported to suppress tumor growth.^{29,30} In addition, the functions of *RNF11*, foocen, and TB2-like 1 genes belonging to type I group are currently unknown.^{31,32} On the other hand, we identified eight genes suppressed by both RET-MEN2A and RET-MEN2B mutant proteins (type IV genes). Several genes such as type I collagen, lysyl oxidase, TIMP3, and annexin I genes are down-regulated in cancer cells and have been implicated in tumor suppression.^{33–35} Thus, it

seems likely that the changes of expression of certain type I and type IV genes directly correlate to tumor development or progression.

MEN2A- or MEN2B-inducible genes (type II or type III genes) showed a wide diversity in their possible physiological functions. They include protein kinases (EMK2, PKA-RI),^{36,37} cell adhesion molecule (ITGA6),^{38,39} microtubule-associated protein (TACC3),⁴⁰ protease (neuropilin),⁴¹ and translation initiation factor (EIF4G3).⁴² Of 11 type II or type III genes identified, we focused on the STC1 gene because it was reported that it may be involved in the early skeletal development²⁰ that is affected in MEN 2B patients.

STC was originally identified as a hormone that is synthesized and secreted by the corpuscles of Stannius, unique endocrine glands associated with kidney and embryologically derived from nephric ducts.⁴³ Human STC1 was cloned in a study aimed at identifying genes involved in the control of cellular proliferation, using a simian virus 40 early region-transfected human fibroblast culture.⁴⁴ Bacterial or Chinese hamster ovary cell-synthesized recombinant human STC was found to reduce renal phosphate excretion in rats, and to reduce calcium absorption and increase phosphate absorption in the rat and pig duodenum.⁴⁵ These findings suggested that mammalian STC acts as a regulator of calcium and phosphate homeostasis in an autocrine/paracrine manner. In addition, the expression pattern of STC1 in mouse embryos was indicative of a role in early skeletal patterning and joint formation.^{20,46–48}

Our current studies using the stable transfectants and Tet-Off cells demonstrated that STC1 was highly induced by the RET-MEN2B mutant protein. The high level of its expression was also observed in MTC from MEN 2B patients. Although no *RET* mutations were found in two sporadic MTCs examined in this study, the investigation of STC1 expression in sporadic MTCs with the *MEN2B* mutation could be important to show the correlation between its expression and the *MEN2B* mutation. In addition, given that mammalian STC is involved in early skeletal development, it is possible that high levels of STC1 expression during embryogenesis may be associated with marfanoid skeletal changes developed in MEN 2B. However, to elucidate this relation, it will be necessary to investigate which tissues and cells during human embryogenesis express STC1 whose expression level is influenced by the *MEN2B* mutation. STC1 was also reported to be expressed in a variety of tumors including lung cancer, pheochromocytoma, neuroblastoma, osteosarcoma, and fibrosarcoma and to be useful as a molecular marker for their micrometastasis.^{49–53} Thus, a high level of STC1 expression in MTC carrying the *MEN2B* mutation may affect biological properties of tumor cells. Further investigation of STC1 expression and functions in human tissues may provide a new insight into the development of MEN 2B phenotype.

In addition to the skeletal development, several physiological roles for STC1 were suggested. We showed that STC1 was induced in neuronal cells in response to GDNF stimulation, suggesting that it plays a role in the GDNF/RET signaling pathway. Because STC1 is expressed in

brain neurons and functions as a molecular guard of neurons during cerebral ischemia as observed for GDNF,^{54–56} this may imply a physiological link between STC1 and GDNF/RET signaling in the nervous system. Moreover, the fact that the high STC1 expression was detected in ureteric bud/collecting duct cells of E14.5–18.5 mouse metanephric kidney and overlapped with RET expression at this stage suggests the possibility that STC1 expression and GDNF/RET signaling cooperatively function in kidney development.^{3–6,57–59} Future studies will clarify the interesting roles of STC1 in the GDNF/RET signaling pathway as well as in tumor biology.

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