Cdk5 Regulates STAT3 Activation and Cell Proliferation in Medullary Thyroid Carcinoma Cells* †

The biological behaviors of thyroid cancer are varied, and the pathological mechanisms remain unclear. Some reports indicated an apparent aggregation of amyloid accompanying medullary thyroid carcinoma (MTC). Amyloid aggregation in neurodegeneration leads to hyperactivation of Cdk5 and subsequent neuronal death. Based on the connection with amyloid, the role of Cdk5 in MTC is worthy of investigation. Initially, the expression of Cdk5 and its activator, p35, in MTC cell lines was identified. Cdk5 inhibition by specific inhibitors or short interfering RNA decreased the proliferation of MTC cell lines, which reveals the importance of Cdk5 in MTC cell growth. Although p35 cleavage has been considered as an important element in neurodegeneration, it seems that p35 cleavage was not a major cause in Cdk5 activity-dependent MTC cell proliferation because neither Cdk5 activity nor cell growth was affected by the inhibition of p35 cleavage. Clearance of amyloid by antibody neutralization indicated that MTC cell proliferation was supported by calcitonin-derived extracellular amyloid and subsequent Her2 and Cdk5 activation. Significantly, the STAT3 pathway was involved in Cdk5-dependent proliferation of MTC cells through Ser-727 phosphorylation. In addition, Cdk5 inhibition reduced nuclear distributions of both the Cdk5-p35 complex and phospho-STAT3 in MTC cells. Finally, Cdk5 inhibition retarded tumor formation in vivo accompanying the reduction of phospho-STAT3. Our findings suggest the first demonstration of a novel and specific role for Cdk5 kinase in supporting the proliferation of the medullary thyroid carcinoma cells and could shed light on a new field for diagnosis and therapy of thyroid cancer.

Medullary thyroid carcinoma (MTC) is transformed from parafollicular C cells, which are primarily responsible for secretion of calcitonin in the thyroid gland. An abnormal increase of blood calcitonin is a common symptom for MTC, a type of carcinoma that may be associated with MEN2 (multiple endocrine neoplasia-type 2). MEN2 occurs along with pheochromocytomas or adrenal medullary hyperplasia, in which many endocrine glands such as the thyroid, adrenals, and parathyroid are affected simultaneously in a patient. MTC has been demonstrated to be associated with amyloid fibrils aggregated in the thyroid and adjacent tissues. Amyloid is a self-aggregated polypeptide and tends to form insoluble fibrils that are extracellularly deposited in many protein-folding disorders such as Alzheimer disease (AD) and other amyloidoses because of overexpression, proteolytic digestion, or mutations of its precursor protein. In addition to typical amyloid-related diseases, type II diabetes, myocardial infarctions, and several kinds of cancers were found to be associated with amyloid aggregation. In these cases, non-neoplastic and malignant tumors of the breast, lung, squamous cell carcinoma, and MTC are all associated with amyloid.

Cdk5 (cyclin-dependent kinase 5) is a member of a small serine/threonine cyclin-dependent kinase family. Like other cyclin-dependent kinase members, Cdk5 needs to bind with a regulator to perform kinase activity. One major regulating partner for Cdk5 is p35, which was first reported in postmitotic neurons. The crucial role of the Cdk5-p35 complex is to support the development of the central nervous system. In addition to the physiological functions of Cdk5-p35 in neurons, Cdk5 appears to be deregulated by association with p25, a calpain-dependent cleavage product of p35 in AD neurons. p25 protein is more stable than p35 and could further stimulate Cdk5 kinase activity. Under AD pathological circumstances, an amyloid aggregation-dependent increase of intracellular calcium raises calpain activity. It has been hypothesized that the Cdk5-p25 complex hyperphosphorylates Tau (a microtubule-associated protein), thereby reducing the association of Tau with microtubules and subsequently resulting in neuronal death. In addition to the roles of Cdk5-p35 in the central nervous system, numerous extraneuronal functions of Cdk5-p35 have been discovered in recent years. For example, the distribution of Cdk5-p35 was reported in several types of endocrine cells, such as Leydig cells, Sertoli cells, and pancreatic β-cells, in which Cdk5 kinase is active and functioning. With regard to cancer research, only a few papers as well as our report describing the apoptotic role of Cdk5 in prostate cancer cells have indicated that Cdk5 may affect leukemia
cells. So far, there is no evidence indicating that Cdk5 would support cancer cell proliferation. According to our findings, we suggest that Cdk5 positively regulates the proliferation of MTC cells in which amyloid aggregation is an important supporter of routine activation of Cdk5 through Her2. We also provide evidence to indicate that Cdk5 at least maintains MTC cell proliferation through activating the STAT3 pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Constructs, and Transfection—Human MTC cell line (TT, ATCC CRL-1803) was kindly provided by Professor Paulus S. Wang, Department of Physiology, National Yang-Ming University, Taipei, Taiwan. Mouse MTC cell line (MTC-M, ATCC CRL-1806) was purchased from Biosource Collection and Research Center, Taiwan. TT cells were cultured in RPMI 1640 medium (Sigma) plus 10% fetal bovine serum (Biological Industries, Israel) at 37 °C in a humidified atmosphere at 5% CO2. MTC-M cells were cultured in RPMI 1640 medium (Sigma) plus 5% fetal bovine serum (Biological Industries) and 10% horse serum (HyClone) at 37 °C in a humidified atmosphere of 5% CO2. siRNA-cdk5, siRNA-her2, and nonspecific control of siRNA were purchased from Upstate (Cdk5 siRNA assay kit, 60-097; Her2 siRNA assay kit, 60-016; Upstate), which are SMARTpoolTM containing four pooled SMARTselected siRNA duplexes. Human p35, cdk5, and stat3 expression plasmids were constructed by RT-PCR amplification of the human p35, cdk5, and stat3 coding sequences. For p35 and stat3 constructs, both coding sequences were inserted into the pcDNA4 vector (Invitrogen) by TA cloning; for the cdk5 construct, the cdk5 coding sequence was inserted into pcDNA4-egfp vector by TA cloning. The construct of stat3-S727A mutant was generated by PCR-based mutagenesis. The integrity of the construct was verified by DNA sequencing and immunoblotting detection of the corresponding expressed proteins after transfection. Human her2 expression plasmid was a gift from Professor Mien-Chie Hung, Department of Molecular and Cellular Oncology, MD Anderson Cancer Center, University of Texas. Introductions of siRNAs, p35, and cdk5 plasmids into cells were performed by using FuGENE (Roche Applied Science) with 50 pmol of siRNA/106 cells and 1 μg of DNA/105 cells.

Cell Proliferation Assay—The modified colorimetric 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was manipulated to quantitate the proliferation of TT cells. Yellow MTT compound (Sigma) was converted by living cells to form blue formazen, which is soluble in dimethyl sulfoxide (Me2SO). The intensity of blue staining in culture medium proportionately represented the number of living cells and was measured by an optical density reader (Anthos-2001, Austria) at 570 nm (8, 21).

Immunoprecipitation and Immunoblotting Analyses—Cell lysate was produced in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 137 mM NaCl, 50 μg EDTA, protease inhibitor mixture (Roche Applied Science), and 1 mM phenylmethylsulfonyl fluoride) for immunoblotting or extract buffer (100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3PO4, 2 mM Na2VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture) for immunoprecipitation (8, 21). Immunoprecipitates were collected by binding to protein G Plus/protein A-agarose (30 μl, IP05; Calbiochem). For subcellular protein isolation, cells were collected and washed in PBS. Pelleted cells were resuspended in buffer A (0.1X PBS, pH 7.4, 0.4 mM Na3HPO4, 0.15 mM KH2PO4, 13.5 mM NaCl, 0.25 mM KCl, 0.5% Triton X-100, 2 mM MgCl2, and protease inhibitor mixture, which dissolves cell membranes). Nuclei fractions were pelleted, and the supernatant was harvested as the cytosolic fraction. Nuclei fractions were washed three times with buffer A before lysis in buffer B (0.0625 x Tris, 25 mM EDTA, 25 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromphenol blue, and 5% β-mercaptoethanol) (22). Protein samples were analyzed by direct immunoblotting (30 μg/lane) or blotting after immunoprecipitation (0.5–1 mg/immunoprecipitation). The antibodies used included anti-Cdk5 (05-364; Upstate and sc-750; Santa Cruz Biotechnology), anti-phospho-Cdk5 (sc-12918; Santa Cruz Biotechnology), anti-p35 (sc-820; Santa Cruz Biotechnology), anti-actin (MAB1501; Chemicon), anti-tubulin (sc-5286; Santa Cruz Biotechnology), anti-STAT3 (610189; BD Biosciences), anti-phospho-Ser-727-STAT3 (612542; BD Biosciences), anti-phospho-Tyr-705-STAT3 (612356; BD Biosciences), anti-phospho-histone H1 (O6-597; Upstate), anti-c-Fos (sc-52; Santa Cruz Biotechnology), anti-JunB (sc-73; Santa Cruz Biotechnology), anti-phospho-Her2 (Tyr-1221/1222) (2243; Cell Signaling), anti-Her2 (O6-562; Upstate), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch). ECL detection reagent (PerkinElmer Life Sciences) was used to visualize the immunoreactive proteins on membrane (polyvinylidene difluoride; PerkinElmer Life Sciences) after transferring by Trans-Blot SD (Bio-Rad).

In Vitro Cdk5 Kinase Activity Analysis—Kinase assay was performed by washing immunoprecipitates three times with assay dilution buffer (five-fold, 20-145; Upstate). The protein G Plus/protein A-agarose beads with Cdk5 protein (15 μg) was incubated with 5 μl of assay dilution buffer containing 2.5 μg of histone H1 (14-155; Upstate) and 10 μl of magnesium/ATP mixture (20-113; Upstate) in a final volume of 40 μl at 30 °C for 30 min. The strength of phospho-histone H1 was identified by immunoblotting with specific anti-phospho-histone H1 antibody (O6-597; Upstate) (8, 21).

Clearance of Extracellular Peptides—Antibodies including anti-calcitonin (1:50, H-66; Santa Cruz Biotechnology), anti-IAPP (1:100, 0486-4989; Biogenesis, UK), and anti-β-amylloid (20 μg/ml, AB5408; Chemicon) were administered into culture medium of TT cells for 3 days. Normal rabbit IgG was used in the control group. After treatments, attached cells were washed two times with PBS and replaced by fresh medium without antibodies. Cell proliferation and Cdk5 kinase activity were analyzed after medium replacement for an additional 2 days. EGTA (0.2 mM) was used as a Ca2+ chelator.

Calpain Activity Assay—TT cells were harvested in calpain lysis buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 0.1% Nonidet P-40), and cell debris was removed by centrifugation as described above (23). Protein (30 μg) from each sample was loaded in quadruplicate to a 96-well plate and the reaction initiated with 130 μM substrate without the addition of exogenous calcium. The substrate for this assay, N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin, is a nonfluorescent peptide that
strongly fluoresces after cleavage by calpain. The increase in fluorescence intensity was recorded at 30 °C for 2 h in a Bio-Tek FL600 microplate fluorometer and normalized to total protein.

**Xenografted Tumor Growth in Nude Mice**—Cultured TT cells (10⁶ cells per mouse) with stable transfection of nonspecific shRNA (modified from pKD-NegCon-v1, 62-002; Upstate) and shRNA-cdk5 (modified from pKD-cdk5-v3, 62-059; Upstate) were subcutaneously injected into the back of 5-week-old BALB/c athymic nude mice (n = 8 for each group, purchased from National Laboratory Animal Center, Taiwan). After 5 weeks, the mice were sacrificed and the tumors were resected. The tumor tissues were subjected to immunoblotting. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taiwan.

**Statistics**—All values are given as the means ± standard error of the mean (S.E.M.). In all cases, Student’s t test was used. A difference between two means was considered statistically significant with p < 0.05.

**RESULTS**

Identification of Functional Cdk5 and p35 Proteins in Human TT Cells—Because there was no evidence to show the expression of Cdk5 and p35 proteins in the thyroid tissue, we used variant loading amounts of total proteins to verify the expression levels of Cdk5 and p35 proteins in TT cells by immunoblotting with specific antibodies for Cdk5 (Upstate) and p35 (Santa Cruz Biotechnology). IMR-32 (human neuroblastoma) and MRC-5 (human embryonal lung fibroblast), respectively, were used as positive and negative control cells. In the results, the protein expression patterns of Cdk5 and p35 in TT cells were identified (Fig. 1A). In addition, in vitro kinase assay was also performed to indicate the presence of active Cdk5-p35 complex (Fig. 1A).

Cdk5 Kinase Is Important for Supporting TT Cell Proliferation—To understand the biological function of Cdk5 kinase in TT cells, two specific inhibitors of Cdk5 (roscovitine (RV) and butyrolactone-I (BL-I)) were utilized (21, 24). The results indicated that TT cell proliferation was significantly diminished by the treatment of inhibitors for 5 days (Fig. 1B). Furthermore, the treatment of commercial siRNA-cdk5 (Upstate) for 7 days after transfection also resulted in the significant decrease of TT cell proliferation paralleled with the reduction of both protein expression and kinase activity of Cdk5 (Fig. 1C). This evidence demonstrates that the biological function of Cdk5 in TT cells might involve supporting tumor cell growth.

p25 Is Not a Major Factor in Cdk5-dependent TT Cell Proliferation—Calpain-dependent p35 cleavage in neuronal cells has been considered to be a major cause in amyloid-dependent hyperactivation of Cdk5 (7, 8). In our previous results, p25 formation could be induced by drug treatment in prostate cancer cells and caused Cdk5-related apoptosis (21). Therefore, the involvement of p25 in Cdk5-dependent TT cell proliferation was then identified. Calpeptin, a specific inhibitor of calpain (12, 21), was treated in cell culture medium, and p25/p35 proteins were detected by immunoblotting with one single specific antibody that was designed to recognize both p35 and p25 on the C-terminal sequence (sc-820; Santa Cruz Biotechnology) (8, 21). Most unexpectedly, the results showed that the basal level of p25 protein in TT cells was very low compared with p35 and insensitive to calpeptin treatment (Fig. 2A). Meanwhile, calpeptin could not significantly affect Cdk5 kinase activity and TT cell proliferation (Fig. 2A and B). These results imply that p25 is probably not a major factor in supporting Cdk5 activation and TT cell proliferation.

Clearance of Extracellular Calcitonin-derived Amyloid Reduces TT Cell Proliferation through Regulating Her2 and Cdk5 Activity—According to our hypothesis, it is possible that the aggregation of extracellular amyloid, which derives from the TT cell-secreted calcitonin, is an important regulator for

**FIGURE 1.** Cdk5 and p35 are important for proliferation of human TT cells. A, lysates of TT cells were immunoblotted by specific antibodies of Cdk5 and p35 with variant loading amounts of total proteins, whereas actin was used as an internal control. The proteins from IMR-32 and MRC-5 were used as the protein-expression controls. In vitro kinase activity analysis of Cdk5 was performed to show Cdk5 kinase activity as described under “Experimental Procedures.” B, cell proliferation, protein expression, and Cdk5 kinase activity of TT cells were respectively measured by MTT assay (n = 4), immunoblotting, and kinase assay after 5-day treatments of Cdk5 inhibitors (RV, 5 μM; BL-I, 10 μM). C, siRNAs-cdk5 (12.5, 25, and 50 pmol/10⁵ cells) were introduced into TT cells by FuGENE transfection. Cell proliferation, protein expression, and Cdk5 kinase activity were measured after introduction of siRNA for 7 days. For the results of cell proliferation, control value was for 100%; * and **, p < 0.05 and p < 0.01 versus control group. Lane N represents the nonspecific siRNA treatment group. Lane C represents the control group. IP, immunoprecipitation.
Cdk5 activation and TT cell proliferation. To eliminate the influence of extracellular amyloid, neutralizing antibodies were used to capture the amyloid around TT cells. The effects on cell proliferation were then measured. The data indicated that antibodies of both calcitonin and islet amyloid polypeptide (IAPP) could decrease TT cell proliferation, but the antibody of neuronal/amyloid did not (Fig. 3A). Paralleling results of affected Cdk5 activity to cell proliferation were also found (Fig. 3B). The p35 cleavage was unchanged just like the results of calpeptin treatment in Fig. 2A (Fig. 3B). Because extracellular amyloid could result in the increase of intracellular calcium in neuronal cells (11, 12), Her2 kinase might be regulated because of calcium-dependent activation of calmodulin (26). Accordingly, we found that elimination of extracellular calmodulin could diminish Her2 activity determined by specific phosphorylation (Fig. 3C). In addition, the interaction between Her2 and Cdk5 protein was first identified (Fig. 3D). We also found that phosphorylation and activity of Cdk5 were altered with Her2 activity (caused by protein overexpression or knockdown). Administration of calcitonin antibody could effectively reduce Her2 and Cdk5 activity (Fig. 3E, left panel). Importantly, TT cell proliferation was found to correlate with Her2 and
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Cdk5 activity (Fig. 3E, right panel). Taken together, these results indicate that the extracellular calcitonin-derived amyloid might play an important role in regulating TT cell proliferation through manipulating Her2 and Cdk5 activity.

**STAT3 Pathway Involves Cdk5-p35-dependent Proliferative Signals**—The relationship between Cdk5 and STAT3 has been reported in neurons (27). Therefore, the involvement of STAT3 in Cdk5-dependent TT cell proliferation was investigated. Overexpression of p35 was used as a stimulator for Cdk5 (Fig. 4A). We identified the interaction between Cdk5 and STAT3 in TT cells (Fig. 4B) as well as the Cdk5-dependent phosphorylation of STAT3 on Ser-727 (Fig. 4C). The downstream protein expressions, c-Fos and JunB, reported by Fu et al. (27), were also regulated by Cdk5 activity in TT cells (Fig. 4C). TT cell proliferation corresponded to the affected Cdk5 activity (Fig. 4D). However, Tyr-705 phosphorylation of STAT3 did not correlate to Cdk5 activity (Fig. 4C). Additionally, mutant STAT3 (serine 727 to alanine) could dominant-negatively decrease TT cell proliferation not only in the basal level of p35 protein but also in overexpression of p35 (Fig. 4E). The results in Fig. 4F indicate the protein expression of treatments in Fig. 4E. These results suggest that STAT3 pathway was involved in Cdk5-supported TT cell proliferation.

**Cdk5-STAT3 Pathway Is Also Important to Proliferation of Mouse MTC Cell Line**—To verify our findings in human MTC cell line, another MTC cell line (mouse MTC-M) was used. The results indicated that Cdk5 knockdown decreased MTC-M cell proliferation (Fig. 5A). In addition, there was no apparent p25 formation with or without calpeptin treatment in MTC-M cells, and cell proliferation was also unaffected (Fig. 5B). Overexpression of wild-type STAT3 was able to increase proliferation of MTC-M cells, whereas mutant STAT3 (S727A) decreased it (Fig. 5C). Taken together with previous results, we strongly suggest the role of Cdk5-STAT3 for regulating MTC cell proliferation.

**Cdk5 Kinase Activity Affects Its Intracellular Distribution in TT Cells**—Several lines of evidence have demonstrated that Cdk5 primarily localizes in the cytoplasm and regulates neuronal cytoarchitecture (28). In the neurodegenerative model, deregulated Cdk5 possibly shuttles into neuronal nuclei and causes downstream pathological events with transcription factors (29). Based on our present results, the activation of STAT3, which is a transcription factor for transducing the signals into nuclei, involves Cdk5-conducted proliferative signals in TT cells (Fig. 4). Therefore, it is of interest to understand the subcellular distribution of functional Cdk5 and STAT3 in TT cells. The live localizations of exogenous Cdk5-EGFP fusion protein in TT cells were monitored before and after treatments with Cdk5 inhibitors (RV and BL-I) and anti-calcitonin antibody. The cdk5-egfp-containing plasmids were introduced into TT cells by transient transfection (FuGENE, Roche Applied Science). We found the steady-state distribution of Cdk5 fusion protein was located within whole cells (Fig. 6A). After treatments with Cdk5 inhibitors and anti-calcitonin antibody for 5 days, Cdk5 fusion protein significantly shuttled from the nucleus of TT cells to the cytosol as compared with the control group (Fig. 6A, upper panels). After treatments with Cdk5 inhibitors and anti-calcitonin antibody for 5 days, Cdk5 fusion protein significantly shuttled from the nucleus of TT cells to the cytosol as compared with the control group (Fig. 6A, lower panels). In addition to the images of fusion protein, we performed the protein fractionation (nuclei/cytosol) to validate above the findings. The results indicated that the levels of Cdk5, p35, and phospho-STAT3 all dropped in the nuclear fraction after treatments with RV (for 5 days) and siRNA-cdk5 (for 7 days) (Fig. 6B). Actin and histone H1 proteins were indicators for cytosolic and nuclear fraction, respectively.

**Cdk5 Inhibition Reduces Tumor Formation in Vivo**—To identify how importantly Cdk5 plays in the tumor formation of medullary thyroid carcinoma, TT cells (10^6 cells per mouse) containing nonspecific shRNA (mock group) or shRNA-cdk5 were subcutaneously injected into the back of nude mice (n = 8 for each group). After 5 weeks, tumor formation of the shRNA-cdk5 group was significantly smaller than the
mock group, suggesting that Cdk5 inhibition did affect tumor formation in vivo (Fig. 7A). Fig. 7B demonstrates the expression levels of Cdk5 in both cell lines and tissue lysates of three individual tumors. The reduction of phospho-STAT3 in tumor tissues was observed (Fig. 7B). Taken together with the results in Fig. 4, we provide the evidence that the STAT3 pathway is a possible mechanism that Cdk5-p35 utilizes to pass the proliferative signals into TT cell nuclei and to regulate tumor formation (Fig. 7C).

**DISCUSSION**

MTC is a type of thyroid cancer that can be sporadic or associated with MEN2. In addition to the direct measurement of serum calcitonin levels, several studies have been published concerning the preoperative diagnosis of MTC using PCR-based methods to offset the nonconclusive cytological examination or when cytological information is not in agreement with clinical data (30–32). Our results first indicate that Cdk5 activity is important to MTC cell proliferation through the STAT3 pathway (Fig. 7C). Thus, the revealing function of Cdk5 in MTC makes Cdk5 itself a potential indication and therapeutic target of thyroid cancer.

Previously, researchers believed that Cdk5 played a unique role in the central nervous system. However, concepts of the biological functions of Cdk5 have diversified in recent years (28). Additionally, some reports have indicated that amyloid aggregation is present in either Cdk5-related neurodegenerative cells or surrounding tissues of MTC cells (3). Correlation of the structural formations of amyloid aggregation between neurodegenerative tissues and thyroid carcinoma tissues has also been suggested (33, 34). Therefore, it is of interest to explore whether Cdk5 would be involved in MTC cell proliferation and whether this event is amyloid-dependent. The TT cell line, established from a specimen obtained by needle biopsy from a...
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77-year-old female with MTC, is a popular tool for investigating MTC (35, 36) and was utilized throughout our experiments. In the first instance, both Cdk5 and p35 protein expressions and kinase activity were identified by us in TT cells, although the levels were lower than those in human neuroblastoma (Fig. 1A). To verify the importance of Cdk5 activity in TT cell proliferation, two commonly used inhibitors (Fig. 1B, RV and BL-1) (21) and siRNA-cdk5 (Fig. 1C) were applied. As the data show, not only Cdk5 inhibitors but also siRNA-induced inhibition of Cdk5 protein expression could effectively alter Cdk5 activity and TT cell proliferation. A similar result was also shown in mouse MTC-M cells (Fig. 5A). This reveals that MTC cell proliferation is sensitive to Cdk5 activity change. Moreover, minute compensatory increases of p35 protein expression were observed after inhibition of Cdk5 activity by 5-day treatment of either Cdk5 inhibitors or siRNA (Fig. 1B, lanes 3 and 5, and Fig. 1C, lanes 3 and 4). To rule out the possibility of TT cell death caused by Cdk5 inhibition, cell survival analyses were performed, and no significant cell death was observed after treatment with Cdk5 inhibitor (data not shown). The above findings illustrate that the Cdk5 kinase, a new player in cancer biology, is important for supporting TT cell proliferation.

Although the role of p25 in the initiation of Alzheimer disease is still controversial (37, 38), the involvement of p25 formation in TT cell proliferation was investigated. p25 is suggested as a more stable and potent activator for Cdk5 in response to a rise of intracellular calcium (10, 12), by which p25 possibly supports the basal level of TT cell proliferation. Most unexpectedly, a much lower production of p25 was observed in both human TT and mouse MTC-M cells, and no influences on cell proliferation were made by the treatment of the calpain inhibitor calpeptin (Fig. 2 and Fig. 5B). We further conducted a calpain activity assay to evaluate the efficiency of calpeptin. The data indicated that calpeptin treatment in TT cells effectively inhibited ionomycin-induced calpain activation after a 6-h treatment (the effect of ionomycin was quick and could lead to cell apoptosis in long term treatment); however, the basal level of calpain activity was too low to be affected by a 5-day treatment of calpeptin (EGTA as a negative control in this assay; see supplemental Fig. S1). To figure out the role of p25 in TT cells, the application of siRNA-p35 here is not a good idea because it could knockdown both p35 and p25 protein expressions at the same time, and TT cell proliferation would certainly be affected. Alternatively, we performed p25 overexpression in TT cells, and cell proliferation was then evaluated. The results reveal that p25 overexpression did not elevate TT cell proliferation although Cdk5 was activated (supplemental Fig. S2). Although p25 overexpression resulted in overactivation (deregulation) of Cdk5 compared with p35 overexpression, it seems that p25 overexpression was incapable of increasing TT cell proliferation. Our speculation is that Cdk5 was deregulated by p25 and played a different role in TT cells. Therefore, it is possible that the maintenance of TT cell proliferation by Cdk5 may be through a p25-independent pathway.

According to the above results, Cdk5 is somehow active to manage TT cell proliferation through a p25-independent pathway. Whether extracellular amyloid aggregation is a trigger to activate Cdk5 in TT cells was then tested. Administration of neutralizing antibodies against amyloid in the cultures was used to eliminate the amyloid influence. Anti-IAPP has been reported to specifically recognize the amyloid aggregation around TT cells (25). Thyroid T cell-secreted calcitonin has been demonstrated to be the sole constituent of extracellular amyloid in MTC tissues (34). Therefore, both anti-calcitonin and anti-IAPP were applied to eliminate the precursors of amyloid and aggregated amyloid, respectively. The results demonstrate that elimination of extracellular amyloid did decrease TT cell proliferation along with the parallel drops in Cdk5 kinase activity, but there was no influence on p25 formation (Fig. 3, A

FIGURE 7. Cdk5 inhibition retards tumor formation in vivo. A, TT cells stably transfected with nonspecific shRNA (mock group) or shRNA-cdk5 were subcutaneously injected into nude mice (n = 8 for each group). After 5 weeks, the weights of tumors were measured. *, p < 0.05 versus mock group. B, levels of Cdk5 protein and phospho-Ser-727-STAT3 in lysates of TT cells and three individual tumors were measured by immunoblotting. Actin was an internal control for protein expression. N and S represented the groups of nonspecific shRNA and shRNA-cdk5, respectively. C, schematic representation demonstrates that the novel mechanism of amyloid-dependent Her2/Cdk5 activation supports the proliferation of medullary thyroid carcinoma cells which is, at least, through the STAT3 pathway.
and B). Treatment of anti-β-amyloid could not affect TT cell proliferation, suggesting the unique feature of the calcitonin-derived amyloid around TT cells. In addition, because p35 is required for Cdk5 activity to support TT cell proliferation, the effects of neutralizing antibodies at low expressing levels of p35 are of investigative interest. The data indicated that partial knockdown of p35 expression by siRNA reduced TT cell proliferation (supplemental Fig. S3). Treatments of amyloid-neutralizing antibodies with siRNA-p35 led to a similar inhibitory pattern of TT cell proliferation to the existence of endogenous p35. These results reveal that amyloid elimination could decrease TT cell proliferation through Cdk5 inhibition regardless of p35 protein expression levels. Furthermore, the results in Fig. 3B demonstrated that treatments of amyloid-neutralizing antibodies did not affect p35 expression and p25 formation; p25 overexpression did not increase TT cell proliferation (supplemental Fig. S2). Taken together, we suggest that amyloid affects TT cell proliferation through a p25-independent pathway. Although p25 did not play a role in linking amyloid to Cdk5, amyloid-dependent activation of Her2 was first reported to phosphorylate/activate Cdk5 and to support TT cell proliferation (Fig. 3, C–E) (26, 39).

After understanding the motive power of Cdk5-dependent proliferation of TT cells, the next question is as follows. How does Cdk5 work on cell proliferation? Coincidentally, STAT3, which correlates to cell survival, also functions as a co-worker with Cdk5 to pass signals into the nucleus (27, 40). In this process, Cdk5-dependent activation of STAT3 in both mice neurons and muscle cells takes place through the specific STAT3 phosphorylation on Ser-727 (27). In addition, the role of STAT3 has also been verified in familial MTC (41). Therefore, the involvement of the STAT3 pathway in Cdk5-related TT cell proliferation was investigated. Interestingly, not only the phospho-Ser-727-STAT3 but also its downstream gene expressions (c-FOS and JUNB (27)) were regulated by Cdk5 activity, and the expression patterns paralleled the proliferation of TT cells. Importantly, mutant STAT3 (S727A) could effectively prevent p35-induced human TT and mouse MTC-M cell proliferation (Fig. 4E and Fig. 5C), which suggests the role of STAT3 in Cdk5-dependent tumor cell growth. Although c-FOS and JUNB are also targets for other pathways, we found that, at the very least, Ras-related mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway did not intervene in the signal transduction of Cdk5-STAT3-c-fos (supplemental Fig. S4). Additionally, although phospho-Tyr-705-STAT3 was considered as an important indication for STAT3 activation (27), we did not find a correlation between Cdk5 activity and the regulation of phospho-Tyr-705-STAT3 in our experiments (Fig. 4C). Again, this is the first report to indicate that Cdk5 transduces the signals into the nuclei of cancer cells by activating a transcription factor, STAT3, in order to maintain cancer cell proliferation.

Furthermore, Cdk5 has been thought to shuttle into the nucleus and phosphorylate MEF2, thereby affecting neuronal fate (29). According to our data, STAT3 is a possible transducer for Cdk5 to regulate TT cell proliferation. Thus, it is important to show the subcellular distribution of Cdk5 in TT cells by monitoring the images of Cdk5 protein after manipulating Cdk5 activity. Interestingly, we found that there is an interaction between Cdk5 activity and its nuclear localization in TT cells (Fig. 6). This is the first report indicating that Cdk5 activity could affect its own subcellular localization in cancer cells. Moreover, the decreasing distribution of phospho-Ser-727-STAT3 in the nuclear fraction of TT cells caused by Cdk5 inhibition corresponded with the nuclear distribution of Cdk5-p35. This observation suggests that inactive Cdk5 is disabled to maintain the nuclear distribution of Cdk5-p35 and phospho-STAT3 and thus retards the proliferation of TT cells. Taken together, the activation of STAT3 is at least one medium for Cdk5 to manage TT cell proliferation.

Finally, to elucidate the role of Cdk5 in cancer biology of medullary thyroid carcinoma, we conducted the xenografts of TT cells in nude mice. Corresponding to the in vitro results, Cdk5 inhibition retarded tumor formation in which the phosphorylation of STAT3 on Ser-727 was reduced (Fig. 7, A and B). In conclusion, our results are the first demonstration of the biological function of Cdk5 in cancer cells, in which extracellular amyloid triggers Her2/Cdk5 activation and supports p25-independent MTC cell proliferation, at a minimum, through the activation of the STAT3 pathway (Fig. 7C). We also demonstrate the correlation between functional Cdk5 kinase and its subcellular distribution in MTC cells. Thus it is possible that Cdk5-p35 complex will become a new target for diagnosing and treating thyroid cancer in the near future.

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