A new animal model for the identification of novel signaling mechanisms mediating medullary thyroid carcinoma

Karine Pozo[1], Emely Castro-Rivera[2]; Chunfeng Tan[2]; Florian Plattner[2]; Roswitha Pfragner[3]; Herb Chen[4]; Fiemu Nwariaku[1] and James A. Bibb[2]

1. Department of Surgery, 2. Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX 75390, USA; 3. Center for Molecular Medecine, University of Graz, Austria; 4. Endocrine Surgery Research Laboratory, University of Wisconsin Carbone Cancer Center, Madison, WI 53792, USA

1. Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumor representing over 14% of thyroid cancer-related deaths. MTC originates from calcitonin-producing parafollicular cells (or C-cells) of the thyroid, and it metastasizes frequently to regional lymph nodes, lung, liver and brain. MTC has an indolent course and late detection is associated with a high rate of mortality. Total thyroidectomy is the only effective treatment for this disease. Some MTC (25%) are hereditary as component of multiple endocrine neoplasia-2 (MEN-2) and arise from well characterized mutations in the proto-oncogene RET, which encodes for a receptor tyrosine kinase. The majority of MTC cases are sporadic and the underlying causes are not known. Therefore understanding the molecular pathways mediating MTC is crucial for the development of novel therapies.

Cyclin-dependent kinase 5 (cdk5) is a proline-directed serine/threonine kinase that is activated by binding of its cofactor, p35. Cdk5 becomes dysregulated when calpain cleaves the first 98 amino acids of p35 to produce p25. The p25/cdk5 complex translocates to the cytoplasm and nucleus where it phosphorylates aberrant substrates. Although cdk5 is expressed ubiquitously, its physiological function has been predominantly studied in the central nervous system (CNS). Cdk5 is essential to the CNS development and to brain function. Growing evidence suggests that cdk5 plays a key role beyond the nervous system. For example, cdk5 is involved in modulating insulin secretion in pancreatic b cells and in regulating cell cycle and oncogenesis.

Here we present a new mouse model in which conditional expression of p25 in thyroid C-cells leads to tumorigenesis and lethal MTC. Furthermore, we use this animal model to identify the signaling pathways by which Cdk5-p25 mediates MTC .

2. Cdk5 and p35/p25 are expressed in human thyroid and MTC samples

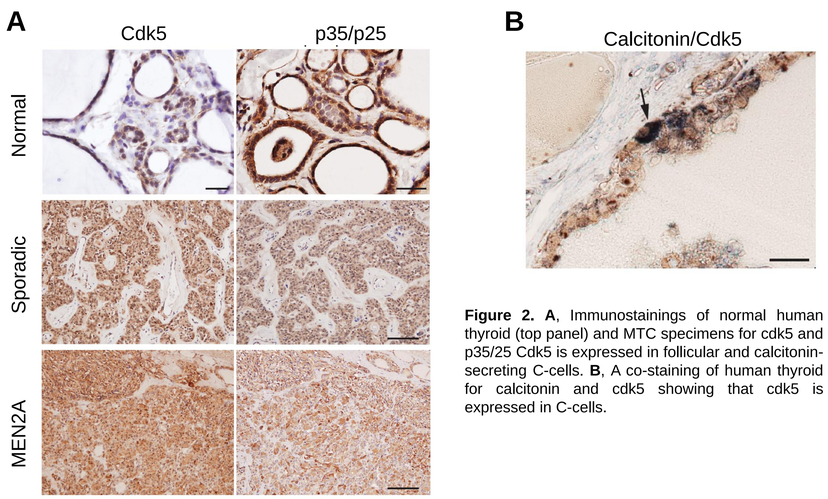

Figure 2. A, Immunostainings of normal human thyroid (top panel) and MTC specimens for cdk5 and p35/25 Cdk5 is expressed in follicular and calcitonin-secreting C-cells. **B**, A co-staining of human thyroid for calcitonin and cdk5 showing that cdk5 is expressed in C-cells.

3. Cdk5 activity regulates growth and proliferation of cell lines derived from human

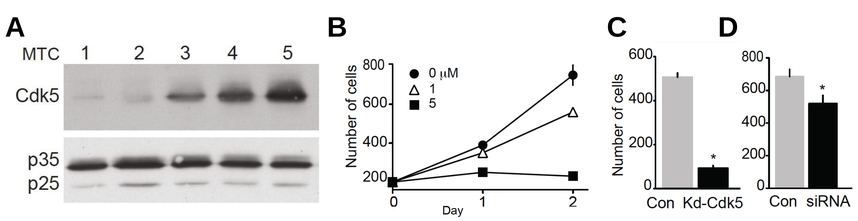

Figure 3. A, Immunoblots showing that Cdk5 and p35/p25 are expressed in cell lines derived from human MTC. **B**, Pharmacological inhibition of Cdk5 blocks cell growth and proliferation. Interfering with Cdk5 activity by expressing **C**, a dominant-negative, kinase-dead Cdk5 construct or **D**, by knocking-down Cdk5 slows human MTC cell growth.

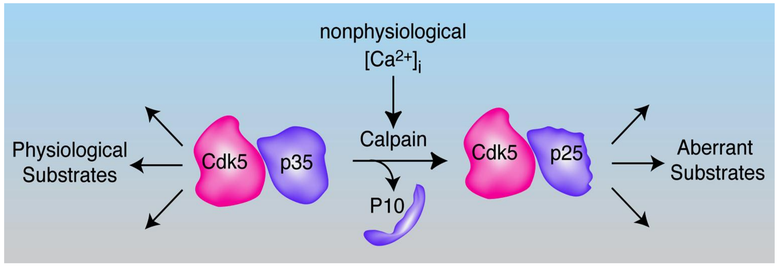

Figure 1. Schematic of Cdk5 in its physiological and aberrant forms.

4. An inducible, bitransgenic animal model for MTC

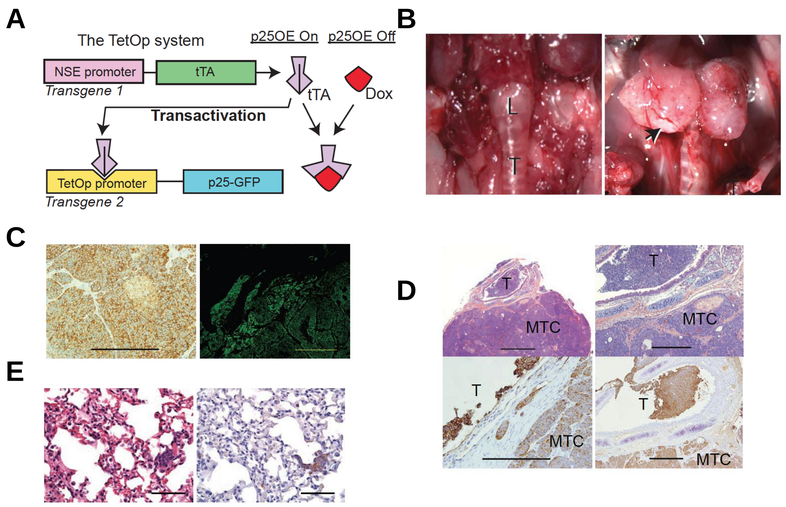

Figure 4. A, Schematic of the bitransgenic system showing activation of TetOp promoter-driven p25-GFP expression by doxycycline (Dox)-controlled Tta. Transgenic tTA expression is driven by the NSE promoter. **B,** p25 overexpression causes thyroid tumor growth. **C**, positive immunostaining with calcitonin classifying the p25OE tumorsas MTC (left), GFP immunofluorescent staining of p25OE mice thyroid tumor (right). **D,** vascular invasion in thracheal smooth muscle by neoplastic C-cells. **E**, metastatic C-cells within alveolar walls of the lung (m), positive calcitonin immunostain of a section contiguous to the section in m (n).

5. An inducible and arrestable animal model for MTC

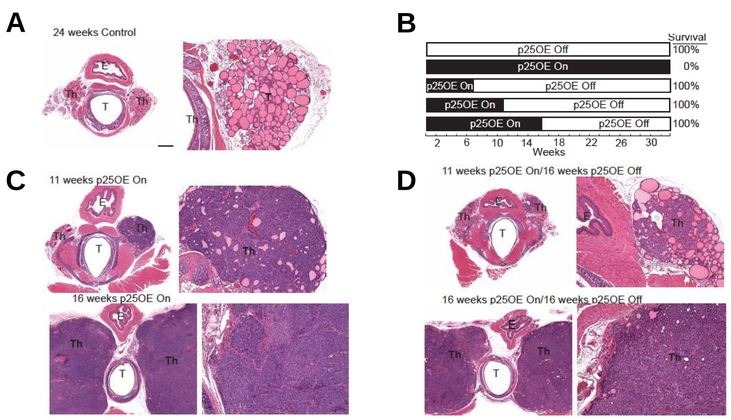

Figure 5. Progression and arrest of medullary thyroid cancer tumors in p25OE mice. Hematoxylin and eosin staining of thyroid in **A**, control, **C**, p25OE mice that have been off doxycycline (p25On) as indicated, **D,** p25OE mice that have been off doxycycline as indicated, then reintroduced to doxycycline for 16 more weeks (p25 off). Note that tumor growth is arrested when p25 is off. B, no mice survive below 32 weeks off doxycycline (p25On). Arrest of p25 overexpression results in mouse survival.

6. p25-mediated Cdk5 activation in p25OE mice causes MTC tumor growth

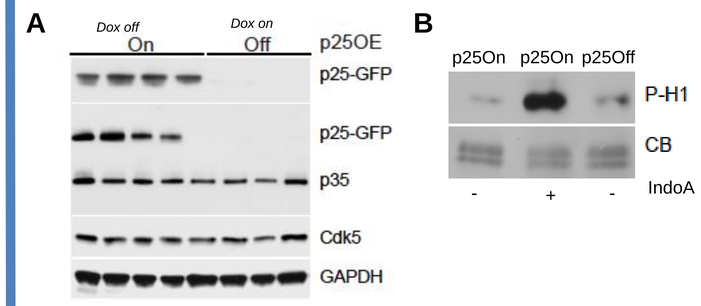

Figure 6. A, p25 overexpression is controlled by addition or removal of doxycyclin and B, is accompagnied by increased Cdk5 activity. **A**, Immunoblots of lysates from p25OE mice deprived of doxycyclin for 16 weeks (p25On) or deprived of doxycyclin for 16 weeks and re-introduced to doxycyclin for 4-6 weeks (p25off). **B,** Cdk5 was immunoprecipitated from tumors from mice 'off doxycycline' or 'off doxycycline/re-introduced to doxycycline' and used to phosphorylate *in vitro* histone H1. The immunoblot shows that histone H1 is phosphorylated by Cdk5 immunopurified from tumors overexpressing p25 but not by Cdk5 isolated from tumors in which p25expression is off. Cdk5 activity is inhibited by the inhibitor IndoA (25 mM)

7. Retinoblastoma protein is inactive in MTC

Figure 7. Retinoblastoma protein (Rb) phosphorylation is correlated with p25 overexpression and Cdk5 activity. A, Immunoblots from lysates from mouse tumors overexpressing p25 or not as in Figure 6. **B,** Immunoblots from lysates of human MTC-SK cellstransfected with dominant negative Cdk5 (Dn) or empty vector (Mock)

8. Rb is a downstream target of Cdk5 in MTC

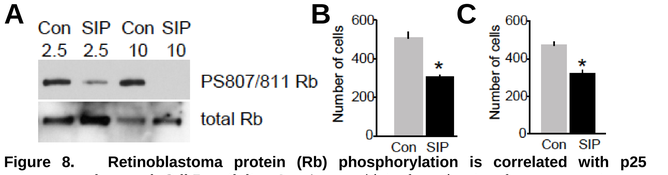

Figure 8. Retinoblastoma protein (Rb) phosphorylation is correlated with p25 overexpression and Cdk5 activity. A, Immunoblots from lysates from mouse tumors overexpressing p25 or not as in Figure 6. **B,** Immunoblots from lysates of human MTC-SK cellstransfected with dominant negative Cdk5 (Dn) or empty vector (Mock)

9. Conclusions

- Cdk5 is present in normal thyroid and in neoplastic MTC tumors.
- Inhibition of Cdk5 activity blocks proliferation of human MTC cell lines.
- NSE promoter-driven p25 overexpression and associated Cdk5 activity results in rapid MTC tumorigenesis in mice.
- Retinoblastoma protein is highly phosphorylated following p25 overexpression. Interfering with Cdk5 activity results in decreased phosphorylation of retinoblastoma protein.
- We have developped a novel and clinically relevant animal model of MTC to study the mechanisms of tumorigenesis and test new treatment strategies.

10. Acknowledgments

We thank Gabriel Mettlach for expert technical assistance, Dr. Stanley Sidhu for providing thyroid and MTC specimens. The NSE/tTA mice and TetOp/p25-GFP were provided by Drs Eric Nestler an Liu Tsai, respectively. This research was made possible by the American Cancer Society (JAB), the NIH (JAB, FN), and a North American NeuroEndocrine Tumor Society Early Career Development Award to KP.