Multistep tumorigenesis of medullary thyroid carcinoma in man and transgenic mouse models

Meerstaps tumorgenese van medullair schildkliercarcinoom in de mens en transgene muis modellen

(met een samenvatting in het Nederlands)

Proefschrift

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Dennis Stanley Acton

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Promotor: Prof. Dr. C. J. M. Lips[1]

Copromotor: Dr. J. W. M. Höppener[2]

[1]Department of Clinical Endocrinology,
Division of Internal Medicine and Dermatology

[2]Department of Metabolic and Endocrine Diseases,
Division of Medical Genetics

University Medical Center Utrecht

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voor

Annet, Igor en Noëlle

mijn Ouders
Leendert Marcellus Acton (1935-1990)
Eugenia Agnes Acton-Markus

mijn Voorouders

Chapter 1

Introduction

Dennis S. Acton
Department of Clinical Endocrinology, Division of Internal Medicine, University Medical Center Utrecht

1 Multiple Endocrine Neoplasia type 2 and medullary thyroid carcinoma

1,1 The MEN Syndromes

The appearance of tumors in various endocrine organs in the same individual is referred to as Multiple Endocrine Neoplasia Syndrome (MEN syndrome). MEN syndromes are dominantly inherited autosomal traits (Steiner *et al*., 1968), meaning that on the average 50% of the offspring of disease gene carriers will be affected. Two main types can be distinguished: MEN type 1 is characterized by the combined occurrence of tumors in the pituitary gland and pancreatic islets, and parathyroid adenomas. MEN type 2 is characterized by the combination of medullary thyroid carcinoma (MTC), a malignant tumor that originates from the calcitonin (CT) producing and secreting C-cells in the thyroid gland (Horn, 1951) and pheochromocytomas, tumors originating from the chromaffin cells of the adrenal medulla.

The MEN 2 syndrome was first described by Sipple (1961), and is also referred to as Sipple's syndrome. MEN 2 can be subdivided in different subtypes: MEN 2A in which MTC and pheochromacytoma occur in combination with parathyroid adenomas and MEN 2B which is characterized by MTC and pheochromacytoma combined with mucosal ganglioneuromas (colon, lips and tongue) (Williams and Pollack, 1966; Schimke *et al*., 1966; Gorlin *et al*., 1968; Chong *et al*., 1975). A third type of MEN 2 concerns families in which MTC is the only clinical manifestation and which is referred to as familial MTC (FMTC).

The MEN 2B syndrome is a rare form of MEN (Williams and Pollack, 1966). Most of the patients with the syndrome are *de novo* mutants (neither of the parents is affected), but these patients transmit the trait in an autosomal dominant manner. In addition to the tumors that are also seen in the MEN 2A syndrome, MEN 2B patients are characterized by a typical appearance, which is an aid for early diagnosis. Numerous skeletal abnormalities have also been described (Khairi *et al*, 1975). Patients will grow abnormally tall; have a low body weight and poor development of body musculature (Carney *et al*., 1976). The face, with blunt features and bumpy lips, is even more specific. This appearance of MEN 2B patients is referred to as marfanoid habitus because it resembles the appearance of patients with Marfan disease. Often the earliest sign in childhood of the MEN 2B syndrome are small submucosal ganglioneuromas covering the tongue and severe obstipation.

1.2 Histopathological aspects of MTC

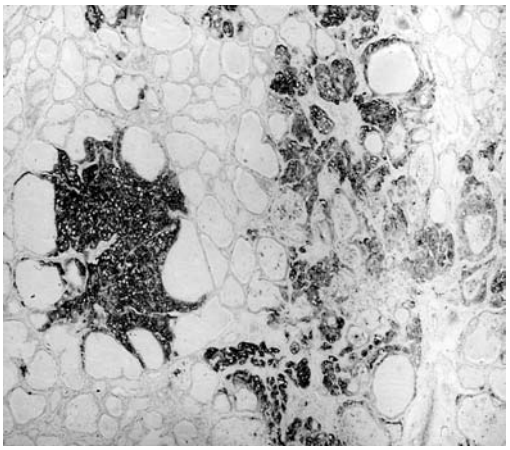
MTC comprises approximately 5% of all thyroid neoplasms and is found as a sporadic and solitary tumor in 75-80% of

the cases (Kebebew *et al*., 2000). In addition, MTC is the most important clinical manifestation of the MEN 2 syndromes (Sipple 1961). MTC originates from the thyroid gland C-cells which are easily recognized in thyroid gland tissue sections by immunohistochemical detection of CT. In the normal human thyroid gland, C-cells are dispersed within follicles, located between the basement membrane and the epithelium of follicles, either as single cells or in small groups. C-cells are not evenly distributed throughout the gland, but are mainly concentrated in the upper two thirds along the central axis of the lateral lobes. In adults, about one in thousand cells of the thyroid gland is a C-cell (Wolfe *et al*., 1981).

1.3 Diagnosis of MTC

In familial MTC, tumors develop bilaterally and multicentrically on a background of pre-existing C-cell hyperplasia (CCH), which may be the first manifestation of the inherited disorder. Diagnosis of CCH is bases on microscopic criteria: the presence of increased numbers of diffusely scattered C-cells (> 7 per follicle). Initiation of monoclonal C-cell proliferation (clusters of 20 or more C-cells per visual field at a magnification of x200) can be considered as a second event resulting in the development of adenomas and micro-carcinomas (photo 1A and B, Lips *et al*., 1994). As the proliferation progresses, the C-cells completely encircle, compress, and displace the follicular epithelium, ultimately producing solid intrafollicular aggregates of C-cells forming nodular hyperplasia. During the next phase of MTC-development, C-cells break through the basement membrane and invade the interstitium. From this stage on the tumor is referred to as carcinoma (photo 1A).

A



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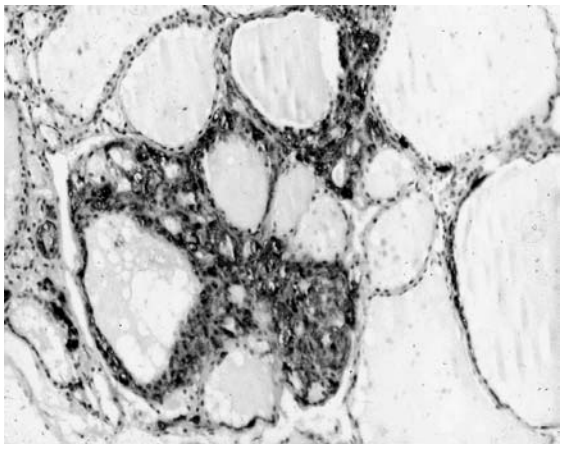

Photo 1

A: Immunohistochemistry for CT demonstrating microscopic carcinoma and areas of CCH (dark staining cells) in the thyroid gland from a MEN 2A patient of 5 years of age at operation.

B: Larger magnification of CCH area (more than 7 C-cells per follicle).

At a later stage, the carcinomas reveal solid areas of large rounded epithelial cells, divided by fibrous septa. Tumor cells show nuclear pleiomorphism and frequent mitosis indicative for malignancy. Amyloid deposits with necrotic cell remnants and cyst formation and capsularisation of the tumor are observed.

Sporadic MTC patients usually present at the clinician with a palpable neck mass, and approximately 15-30% of the patients have symptoms of respiratory complaints such as hoarseness, dysphasia, weight loss and diarrhea. MTC diagnosis is made after fine needle aspiration of tumor cells and positive cytology as indicated by CT immunohistochemistry and measurements of plasma CT levels. For members of MEN 2 families, periodic screening is performed to detect early stages of MTC development. Since C-cell proliferation almost always preludes MTC in MEN 2 patients, screening of family members with a C-cell stimulation test (pentagastrin or calcium mediated) is often used for identifying supra-normal CT secretion at an early stage of MTC development. The results of the calcium and/or pentagastrin stimulation tests are important for the decision whether to perform surgical treatment or not. When after stimulation, the serum concentration of CT increases by more than three times the basal level, surgery is indicated. However, prophylactic surgery revealed the presence of microscopic MTC in MEN 2 gene carriers already at a young age (Arts *et al*., 1999). Disease gene carriers may be divided into three risk groups, depending on the nature of the genetic predisposition for MTC. The highest risk concerns children with a predisposition for MEN 2B. The prognosis of MEN 2B is poor, primarily due to the progression of MTC. Especially in the MEN 2B syndrome, these tumors tend to develop at an early age and spread to other organs (Jackson *et al*., 1992; Sizemore *et al*, 1992). For these children treatment during the first year of life can be considered because early metastases of MTC have been reported, but other surgeons prefer to delay operation until the child is older (up to 5 years). The second highest risk group includes patients with predisposition for MEN 2A. These patients are treated before the age of 6 years. In patients with predisposition for FMTC treatment may be postponed until an abnormal response in the CT secretion test is observed as tumors in these patients generally develop at later age, grow more slowly and behave less aggressively (Machens *et al*., 2003).

1.4 Treatment modalities for primary MTC

Surgery is the only treatment of choice for primary MTC (Cohen and Moley, 2003). Currently a total thyroidectomy with central neck exploration of the lymph nodes is performed. However, many patients have been treated with partial thyroidectomy in the past.

Basal plasma CT level can give a prediction for the presence or absence of local or distant metastases. Pre-operative CT measurement proved to have significant predictiveness as was reported in a study showing that from the patients with pre-operative plasma CT<50ng/L 98% had post-operative CT normalization, indicating the absence of distant metastases, whereas 59% of the patients with plasma CT>50ng/L had post-operative persistent disease (Cohen *et al*., 2000).

Cure rates after primary surgery correlates with the stage of tumor development at diagnosis. The staging is based upon the size of the tumor, lymph node involvement and involvement of distant organs (table1, DeGroot, 1975).

Table 1. Staging of medullary thyroid carcinoma

Stage	Features
I	MTC diameter < 1 cm and tumor restricted to thyroid gland
II	MTC diameter > 1cm and locally invasive
III	MTC with metastases in regional lymph nodes
IV	MTC with metastases at distance to liver, lungs, and/or bones

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At this moment, the cure rate of primary operation is almost 90% if the tumor is restricted to the thyroid tissues (stage I/II). However, if the tumor has already spread to regional lymph nodes (stage III) cure is observed in less than 35% of the cases. Stage IV patients cannot be cured (Modigliani *et al*., 1998).

1.5 Re-operation of persistent MTC

For patients with persistent disease a post-surgical metastatic work-up is performed to demonstrate if remnant thyroid tissues and regional lymph nodes still contain tumors or to rule out metastases at distance. The most frequently used methods are, ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI), angiography of the liver, 99mTc-pentavalent dimercaptosuccinic acid (DMSA), 123I-meta-iodobenzyl guanidine (MIBG), 111In-penteotride and 6-[18F]-fluorodopamine positron emission tomography (PET). The many different techniques used are a reflection of the fact that none of the mentioned methods has sufficient sensitivity and specificity. If there is no indication for distant metastases, re-operation of the neck region can be considered to remove cancerous remnant thyroid tissues and local lymph nodes with total neck exploration and sternotomy (Gimm and Dralle, 1997). The success rate of such aggressive re-operation strategies is less than 25%. However, if during preoperative examination the tumor appeared to be already spread to regional lymph nodes, cure after re-operation is observed in only 9% of the cases (Fleming *et al*., 1999). Considering the high rate of complication (50% of the cases) reported upon extensive re-operation (Gimm and Dralle, 1997), a restraint strategy is warranted if regional lymph node metastases are detected after a primary operation. Some clinicians prefer an expectative “wait and see” strategy in patients who have negative visualization in the presence of elevated postoperative CT levels as prognosis in these patients can be favorable (van Harden *et al*., 1990, figure 1)

1.6 Metastatic MTC

Distant metastases are the main cause of death in MTC patients. Affected organs include lung, liver, bones and more rarely the brain. Lung metastases are often dispersed diffusely throughout both lungs. Liver metastases may be small and show features of hepatic haemangiomas and bone metastases are generally osteolytic or osteoblastic. The survival at ten years after initial discovery of the metastases is 20%, but patients have been reported to survive up to 20 years or more, even without any systemic treatment (Bergholm *et al*., 1997).

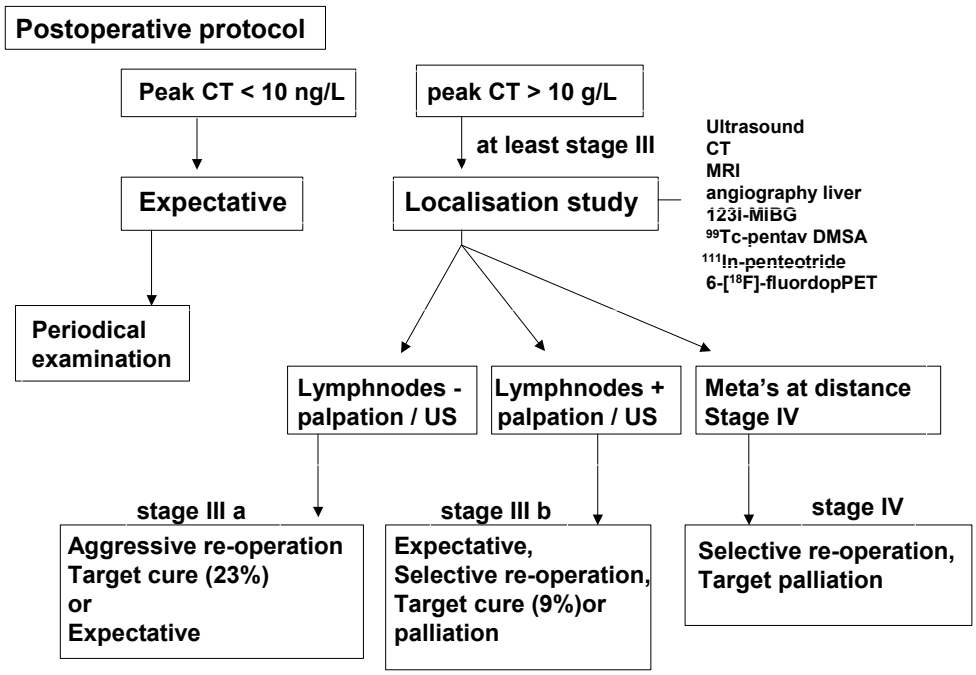

Figure 1. Protocol for post-operative follow-up of MTC patients.

1.7 Current treatment modalities for metastatic MTC

Currently no effective therapy is available for MTC metastases. MTC is not sensitive to regular treatment with cytostatic therapy. No tumor responses have been obtained with etoposide. Symptomatic improvement in some patients was reported for the treatment with various combinations of 5-fluoroacil (5-FU), dacarbazine, streptozocin, cyclo-phosphamide and vincristine, but no benefit was found on survival rates (Schlumberger *et al*., 1991). High-dose doxorubicin treatment only resulted in major toxic effects (Nocera *et al*., 2000).

Contrary to the papillary thyroid tumors that originate from the epithelial cells, radiotherapy with radio-labeled iodine cannot be applied to MTC, because the thyroid C-cells can not internalize iodine and are therefore refractory to this form of therapy. Thus, for MTC therapy other ways to target the tumor cells with radioactivity are necessary. External radiation therapy for MTC is indicated for metastases not amenable to surgery, such as bone, spine or brain metastases. Radioactive iodine covalently linked to MIBG does not seem to offer significant benefit. Treatment modalities with somatostatin analogues labeled with Yttrium90 or other radio-nucleotides in selected patients are currently under evaluation. Radiolabeled monoclonal antibodies (mobs) directed against carcinoembryonic antigen (CEA) have been used to image MTC in humans for more than 15 years. It was not until 1995 that the use of murine I-131 labeled anti-CEA mAbs for MTC therapy was reported (Juweid *et al*., 1996). However, bone marrow toxicity due to a high radiation dose to the bone marrow is one of the major drawbacks of the use of mAbs in nuclear medicine therapy. Furthermore, treatment with bi-specific antibodies directed against CEA and DTPA and with DTPA-labeled 131 Iodine appeared poorly effective in patients with large metastases (Kraeber-Bodere *et al*., 2003).

Figure 2. Ligands and co-receptors of the RET receptor tyrosine kinase

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2 RET receptor tyrosine kinase gene in human cancers

2.1 The RET proteins

The *RET* proto-oncogene (Takahashi *et al*., 1988) encodes a receptor tyrosine kinase, which is a transmembrane receptor endowed with intrinsic ligand-stimulated tyrosine kinase activity. The extracellular portion of RET contains four cadherin-like repeats, a calcium binding site and a cysteine-rich domain (Manie *et al*., 2001). The RET receptor tyrosine kinase functions as the receptor for the GDNF family of neurotrophic factors (Trupp *et al*., 1996) in conjunction with GFR(alpha) co-receptors (Treanor *et al*., 1996, Jing *et al*., 1996). The RET protein can form multicomponent receptor complexes with each of four high affinity glycosyl-phosphatidyl-inositol (GPI)-anchored components (GFR(alpha)1-GFR(alpha)4). These complexes serve as receptor for the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs). There are currently four known GFLs: GDNF, neurturin, artemin and persephin. For each GFL there is a preferred GFR(alpha) receptor, to which the GFL binds with highest affinity and most potently activates the RET tyrosine kinase (Airaksinen and Saarma, 2002, figure 2).

Upon ligand binding, dimerisation of RET molecules occurs which juxtaposes two intracellular catalytic domains. This allows mutual trans-phosphorylation (auto-phosphorylation) of tyrosine residues (figure 3).

Expression of the *Ret* gene involves alternative splicing that results in two major protein isoforms of 1072 and 1114 amino acids (RET9 and RET51), respectively, which differ in the amino acid sequences immediately downstream from glycine residue 1063. A third isoform, a putative RET43 protein, has been reported, but no further analyses have been performed (Myers *et al*., 1995). The intracellular domain of RET contains at least 12 auto-phosphorylation sites. Sites Y1090 and Y1096 are present only in the RET51 isoform. Studies of mice with targeted disruption of only the RET9 or the RET51 isoform demonstrated that the RET9 isoform is crucial for normal development (de Graaff *et al*., 2001). The phosphorylated tyrosine's serve as docking sites for intracellular signaling proteins that carry src-homology 2 domains and phospho-tyrosine binding domains. These proteins, in turn, invoke intracellular signaling cascades that ultimately lead to gene expression modulation and biological

responses (Schlessinger and Lemmon, 2003, figure 4). The RET-binding, intracellular signal transduction proteins include Grb7/10, phospholipase Cγ, c-Src and Grb2, the latter of which is unique to RET51. Formation of protein complexes at tyrosine residue 1062 leads to stimulation of Ras/ERK and PI3K/Akt cascades. Proteins that bind Y1062 include Shc, ShcC, IRS1/2, FRS2, DOK1/4/5 and Enigma (Ichihara *et al*., 2004). The Y1062 residue is not only essential for normal development but it is also a prerequisite for the transforming ability of RET-derived oncogenes (Murakami *et al*., 2002; Ichihara *et al*., 2004).

2.2 Developmental function of RET

RET plays a critical role in the development of the enteric nervous system and in kidney organogenesis (Schuchardt *et al.,* 1994).). Mice lacking functional RET develop to term but die within 24 hours after birth due to the absence of an enteric nervous system, as seen in Hirschsprungs disease, and renal dysgenesis or agenesis. In the kidney rudiments that were observed in some homozygous null mutant mice, no functional regions as cortex and medulla could be recognized, and large regions of undifferentiated mesenchyme were present. Tissue recombination studies with mesenchyme and ureteric buds from wild type and *RET* deficient mice indicated that the renal defect is caused by non-responsiveness of the buds to signals from the nephrogenic mesenchyme (Schuchardt *et al.,* 1996).

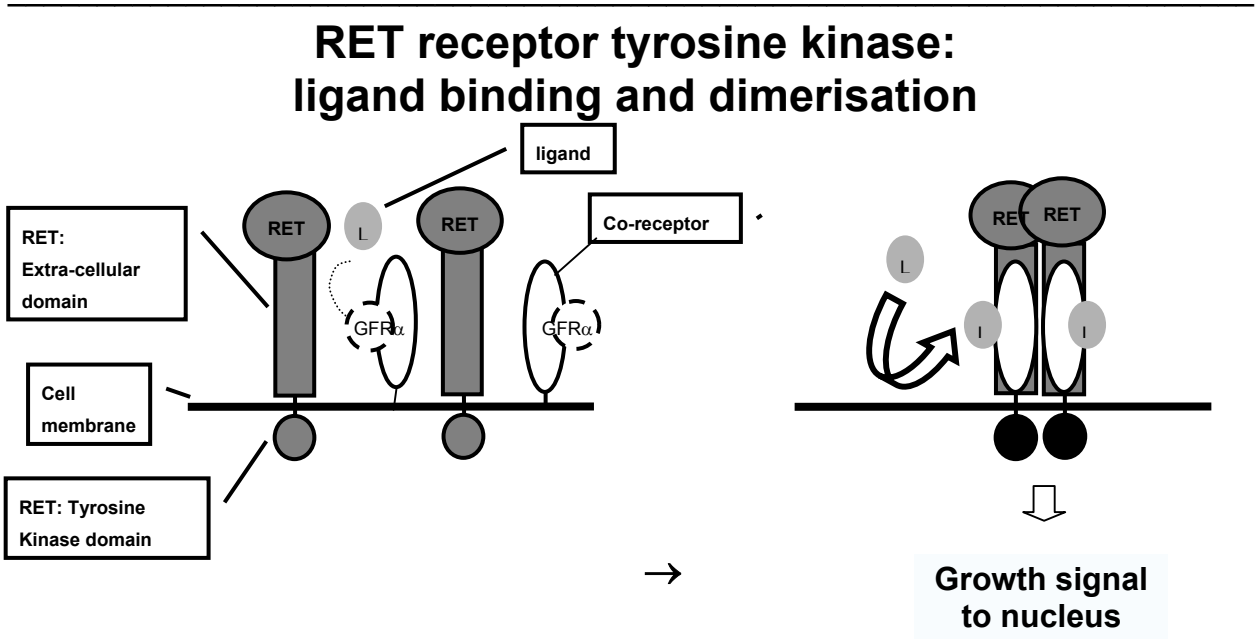

Figure 3. Receptor stimulation of RET.

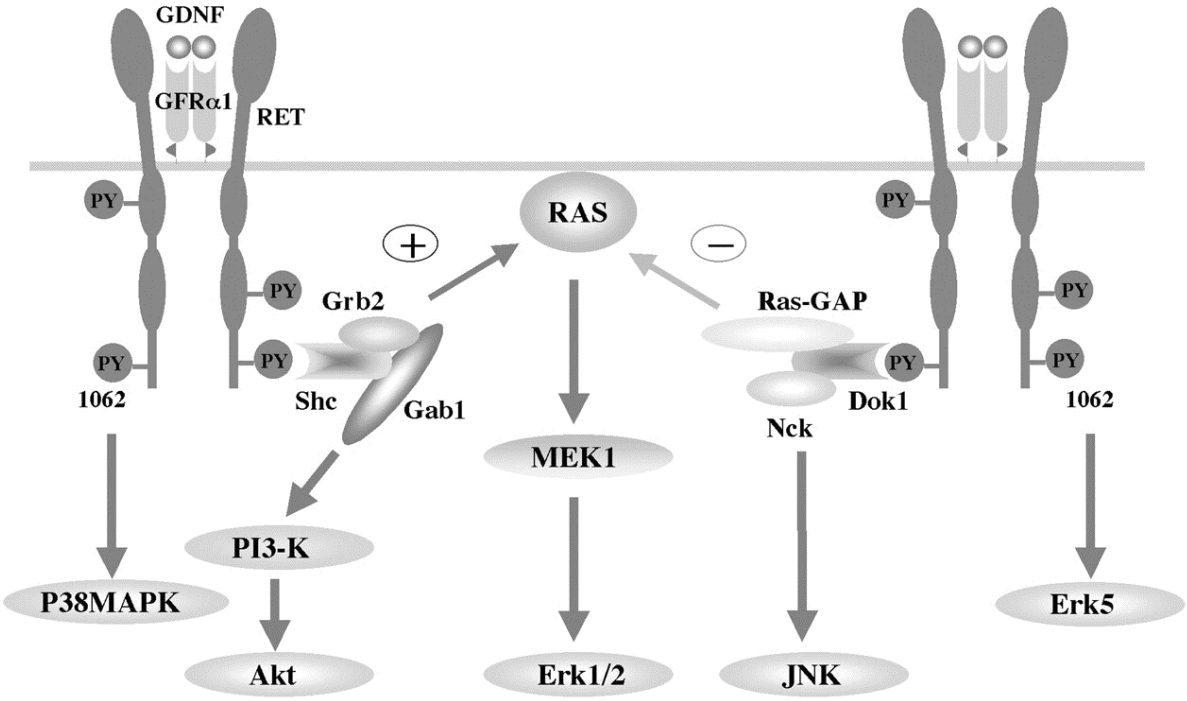

Figure 4. RET signal transduction pathway (from Murakami *et al*., 2002)

GDNF is a survival factor for central nervous system motoneurons and midbrain dopaminergic neurons. Several biochemical and experimental approaches have verified that GDNF is a ligand for RET (Trupp *et al.,* 1996). Mice with a targeted deletion of *GDNF* show a phenotype identical to that of *RET* knock-out mice (Moore *et al.,* 1996). GDNF does not bind to RET directly, but through the interaction with a GFRα, a GPI-linked cell surface receptor lacking a transmembrane or intracellular domain (Jing *et al.,* 1996, Treanor *et al.,* 1996). The GDNF-GFRα-RET complex has been shown to regulate ureteric branching morphogenesis (Sainio *et al.,* 1997). The expression of the components from this complex is tightly regulated during embryonic kidney development (Sainio *et al.,* 1997) and mice deficient for GFRα also present with renal defects (Enomoto *et al.,* 1998). Misexpression of RET in the kidney during embryonic development in *Hoxb7-RET* transgenic mice was shown to result in abnormal embryonic kidney development (Srinivas *et al.,* 1999). Studies of mice with expression of only the RET9 or only the RET51 isoform demonstrated that the RET9 isoform is crucial for kidney organogenesis, whereas absence of RET51 does not influence kidney development (de Graaff et al., 2001).

We have generated *CALC-WT-RET* transgenic mice that express a wild type human *RET* proto-oncogene, that encodes the RET9 isoform, under control of a human CT promoter. The *CALC-WT-RET* mice had sustained postnatal expression of RET in the kidneys. The effects of this aberrant RET expression on renal function and disease development are presented in Chapter 2.

2.3 RET rearrangements in papillary thyroid carcinomas

Chromosomal rearrangements producing chimeric oncogenes are frequently associated with human cancer and several lines of evidence suggest that they are involved in tumorigenesis. Transfection of DNA from papillary thyroid carcinoma (PTCs) into NIH3T3 cells, led to the identification of chromosomal rearrangements of the RET gene with foreign sequences. In 1987, the first activated version of the RET oncogene, named RET/PTC (papillary thyroid cancer), was isolated (Fusco *et al*., [1987]). Subsequently, a number of different RET/PTCs have been isolated, where the RET TK domain was found to be fused to different partner genes.

RET/PTC1 originates by chromosome 10 inversion, inv(10)(q11.2q21.2), and results from the fusion of the RET-TK domain and H4 (D10S170) gene, whose function is still unknown. The H4/RET fusion incorporates 101 amino acids of H4, predicted to encode a leucine zipper domain responsible for RET/PTC1 oligomerization and constitutive TK activity (Tong *et al*., 1997). Another rearrangement, containing the N-terminal 150 residues of H4, creates an oncoprotein named RET/PTC1L able to transform NIH3T3 cells with fivefold lower efficiency than RET/PTC1. Its low transforming ability may explain its low frequency in human thyroid carcinomas (Giannini *et al*., 2000)

In RET/PTC2, the RET-TK is fused to the type I alpha regulatory subunit of protein kinase A (RIα) and this fusion gene is generated by a reciprocal and balanced chromosome translocation (Lanzi *et al*., 1992; Bongarzone *et al*., 1993). The resulting 596-aa protein contains the first two-thirds of RIα. The wild-type RIα subunit dimerizes in an antiparallel orientation between Cys-16 and -37 (Bubis *et al*., 1987). RET/PTC2 deletion mutants showed that the RIα dimerization domain is the only portion of RIα required for RET/PTC2 mitogenic activity, thus suggesting that RET TK is activated in RET/PTC2 via the dimerization domain of RIα (Durick *et al*., 1995).

Both RET/PTC3 and RET/PTC4 oncogenes are generated by an intrachomosomal rearrangement with the ELE1α/ARA70 gene.

RET/PTC3 contains the first 238 amino acids of the androgen receptor-associated protein 70 (Santoro *et al*., 1994). Bongarzone *et al*. (1997) identified a short homology sequence (3-7 bp) in the two rearranging genes and a break cluster region (Bcr) in ELE1, in A + T rich regions. The N-terminal coiled-coil domain of ELE1α/ARA70 mediates oligomerization, RET kinase activation and transforming ability (Monaco *et al*., 2001). The RET/PTC3 gene is critical for the development of the solid subtype of PTC. Recently Basolo *et al*. (2002) associated RET/PTC3 rearrangement also with the Tall-Cell Variant (TCV) of PTCs. The finding that RET/PTC3 is present in the aggressive histological PTC subtypes (Solid PTC and TCV) could depend on the more efficient in vitro mitogenic ability and MAPK activation of this RET rearrangement in comparison to RET/PTC1.

In the case of RET/PTC4, in spite of the presence of the same RET/PTC3 breakpoint in exon 5 of ELE1α/ARA70 gene, the sequence of the rearranged genomic DNA showed a different intra-exonic breakpoint in the RET proto-oncogene. Moreover, it has been demonstrated that the exon 5 of ELE1α/ARA70 joined to exon 11 instead of to exon 12 of the RET gene; as a consequence, the RET/PTC4 cDNA sequence is 93 nucleotides larger than the RET/PTC3 one (Fugazzola *et al*., 1996).

After the Chernobyl power plant explosion, an unusual number of thyroid cancers were noted in Belarus and

Ukraine, between 10 and 30 fold higher than in the rest of Europe. Intrachromosomal rearrangements involving RET and the adjacent H4 or ELE1α/ARA70 gene on chromosome 10 (RETPTC3 and 4) are very frequent events (58%) in thyroid cancer of children of the Chernobyl-contaminated zone (Klugbauer *et al*., 1995; Nikiforov *et al*., 1997). In addition RET/PTC3 rearrangement is strongly associated with PTC of short latency and connected with the solid-follicular variant (Thomas *et al*., 1999). Interestingly RET/PTC1 rearrangement has been found to be associated with post-Chernobyl PTC of long latency. The analysis of the PTCs derived from patients in the contaminated zones led to the identification of other rearranged forms of RET, where the RET-TK is fused to seven different donor genes. For instance, the RET/PTC5 fusion partner protein is GOLGA5, a coiled-coil protein expressed on the Golgi surface (Klugbauer *et al*., 1998). RET/PTC6 and RET/PTC7 display rearrangements with the transcriptional intermediary factor 1-alpha and gamma, respectively (Klugbauer and Rabes, 1999). This protein family is able to bind to the ligand-dependent activation function (AF2)-activating domain of the estrogen receptor, RARs, RXRs and vitamin D3 receptor and regulate transcription. Kinectin is the RET/PTC8 partner (Salassidis *et al*., 2000), whereas RFG9, a putative cytoplasmic protein that might be involved in intracellular transport processes, is rearranged with RET to form RET/PTC9 (Klugbauer *et al*., 2000). In RET/PCM-1 the RET-TK-activating sequences belong to a gene coding for a centrosomal protein that displays distinct cell cycle distribution (Corvi *et al*., 2001). The gene involved in the ELKS/RET rearrangement is ubiquitously expressed with the highest expression in heart, placenta, pancreas, thyroid, and testis but the function of the ELKS protein is still unknown (Nakata *et al*., 1999).

2.4 RET mutations in Hirschsprungs disease

Hirschsprungs disease (HSCR), or congenital intestinal aganglionosis, is a relatively common disorder of neural crest migration. HSCR is characterized by a loss of intestinal neurons, and affects about 1 in 5000 live births (Badner *et al*., 1990). HSCR has a strong genetic basis as genetic factors have been implicated in the etiology of this disease in about 20% of cases and a dominant pattern of inheritance has been revealed in several families, although simple Mendelian inheritance is rarely observed. Mutations in the RET gene are responsible for approximately half of familial cases and a smaller fraction of sporadic cases (Romeo *et al*., 1994, Edery *et al*., 1994). Different animal and human genetic studies have identified 5 additional Hirschsprung genes: endothelin 3 (*EDN3*), endothelin B receptor gene (*EDNRB*), glial-cell-line-derived neurotrophic factor (*GDNF*), endothelin converting enzyme (*ECE1*) and the gene encoding the Sry-related transcription factor *SOX10* (reviewed by Parisi and Kapur, 2000). For almost every HSCR gene, incomplete penetrance of the HSCR phenotype has been observed, probably due to genetic modifier loci. The types of mutations found in the *RET* gene, which have been implicated in Hirschsprungs disease, can be placed loosely into two groups. They can be either frameshift or missense mutations which disrupt the structure of the intracellular tyrosine kinase domain (Romeo *et al*., 1994) or missense mutations in exons 2, 3, 5, or 6 affecting the extracellular domain (Edery *et al*., 1994). Mutations of the tyrosine kinase domain are likely to disrupt the intracellular signaling functions of the

molecule, whereas mutations in the extracellular domain may interfere with ligand binding, or impair the ability of the molecule to form functional dimers (Figure 5).

2.5 RET mutations in MEN 2 and MTC

MEN 2 is associated with germline mutations in the RET proto-oncogene, leading to amino acid substitutions in its encoded transmembrane receptor tyrosine kinase. A clear genotype phenotype correlation was observed with respect to the types of MEN 2 (Eng *et al*., 1996A). In MEN 2A the mutations in the *RET* gene affect cystein residues in the extracellular domain of the protein, which results in constitutive kinase activity.

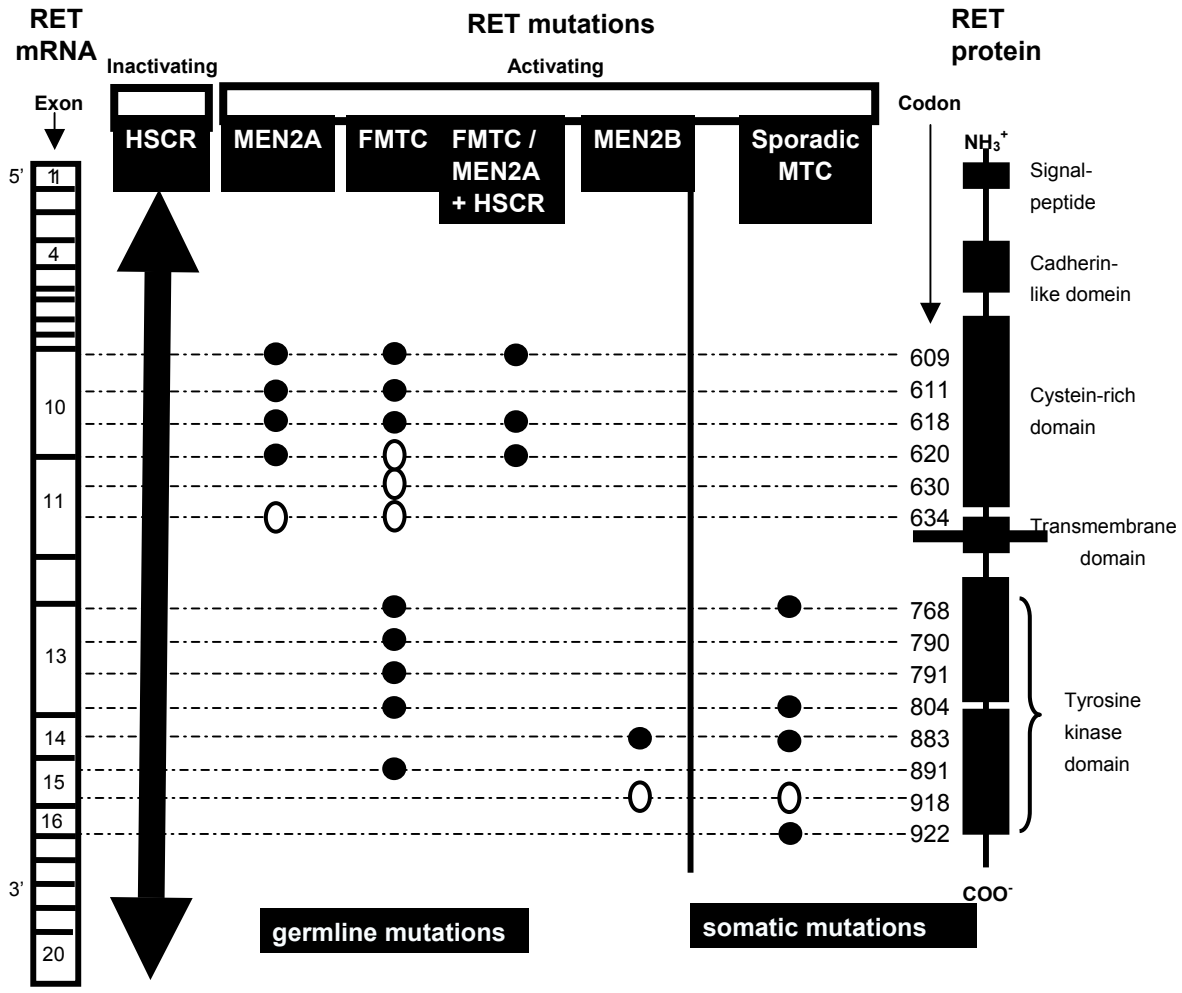

Figure 5. Ret mutation spectrum in MEN 2A, MEN 2B, FMTC, sporadic MTC and Hirschsprungs disease (HSCR). Open circles indicate frequently found mutations, closed circles indicate mutations that are found less frequently. HSCR mutations are frameshift and missense mutations found in the region of the RET gene encoding extracellular and intracellular parts of the RET protein.

Mutations of the cystein residue at position 634 are most frequently found (Mulligan *et al*., 1993, Donis-Keller *et al*., 1993). Mutations associated with MEN 2A cause ligand independent dimerization of RET via the formation of disulphide bonds (Asai *et al*., 1995, Santoro *et al*., 1995). In MEN 2B the most common mutation results in a Met→Thr substitution at aa position 918 in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40 %) of sporadic MTC cases (Hofstra *et al*., 1994, Eng *et al*., 1994, Carlson *et al*., 1994). This MEN 2B mutation appears to modify the substrate specificity of the tyrosine kinase (Santoro *et al*., 1995). A small fraction of MEN 2B patients harbor a germline RET mutation leading to an A883F or an S922F substitution. Germline mutations in FMTC patients affect cysteine residues 611, 618, 620 and 634 in the extracellular domain as well as residues in the intracellular kinase domain leading to E768D, L790F, Y791F, V804L, V804M and S891A substitutions (figure 5).
All MEN 2 mutations convert the *RET* proto-oncogene into a dominantly acting transforming gene in humans, as expression of the non-mutated allele is usually retained in tumor tissue (Landsvater *et al*., 1996), but somatic MEN 2B mutations have been described in tumors from MEN 2A patients (Marsh *et al*., 1996)

3 Cancer susceptibility genes and multistep tumorigenesis in MTC

3.1 Cancer susceptibility genes.

MTC development and cancer in general arise due to mutations occurring in a wide variety of genes. As more cancer susceptibility genes are identified, the question as to how these genes interact with each other to produce the cancer phenotype becomes more difficult to answer. The first step in understanding their interactions is the identification of the genes involved. The very early genetic studies identified cancer as a recessive phenotype. These studies showed suppression of the transformed phenotype of cells after fusion with single human chromosome somatic cell hybrids. The chromosome-specificity of this phenomenon indicated the involvement of different genes. The recessive phenotype of cancer seemed in contrast with the dominant nature of inherited tumor syndromes. Knudson (1971) formulated the two-hit theory stating that in familial cancer an initial mutation on one allele was inherited and that a second mutation in the other allele of that specific gene was somatically acquired in certain cells, giving rise to cancer. The mechanisms by which this occurs include tumor-specific loss of heterozygosity. Mutation or loss of the second allele in specific tissues in familial cancer patients is due to spontaneous mutations. The cancer genes that are involved are referred to as tumor suppressor genes that require biallelic loss of function in order to initiate cancer development.
Oncogenes represent another class of tumor susceptibility genes. Contrary to tumor suppressor genes, oncogenes harbor genetic changes that result in gain of function of the encoded proteins. Oncogenes require mutations in only one allele to exert their effects and therefore act with a dominant nature.
A third class of genes involved in cancer development is the DNA repair genes. Loss of function of these genes leads to greatly increased mutation frequencies of other genes involved in cancer development.
Genetic studies of familial cancers have identified genes and mutations that are involved in the initiation of tumor

development. Genes from all three above mentioned functional categories have been identified as causative in families with familial tumor development. The majority are tumor suppressor and DNA repair genes whilst only two oncogenes, RET and MET, have been associated with cancer syndromes. Kinzler and Vogelstein (1997) introduced the subdivision of cancer susceptibility genes into gatekeeper and caretaker genes. Gatekeeper genes directly control cellular proliferation. Gatekeeper genes act tissue specific and have a function in inhibition of cell proliferation or the promotion of cell death. Caretaker genes are involved in the maintenance of genetic stability and the prevention of undue mutation. Mutations in caretaker genes, which include mismatch repair genes and nucleotide excision genes, do not directly promote cancer initiation. Due to an increased mutation frequency, additional mutations e.g. in gatekeeper genes can occur, which initiate cancer development. The gatekeeper genes involved include both oncogenes and tumor suppressor genes (figure 6).

The RET and MET proto-oncogenes are gatekeeper genes associated with tumor initiation in MEN-2 and hereditary papillary renal carcinoma, respectively. Contrary to all other genes associated with familial cancer syndromes, germline mutations in these genes can directly initiate oncogenesis. The non-mutated allele is usually retained in tumors.

The *APC* tumor suppressor gene functions as a gatekeeper gene involved in the suppression of cancer of the intestinal tract. The *APC* gene is mutated in the germline of familial adenomatous polyposis coli (FAP) patients. Other examples of gatekeeper genes are Rb, MEN-1 and VHL.

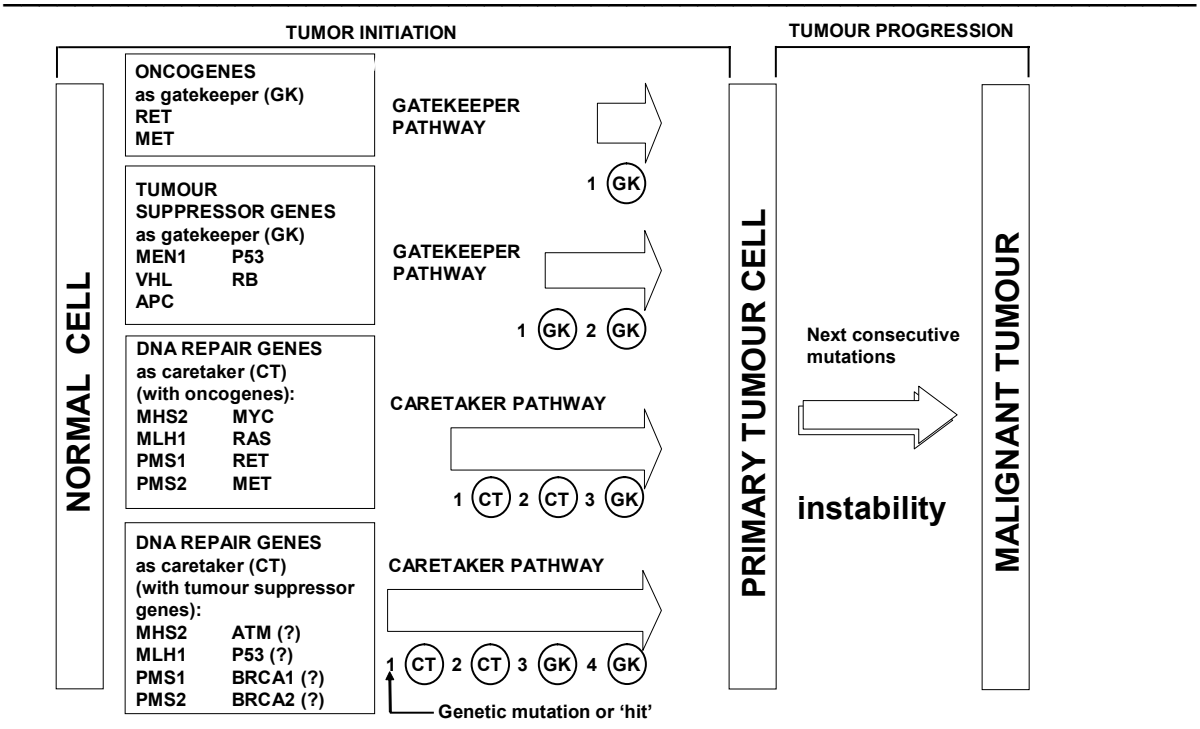

Figure 6. Schematic representation of different genetic pathways for tumor initiation (based on Kinzler and Vogelstein, 1997)

The P53 gene is a tumor suppressor gene that may function both as gatekeeper and as caretaker. Germline mutation of P53 is causative for the Li-Fraumeni syndrome and initiates tumor development in these patients, suggesting a gatekeeper function. Somatic mutations in P53 are obtained during the progression of a wide variety of tumors. Normal P53 has a function in cell cycle control. Following DNA damage P53 determines if a cell will enter programmed cell death (apoptosis) or will survive. The P53 mediated cell cycle arrest occurs to allow time for DNA damage repair. If DNA repair is unsuccessful the cell undergoes apoptosis. By this means survival of a cell with profound DNA damage is prevented. Thus, if in a tumor cell P53 function is lost, this cell can escape this crucial checkpoint and obtain immortality. This clearly points towards a caretaker function for P53. Other caretaker genes include MLH1, MSH2, PMS1 and PMS2 involved in hereditary non-polyposis colorectal cancer.

3.2 Mouse models for multistep cancer development

Cancer research has gained major insights into the complexity of tumor development, in particular into the molecular mechanisms that underlie the progressive transformation of normal cells into highly malignant derivatives. It is estimated that the transformation of a normal cell to a malignant tumor cell is dependent upon a small number of genetic alterations, most likely within the range of four to seven rate-limiting events. Critical events in the evolution of neoplastic disease include the loss of proliferative control, the failure to undergo programmed cell death (apoptosis), the onset of neoangiogenesis, tissue remodeling, invasion of tumor cells into surrounding tissue and, finally, metastatic dissemination of tumor cells to distant organs. Over the past decades, the genetic understanding of tumor development has been greatly enhanced by the direct study of human cancer. Studies on rare forms of familial inherited cancer syndromes have led to successful identification of many tumor-suppressor genes that are crucial to the development of distinct forms of cancer. Mutation or loss of these genes has highly penetrant phenotypic consequences, and germline mutations of these genes predispose the carriers to tumor development. However, the study of cancer genetics in humans has certain limitations. In patients, the molecular analysis of these multiple steps is often hampered by the unavailability of tumor biopsies from all stages of tumor progression. Furthermore, tumor susceptibility in each individual is determined not only by these key players but also by several modifier genes whose activity might also have a key influence on cancer development. The diversity in genetic background and the difficulty in tumor and tissue procurement make it difficult to study tumorigenesis in humans for the purpose of identifying (weak) tumor determinants. By contrast, the mouse offers several distinct advantages and has proved to be a valuable model system for the study of multistep tumorigenesis at two distinct levels: (i) identification of novel genes involved in cancer pathogenesis and (ii) experimental in vivo assessment of the role of different genetic hits in multistep tumorigenesis. Furthermore, mouse models of tumorigenesis allow the reproducible isolation of all tumor stages, including normal tissue, which are then amenable to pathological, genetic and biochemical analyses and, hence, have been instrumental in investigating cancer-related genes and their role in tumorigenesis.

3.3 MTC mouse models

Clues for potential involvement of several oncogenes or tumor suppressor genes in MTC tumorigenesis has come from studies with transgenic mouse strains. Mice carrying a c-mos transgene linked to an MSV-LTR displayed MTC (Schulz *et al*., 1992). Abnormal expression of c-mos mRNA was reported previously in human MTC (Parkar *et al*., 1988), but no mutations of c-mos have been identified so far in sporadic MTC (Eng *et al*., 1996B). Also in mice heterozygous for a mutation that disrupts the retinoblastoma (RB) gene, MTC was detected (Williams *et al*., 1994). In man, however, no loss or major rearrangements of the RB1 gene have been detected in MTC samples (Holm and Nesland, 1994). In a transgenic mouse model with expression of a v-Ha-ras gene driven by the rat CT promoter, MTC development was observed (Johnston *et al*., 1998). In humans, however, c-Ha-ras is not associated with MTC (Moley *et al*., 1991). In conclusion: there appears to be some discrepancy between mice and man with regard to genetic changes involved in MTC development. These discrepancies may be caused by species differences, but are more likely to reflect differences in the initiating events in MTC oncogenesis. The existence of a hereditary form of MTC in man (MEN 2) has allowed the identification of RET as a crucial initiating event, with a dominantly acting nature. Therefore, by generating transgenic mice expressing a human MEN 2-specific RET oncogene, known to be crucial in MTC initiation and development in man, these discrepancies may be overcome. Transgenic mice with C-cell expression of a human RET oncogene with a MEN 2A-specific mutation develop MTC (Michiels *et al*., 1997). In mice with a MEN 2B mutation in the murine RET gene, generated by homologous recombination, only CCH, but not 'full blown' MTC development was reported (Smith-Hicks *et al*., 2000). We generated *CALC-MEN2B-RET* transgenic mice with C-cell specific expression of a human RET gene in which the MEN 2B mutation M918T was introduced. The phenotype of these mice is presented in Chapter 3.

3.4 Additional oncogenic events in multistep MTC development

It is widely accepted that tumorigenesis is a multistep process that involves a series of genetic and epigenetic alterations, such as activation of dominantly acting oncogenes and inactivation of tumor-suppressor genes. These mutations accumulate in the cells and change their behavior from normal growth to unrestrained growth and eventually lead to invasion into surrounding tissue and/or metastasis. This multiple-stage process is revealed by a range of observations, including clinical, epidemiological and laboratory experiments. The process can be extremely complex. For example, for the development of colorectal carcinoma, it has been proposed that at least seven sequential genetic alterations occur, including mutation of the adenomatous polyposis coli (*APC*) tumor-suppressor gene, mutation of Ki-*ras* and loss of *P53* gene function (Kinzler and Vogelstein, 1996). These sequential mutations of key growth-regulatory genes in the somatic cells and their progeny are generally regarded as 'multiple-hits'. We studied several oncogenic events which may play a crucial role as additional oncogenic steps in RET-induced MTC development.

3.5 P53 and MTC

P53 is a transcription factor performing a critical role in the cellular response to stress attacks. There is now ample evidence demonstrating that functional p53 is vital for tumor growth suppression *in vivo* (Jimenez *et al*., 2000). This function of p53 is lost by mutation in more

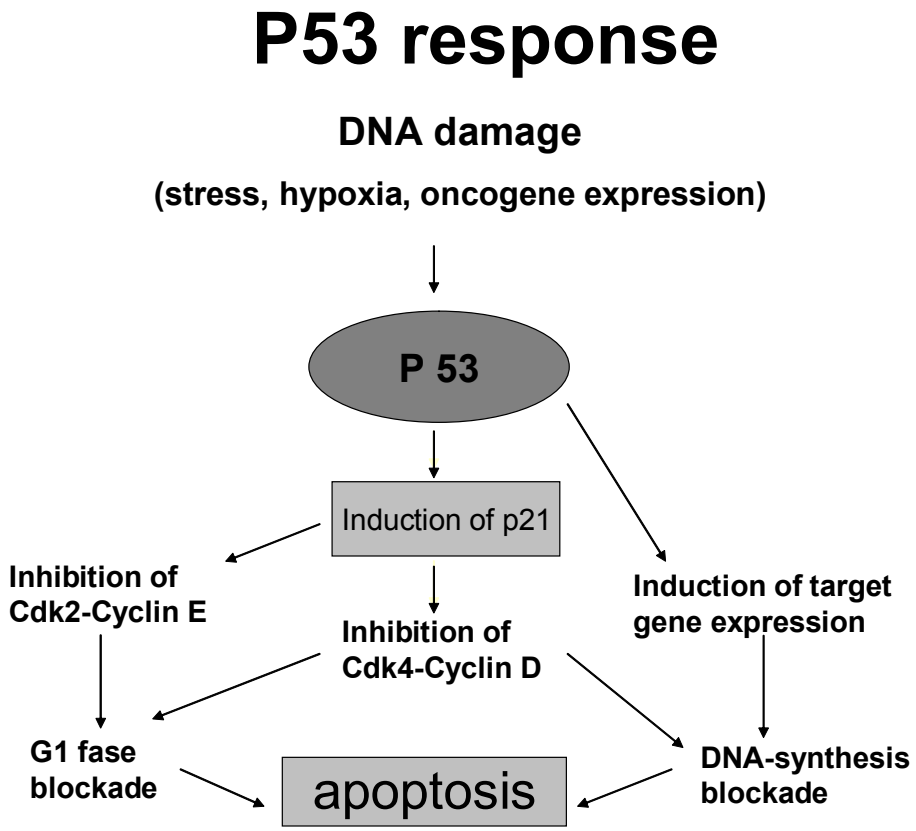

Figure 7. DNA damage responses of P53 leading to cell cycle arrest and apoptosis.

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than 50% of cancers, and the remainder appear to have defective p53 signaling pathways (Vogelstein and Kinzler, 1992). Under normal, non-stressed conditions, p53 is maintained at very low levels. However, upon exposure to the stress of DNA damage, hypoxia, alterations in redox potential and deregulated oncogene expression, p53 is stabilized and activated through post-translational modification. Activated p53 induces growth arrest and/or apoptosis through the induction or repression of specific target genes (reviewed in Vogt Sionov and Haupt, 1999). P53 promotes cell growth arrest in G1 phase, through the induction of the cyclin dependent kinase (CDK) inhibitor $p21^{WAF1}$ (El-Deiry *et al*., 1993). P53 directed pathways of apoptosis are more intricate and have been identified to involve both transcriptional regulation of multiple p53-target genes as well as transcription-independent mechanisms. P53-targets have been identified in both the extrinsic (death receptor) and intrinsic (mitochondrial) pathways of apoptosis (reviewed in Haupt *et al*., 2003). The determinants of the type of response that is provoked in a cell are the subject of intense study. The nature and intensity of the stress, the type of cell and the molecular partners interacting with p53 are all considered to influence the

decision of a cell to either arrest or induce apoptosis in response to a given stress signal (reviewed in El-Deiry, 2003). Through these mechanisms p53 eliminates cells with damage that cannot be repaired thereby preventing the perpetuation of the affected cells.
P53 involvement in human MTC has been reported previously. Several series of human MTC have been studied using immunohistochemistry. P53 immune-reactivity was reported in 5-15% of MTCs analyzed (e.g. Pollina *et al*., 1996). In mice that have, in addition to an inactivating retinoblastoma gene (RB) mutation, also an inactivating P53 mutation, MTC development was promoted (Williams *et al*., 1994). Interestingly, somatic MEN 2A mutations in the murine RET gene were detected in these MTCs, indicating that loss of RB and P53 function and RET activation collaborate in MTC induction and development in mice (Coxon *et al*., 1998). The MEN 2B mutation, however, was not detected in this study.
We studied the potential involvement of P53 in RET induced MTC development by analysis of tumor material from MEN 2 and sporadic patients and from our transgenic mice. By inactivating one or both P53 alleles in our *CALC-MEN2B-RET* transgenic mice, we tried to obtain experimental evidence for a collaborative action of P53 inactivation and activation of RET by M918T mutation in MTC development. These studies are presented in Chapter 4.

3.6 LOH of Chromosome 1p in MTC

Allelic loss of 1p has been described to be associated with MTC development in MEN 2 patients and with sporadic cases of MTC. The first report described loss of heterozygosity for the marker D1S7 located on 1p33-35 in 3 of 8 (6 familial and 2 sporadic) informative MTCs examined (Mathew *et al*., 1987). Others reported association of MTC with heterozygous loss of a marker on 1p35-p34 and a marker on 1p36.3 in 1 of 11 (1 familial and 10 sporadic) cases analyzed (Koshla *et al*., 1991), whereas heterozygous loss of 1p was observed in 3 of 24 (19 familial and 5 sporadic) informative cases of MTC (Moley *et al*., 1992). Cytogenetic analysis of cell lines derived from MTC and primary cultures of MTC specimen revealed relative loss of 1p in 3 of 10 informative cases (Cooley *et al*., 1995). In a more recent study the genome-scanning technique comparative genomic hybridization (CGH) was applied to 9 familial and 29 sporadic cases of MTC and to the TT cell line (Marsh *et al*., 2003). The TT cell line is derived from a MTC from a patient with a C634W MEN 2A RET mutation (Cooley *et al*., 1995). Loss at 1p was observed in 1 of 9 familial MTCs, whereas 7 of 29 sporadic MTC cases showed loss at 1p. Also in the TT cell line loss of 1p was observed. In this study further fine mapping of the regions of 1p deletion was applied resulting in identification of two regions of loss. One located more centromeric (1p13-p22) and one located more telomeric (1p32-pter), indicating that potentially two suppressor genes of MTC development are located on 1p (Marsh *et al*., 2003).
The identity of the tumor suppressor genes located on 1p, involved in the tumorigenesis of MTC, is not known. A possible candidate is the P18/INK4C/CDKN2C (P18) gene, which is located on 1p32. P18 is a member of the family of cyclin dependent kinase (CDK) inhibitors (CDKIs). These CDKIs play an important role in the regulation of cell cycle progression (figure 8). The CDKIs are subdivided in two classes. One is the class of the “kinase inhibitor proteins” (KIP). The other class is the “inhibitors of CDK4” (INK4) of which P18 is a member. P18 binds to CDK4 but to a lesser extent than the preferentially bound CDK6, inactivates their kinase

activity and inhibits their association with cyclin D (Guan *et al*., 1994). Besides P18, other members of the INK4 family are the proteins P16(CDKN2A, INK4A), P15(CDKN2B, INK4B) and P19(CDKN2D, INK4D). The functional similarity of these four proteins is reflected in the highly conserved four ankyrin repeats, which all members share When P18 or another member of the INK4 proteins binds to CDK4 or CDK6, their ability to phophorylate and inactivate the Rb protein is suppressed. As a consequence the Rb protein retains active in its growth-suppressive state and prevents the G1/S transition of cell cycle progression. On the other hand, when P18 is absent or downregulated, the cyclin D is able to activate CDK4 and CDK6, which in turn result in phosphorylation and functional inactivation of Rb. This protein can no longer prevent S-phase entry and therefore cell cycle progression will occur. In line with this, ectopic induction of P18 expression was shown to correlate with cell cycle arrest and apoptosis with dependence on endogenous Rb (Guan *et al*., 1994). Furthermore, deregulation of cell cycle control by loss of function of proteins involved is believed to be a common event for most types of cancers. We studied the potential involvement of loss of P18 function during MTC development in MEN 2 and sporadic patients (Chapter 5).

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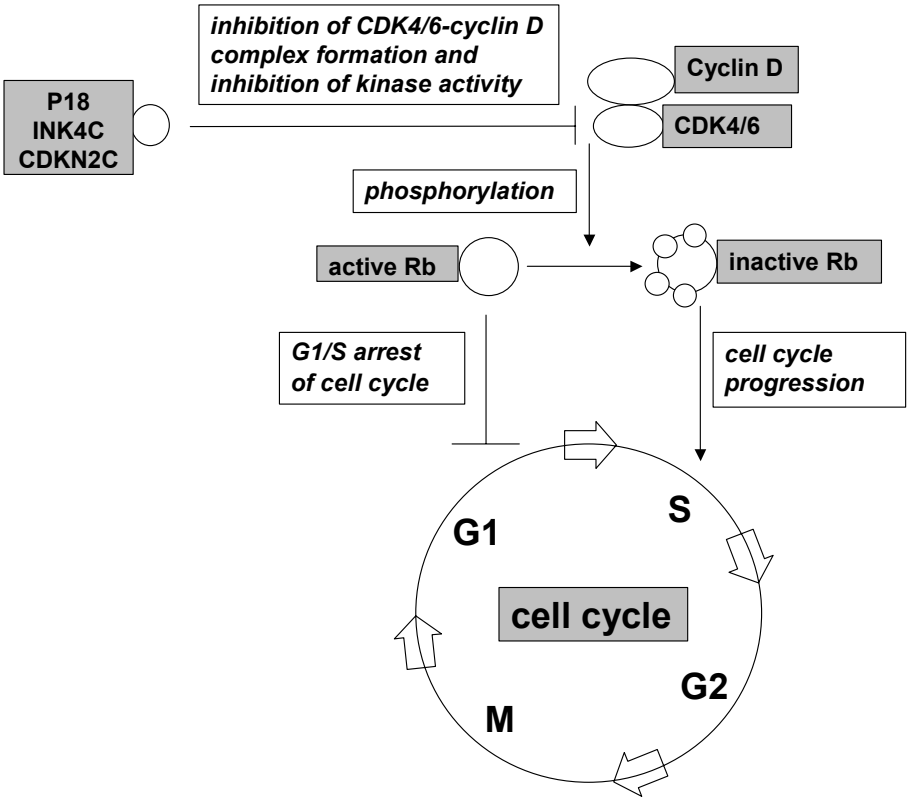

Figure 8. Role of P18 in cell cycle regulation.

3.7 Wnt/beta-Catenin/Tcf signaling in MTC development

Deregulation of the Wnt/beta-Catenin/Tcf signaling pathway has been associated with several forms of cancer (Bienz end Clevers, 2000). This results in high levels of cytoplasmic and nuclear beta-Catenin. In the nucleus, beta-Catenin associates with Tcf4 and forms a transcriptionally active complex (Korinek *et al*., 1997). Tcf1 serves as a negative regulator of this complex (Roose *et al*., 1999). In a normal intestinal epithelial cell, regulation of the transcriptional activity of this complex is mainly achieved by controlling the level of free cytoplasmic beta-Catenin available for nuclear translocation and binding to Tcf4. A quaternary cytoplasmic complex comprising beta-Catenin, the adenomatous polyposis coli protein (APC), conductin-axin and glycogen synthase kinase (GSK)-3B mediates the phosphorylation of beta-Catenin, which can then be degraded by the ubiquitin-proteosome pathway. Deregulation of the Wnt/beta-Catenin/Tcf pathway has not yet been associated with MTC induction or development. Other types of thyroid tumors have been associated with Wnt/beta-Catenin/Tcf signaling. In anaplastic thyroid tumors nuclear beta-Catenin staining was observed (Carcia-Rostan *et al*., 1999). In patients with familial adenomatous polyposis coli (FAP), carrying a germline APC mutation, the papillary type of thyroid gland tumor occurs at a frequency of 1-2%, which is significantly higher compared to the 0.005% found in the general population. In addition, thyroid carcinomas frequently develop in a multicentric fashion in FAP patients (Miyaki *et al*., 2000). This indicates that a germline APC mutation gives a predisposition for this type of thyroid cancer. Nuclear expression of beta-Catenin has been observed in these FAP-related papillary thyroid gland tumors (Kurihara *et al*., 2000). Biallelic loss of the APC tumor suppressor gene in papillary thyroid tumors of FAP patients was observed in the tumor in these patients (Miyaki *et al*., 2000). However, it was recently reported that biallelic loss of the APC gene is not necessary for thyroid tumorigenesis in FAP patients (Cetta *et al*., 2001). A clear explanation for this observation still remains to be found. In the FAP associated papillary thyroid tumors somatic translocations involving the RET gene, which lead to activation of RET kinase activity, are frequently found (Cetta *et al*., 1998). It is of interest that in the tumors which lack the biallelic inactivation of the APC gene, the RET gene is activated by a translocation mechanism resulting in the RET/PTC-1 oncogene. It is therefore tempting to speculate that there may be a gene dosage effect of APC with respect to thyroid tumorigenesis. A phenotypic manifestation of this dosage effect apparently becomes manifest in the presence of an activated RET gene. These data warrant further investigation with respect to RET activation and Wnt/beta-Catenin/Tcf deregulation in MTC. We studied the deregulation of Wnt/beta-Catenin/Tcf signaling in RET induced MTCs from our *CALC-MEN2B-RET* transgenic mice and in RET-induced human MTCs. In addition, we introduced deregulation of Wnt signaling in our *CALC-MEN2B-RET* mice, by crossing them with APC(min) mice (Moser *et al*., 1995). The results of these studies are presented in Chapter 6.

4 Aim of this study and questions

Development of MTC, particularly in the hereditary setting of MEN 2, has been shown to be a multistep event. Analysis of the age of onset data for MTC (Ponder, 1988) indicated that in addition to the mutated RET gene, further tumorigenic events must accrue for carcinoma

development. The identity of these secondary tumorigenic events in MTC development is still largely unknown. The differences observed in severity of MTC progression between individual MEN 2 family members (who have the same germline RET mutation) may indicate that these additional events are important contributors to progression and dissemination. Currently we are in need of new treatment modalities for metastatic MTC as no effective therapy is available.

Aim of the study was to answer some unresolved questions with respect to the multistep tumorigenesis of MTC, which may increase our knowledge of the molecular basis of MTC initiation, progression and dissemination.
I. Can we obtain experimental *in vivo* evidence for the oncogenic potential of the M918T RET mutation most frequently observed MEN 2B patients and in sporadic MTC?
II. Can we identify other oncogenic pathways which, in addition to the RET oncogene, contribute to MTC development?
III. Can we provide experimental evidence for the involvement of identified additional events or pathways?
IV. Can we get indications for preference for specific additional oncogenic events or pathways in RET-induced MTC development?
V. Can this knowledge be applied to other RET-induced tumors such as pheochromocytomas?
VI. Can this provide us with new targets for therapy of MTC?

In the following chapters I describe the generation and characterization of transgenic mice expressing a wildtype *RET* proto-oncogene (Chapter 2) and a mutated *RET* oncogene (chapter 3), the latter as a model for Multiple Endocrine Neoplasia type 2B and sporadic MTC, to study multistep MTC development.
We studied the potential involvement of P53 inactivation (Chapter 4), mutation of the cell cycle regulator P18 (Chapter 5) and deregulation of Wnt/beta-Catenin/Tcf signal transduction pathway (chapter 6) as additional oncogenic events in RET-induced multistep MTC development in human patients and the *CALC-MEN2B-RET* transgenic mouse model.
The results and the implementation of the findings for therapy of MTC and for future research are discussed (Chapter 7).

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Chapter 2
Aberrant renal expression of *RET* proto-oncogene in transgenic mice induces progressive renal cystic disease associated with growth retardation.

Dennis S. Acton[1], Danny Velthuyzen[1], Roel Goldschmeding[2], Sylvia C. van Buul-Offers[3,4], Cornelis J.M. Lips[1] and Jo W.M. Höppener[4,1].

Department of Clinical Endocrinology, Division of Internal Medicine[1],Department of Pathology, Laboratory Division[2] ,Department of Pediatric Endocrinology, Laboratory Division[3],University Medical Center Utrecht, Department of Metabolic and Endocrine Diseases, Division of Medical Genetics[4], University Medical Center Utrecht

ABSTRACT

The *RET* proto-oncogene encodes the RET receptor tyrosine kinase which serves, in conjunction with GFRα's, as the receptor for the GDNF family of neurothrophic factors. Mutations in the *RET* gene are associated with Multiple Endocrine Neoplasia type 2 and Hirschsprungs disease and with sporadic medullary and papillary thyroid cancer. RET is an essential component of signaling pathways required for kidney organogenesis. We generated *CALC-WT-RET* transgenic mice, expressing a wildtype human *RET* proto-oncogene under the control of a calcitonin promoter fragment. Whereas endogenous RET expression in kidneys is restricted to the embryonic stage, transgenic RET expression was also observed in postnatal kidneys. *CALC-WT-RET* transgenic mice developed renal cystic disease depending on the level of RET protein expression in the kidneys. Two founders succumbed to renal cystic disease after 10 months. In several transgenic lines mild renal cystic disease developed after one year. Increasing RET expression, by breeding homozygous or compound heterozygous offspring, resulted in a phenotype of progressive renal cystic disease and growth retardation. Severity of renal failure varied and was strongly correlated with the severity of growth retardation. These findings stress the importance of regulated RET expression in normal kidney development and function, and may indicate a role for RET in human kidney pathology.

INTRODUCTION

The *RET* gene was identified as a proto-oncogene in a classical NIH3T3 transfection assay (Takahashi *et al.,* 1985). The *RET* gene can encode three proteins which differ by 9, 43 and 51 amino acids in the carboxy terminal domain, due to alternative splicing of the pre-mRNA (Tahira *et al.,* 1990, Myers *et al.,* 1995). The RET proteins are transmembrane receptor tyrosine kinases. RET has been associated with several human diseases. Gene rearrangements involving the *RET* gene are frequently observed in papillary thyroid carcinomas. These rearrangements result in proteins in which the tyrosine kinase domain of RET is fused to parts of other proteins (Jhiang *et al.,* 1992, Bongarzone *et al.,* 1994, Santoro *et al.,* 1994). Activating point mutations in the *RET* gene have been found in DNA from patients with the hereditary cancer syndromes Multiple Endocrine Neoplasia type 2A and type 2B (MEN 2A and MEN 2B). The cardinal feature of MEN 2 is development of medullary thyroid carcinoma (MTC), a

tumor originating from the calcitonin-producing C-cells of the thyroid gland. In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein, which results in constitutive kinase activity (Mulligan *et al.,* 1993, Donis-Keller *et al.,* 1993) .In MEN 2B the most common mutation results in a Met→Thr substitution of aminoacid 918 in the intracellular domain of the protein (Hofstra *et al.,* 1994, Eng *et al.,* 1994, Carlson *et al.,* 1994). Inactivating mutations in the *RET* gene have been linked to Hirschsprung's disease, a heterogeneous genetic disorder, which is characterised by congenital absence of neural crest-derived autonomic ganglion cells in the hindgut. As a result there is no innervation of the gut musculature resulting in severe obstipation (Edery *et al.,* 1994, Romeo *et al.,* 1994). Further experimental proof for RET functions came from the analysis of *RET* knock-out mice (Schuchardt *et al.,* 1994). Mice lacking functional RET develop to term but die within 24 hours after birth due to the absence of an enteric nervous system, as seen in Hirschsprung's disease, and renal dysgenesis or agenesis. In the kidney rudiments that were observed in some homozygous null mutant mice, no functional regions as cortex and medulla could be recognised, and large regions of undifferentiated mesenchyme were present. Tissue recombination studies with mesenchyme and ureteric buds from wild type and *RET* deficient mice indicated that the renal defect is caused by non-responsiveness of the buds to signals from the nephrogenic mesenchyme (Schuchardt *et al.,* 1996).

Glial cell line-derived neurothrophic factor (GDNF) is a survival factor for central nervous system motoneurons (Henderson *et al.,* 1994) and midbrain dopaminergic neurons (Lin *et al.,* 1993). Because in many organs GDNF and RET are expressed in adjacent tissues, it was proposed that GDNF might be the ligand for RET. Several biochemical and experimental approaches have verified this notion (Durbec *et al.,* 1996, Trupp *et al.,* 1996). Mice with a targeted deletion of *GDNF* show a phenotype comparable to that of *RET* knockout mice (Moore *et al.,* 1996). GDNF does not bind to RET directly, but through the interaction with GFRα, a glycosyl-phophatidylinositol-linked cell surface receptor lacking a transmembrane or intracellular domain (Jing *et al.,* 1996, Treanor *et al.,* 1996). The GDNF-GFRα-RET complex has been shown to regulate ureteric branching morphogenesis (Sainio *et al.,* 1997). The expression of the components from this complex is tightly regulated during embryonic kidney development (Sainio *et al.,* 1997) and mice deficient for GFRα also present with renal defects (Enomoto *et al.,* 1998). Misexpression of RET in the kidney during embryonic development in *Hoxb7-RET* transgenic mice was shown to result in abnormal embryonic kidney development (Srinivas *et al.,* 1999).

We have generated *CALC-WT-RET* transgenic mice that express the wildtype human *RET* proto-oncogene under control of a human calcitonin promoter. Initially these mice were generated to study effects of RET in the main target organ of MEN 2, the calcitonin producing C-cells from the thyroid gland. These mice served as control to determine the specificity of the effects of a similar transgene, but with a MEN 2B-specific RET mutation, on the development of MTC. Contrary to the *CALC-MEN2B-RET* construct which induced MTC, expression of the non-mutated human RET proto-oncogene in the thyroid gland of *CALC-WT-RET* mice did not induce tumor development in this organ (Acton *et al.,* 2000). The *CALC-WT-RET* mice had sustained postnatal expression of RET in the kidneys. The effects of this aberrant RET expression on renal function and

disease development are presented in this paper.

RESULTS

Expression of transgenic RET and endogenous RET in transgenic founders and transgenic lines.

Eight *CALC-WT-RET* transgenic founders were obtained (table1). Four gave rise to transgenic lines (lines-04, 09, 33 and 49). Of the remaining founders three (F08, F32 and F42) did not transmit the transgene to their offspring. Founder 38 transmitted the transgene to only one female offspring, which succumbed due to complications during her first pregnancy.

Using RT-PCR, transgene-specific RNA expression was detected in the adult thyroid gland as well as in kidney, lung and brain, whereas no expression was observed in the liver (Figure 1a). In the kidneys from mice from all four transgenic lines RET protein expression was demonstrated using immuno-histochemistry. Of these four lines, line-49 had the highest expression in the kidney (table1). RET expression was most prominent in the outer cortex and the medulla. The collecting duct and papillary duct epithelium had highest expression. Lower expression was observed in the distal and proximal tubules, whereas glomeruli did not stain for RET (data not shown). We were unable to detect endogenous RET expression in non-transgenic kidneys using immuno-histochemistry.

With the more sensitive method of RT-PCR with human/mouse *RET* primers we observed *RET* mRNA expression in the kidneys and in brain from *CALC-WT-RET* line 49 mice, as well as in brain from non-transgenic mice. However, no *RET* mRNA was detected in kidneys from non-transgenic mice indicating that endogenous *RET* expression in the adult kidneys is extremely low or absent (Figure 1b).

In order for RET to exert a biological effect in kidneys, its co-receptors GFRα1-4 should also be expressed. With RT-PCR, GFRα1 transcripts were detected in RNA from *CALC-WT-RET* transgenic and non-transgenic adult kidneys. GFRα1 expression was also observed in adult brain, lung and thyroid gland (data not shown), indicating that also in these tissues RET may exert a biological effect.

Table 1. *Founders, transgene transmission, kidney pathology and kidney RET protein expression in CALC-WT-RET mice*

[a] Severity of kidney pathology + mild renal disease after 14 months, ++ renal disease within 12 months, +++ severe renal disease within 10 months, n.a.=not assessed. [b] Relative staining intensity of RET using immuno-histochemistry: + weak, ++ moderate, +++ strong, n.d.=not determined.

Founder Nr.	Sex	Trans-Mission	Line	Kidney Pathology[a]	RET Protein[b]
04	F	4/26	Yes	+	+
08	F	0/2	No	n.a	n.d.
09	M	17/35	Yes	+	+
32	M	0/1	No	+++	+++
33	F	¾	Yes	+	+
38	F	1/28	No	n.a	n.d.
42	M	0/50	No	+++	+++
49	M	8/24	Yes	++	++

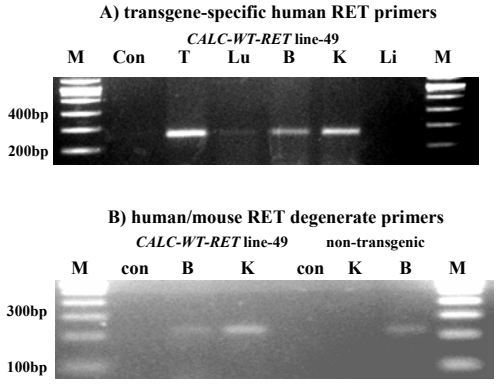


Figure 1. *Transgene expression in CALC-WT-RET line-49 tissues.*
(a) RT/PCR using human RET-specific primers generating a 278 bp product. M=size marker, Con=negative control without template cDNA, T=thyroid gland, Lu=lung, B=brain, K=kidney, Li=liver.
(b) Endogenous and transgenic *RET* expression in brain (B) and kidney (K) from adult *CALC-WT-RET* line-49 and non-transgenic mice. RT-PCR using human/mouse degenerate primers generating a 233 bp fragment both on mouse and human RET mRNA.

Kidney abnormalities in *CALC-WT-RET* transgenic founders

Mice from the four established transgenic lines did not show gross abnormalities or abnormal growth. Also reproduction seemed normal. A kidney phenotype became apparent when two founders (F42 and F32) became ill after 10 months and had to be sacrificed. Autopsy revealed abnormal kidneys with transluminescent macrocysts filled with fluid and giving the kidneys a yellow appearance. In each mouse both kidneys were equally affected. The size of the kidneys was reduced compared to kidneys from non-transgenic, age-matched controls. Histopathology revealed large cysts scattered throughout the whole kidney. Most cysts were of tubular origin. All cysts were lined with a monolayer of epithelium. A diverse phenotype of the cyst epithelium cells was observed. Tightly apposed regular cubical cells with vacuoles, sometimes with a recognisable apical brush border, were often observed. Also cysts with a more columnar epithelium were found. In addition, loosely apposed foamy epithelial cells or flattened cells were seen. Cysts lined with flattened epithelium often contained dark staining proteinaceious fluid, indicative for proteinuria. Some cysts were septated suggesting epithelial hyperplasia. However, no neoplastic outgrowth of the epithelium was evident although occasionally layering of epithelial cells in small hyperplastic foci was seen. Clear glomerular cysts were also found. Some glomeruli displayed varying degrees of sclerosis and atrophy. Atrophy of the tubular and interstitial structures was also seen, as well as areas with mild inflammation and fibrosis (Figure 2a). The renal areas that were not affected by the cysts appeared to have developed normally. Since both founders described above presented with identical bilateral kidney abnormalities, these abnormalities were likely due to expression of the transgene. We examined the level of RET protein in the kidneys from the two founders in comparison to the kidneys from mice from the four transgenic lines and from non-transgenic littermates at an age of 10 months. Using immuno-histochemistry, we detected high levels of RET protein expression in kidneys from both affected founders. RET expression was observed in the epithelium of the nephron tubules, collecting ducts and papillary ducts as well as in the epithelium aligning most of the cysts (Figure 2b). RET expression levels in kidneys from these founders were higher compared to the levels observed in mice from established transgenic lines (table 1).

Severe renal cystic disease in homozygous *CALC-WT-RET* line-49 transgenic mice

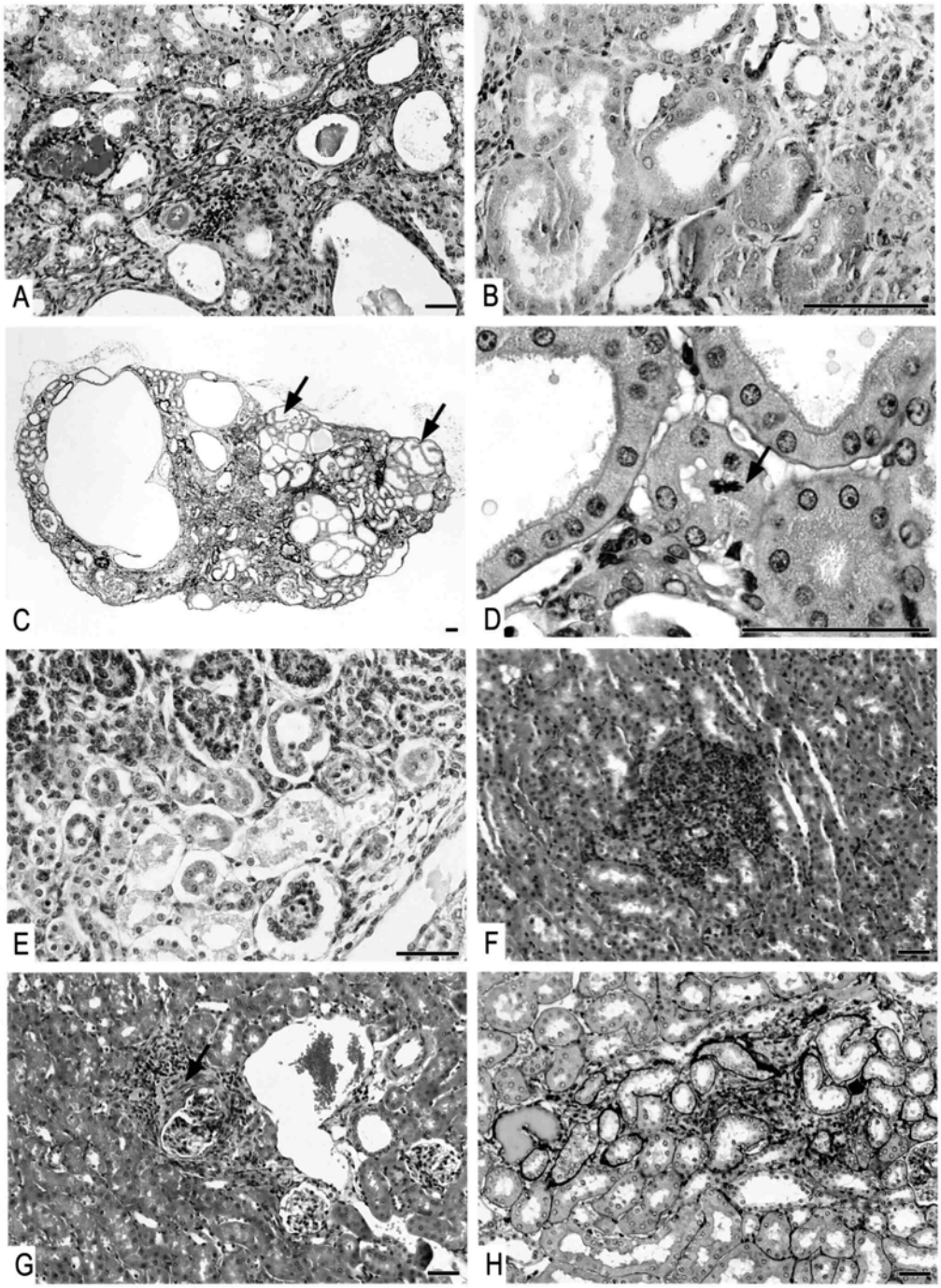
The renal cystic disease was associated with higher expression levels of RET in the kidneys from the affected founders as compared to mice from the established transgenic lines. We therefore studied the effects of higher transgenic RET expression by mating heterozygous mice of each transgenic line to obtain homozygous transgenic mice. No gross abnormalities were observed in homozygous offspring from lines-04, 09 and 33. However, part of the homozygous offspring from line-49 displayed a phenotype of severe growth retardation associated with signs of general illness. Three mice that presented with severe illness and abnormal growth from birth on had to be sacrificed at 2-3 months of age. All three presented with extremely advanced bilateral cystic kidney disease. The kidneys displayed a very high degree of atrophy of the tubular and interstitial structures as judged by the clustering of the basal membranes. The normal architecture of the kidneys of these mice had disappeared almost completely (Figure 2c). Cysts in these severely affected kidneys were large and often septated, suggesting a high proliferation rate of the epithelial cell layer. Ultrathin sections (1μm) were made from kidneys of these mice and 6 slides of each mouse were systematically scanned to count the number of mitotic figures. In the cyst epithelium an average of 9.8 (range 7-14) mitotic figures per slide was detected. Mitotic figures were present not only in the epithelium of the larger septated cysts but also in the epithelium of less affected tubules (Figure 2d). Mitotic figures were not detected at all in slides from non-transgenic kidneys. Despite the high proliferation rate in the epithelium of affected kidneys, no neoplastic outgrowth was observed, which suggested a compensatory high rate of apoptosis. Analysis of apoptotic DNA fragmentation was performed in these homozygous kidneys as well as in age-matched control heterozygous and non-transgenic kidneys. Only in DNA from the homozygous kidneys an apoptotic ladder was observed (data not shown).

Early renal cystic lesions arise in the medulla of the kidney

In trying to understand the pathophysiological mechanism underlying the renal cystic disease in line-49, we examined the earliest phases of cyst formation and expansion. The kidneys from homozygous line-49 new-born mice as well as mice at the age of 4 weeks, 8 weeks and 17 weeks were examined. Histopathology was performed on both kidneys from 3 mice for each age. No clear kidney abnormalities were observed in the newborn mice or in the mice of 4 weeks of age. Immuno-histochemistry for RET was performed on the three new-born homozygous mice as well as on three heterozygous line-49 mice and three non-transgenic new-born mice. In homozygous kidneys high levels of RET protein were present (Figure 2e). Staining was confined to the tubules and to the collecting ducts. In heterozygous new-born kidneys a lower, but still detectable, level of RET protein expression was observed, whereas RET expression was undetectable in the kidneys from non-transgenic new-born mice. These data indicate that the transgene is expressed in the kidneys at birth when endogenous RET protein expression is already downregulated.

Pathological lesions in kidneys were first observed in homozygous mice at 8 weeks of age. Cysts observed in these mice were solely found in the medulla of the kidney. Cysts were ligned by a single layer of flattened epithelium, were filled with dark staining proteinacious deposits

Figure 2. *Histopathology and immuno-histochemistry of CALC-WT-RET kidneys*.
(a) Renal cystic disease in 10 months old founder-32, showing cyst formation, proteineacious deposits and inflammatory areas (PAS). **(b)** RET immuno-histochemistry in 10 months old founder-42 cystic kidney. **(c)** Severely affected kidney from 3 months old homozygous transgenic line-49 mouse, showing atrophy of renal tissue and septation of epithelial cyst aligning (arrows) (PAMS). **(d)** Mitosis (arrow) in tubular epithelium of homozygous transgenic line-49 mouse (PAS). **(e)** RET immuno-histochemistry in kidney from newborn homozygous transgenic line-49 mouse. **(f)** Mononuclear cell infiltration in kidney from 8 weeks old homozygous transgenic line-49 mouse (HE). **(g)** Periglomerular sclerosis (arrow) in kidney from the same 8 weeks old homozygous transgenic line-49 mouse (HE). **(h)** Atrophy of renal tissue and progressive cyst formation in kidney from 17 weeks old homozygous transgenic line-49 mouse (PAMS), scale bar=100μm



and probably originated from the papillary ducts or collecting tubules.In addition to these medullary cysts other lesions were found. Multiple nodular, inflammatory mononuclear cell infiltrates and several areas with periglomerular sclerosis were observed (Figure 2f and g). In kidneys from homozygous mice analysed at 17 weeks of age, cysts were also observed in the cortex. Some of these cortical cysts originated from collecting tubules while others originated from the distal tubules. Cyst formation was associated with surrounding tubular atrophy manifested by basal membrane thickening and clustering (Figure 2h).

Kidneys from heterozygous mice from line-04, line-09, line 33 and line-49 were also examined at later age. Some heterozygous mice from line-49 presented with cysts in the medulla and the cortex from the age of one year on. In these mice, glomerular cysts and atrophy of renal tissues were also observed. In several other heterozygous mice of line-04, line-09 and line-33, lesions comparable with the very early stages of renal cystic disease were observed first at an age of 14-24 months. In control non-transgenic mice no renal cystic disease was observed at this age.

Progressive renal failure in homozygous *CALC-WT-RET* line-49 transgenic mice

From a group of 34 homozygous transgenic offspring from *CALC-WT-RET* line-49, five mice displayed signs of severe illness from birth on, demonstrated severe growth retardation and succumbed before the age of 4 months. Autopsy could be performed on three of these mice sacrificed after 2-3 months (see above) and all three presented with extremely advanced bilateral cystic kidney disease. To investigate renal function, we determined the concentrations of creatinine and urea in plasma from these animals and compared these with the levels of age-matched non-transgenic mice. In non-transgenic mice at 2-3 months of age, mean plasma creatinine concentration was 19.5 +/- 3.8 μmol/l (n=26). In the three homozygous line-49 mice plasma creatinine concentrations of 90, 102 and 98 μmol/l were found, respectively. In these three mice the plasma urea concentrations were also increased to 66.9, 67.5 and 27.3 mmol/l as compared to 5.2+/-1.0 mmol/l for non-transgenic mice (n=26). This indicated that normal renal function in these mice had ceased and that the cystic kidney disease resulted in severe end stage renal failure.

To investigate if renal defects could be detected and quantified also in the less severely affected homozygous mice, we compared the levels of plasma creatinine and urea in the other 29 homozygous mice with those in 47 non-transgenic and 13 heterozygous mice (Figure 3a and b). Comparison of the mean serum concentrations of creatinine and urea between non-transgenic and heterozygous transgenic animals did not reveal differences (18.9+/-3.7 μmol/l versus 17.2 +/-4.3 μmol/l and 6.1+/-1.5 mmol/l versus 6.3+/-1.5 mmol/l, respectively). In homozygous mice, however, both the mean creatinine level and the mean urea level were significantly increased to 35.5+/- 10.1 μmol/l and 10.1+/-4.4 mmol/l respectively ($P<0.001$). In addition, in all individual homozygous mice serum levels were higher than the mean level observed in the non-transgenic group. Comparison of creatinine and urea concentrations in individual homozygous mice revealed a strong linear correlation ($r=0.97$, $p<0.0001$, $n=29$) (Figure 3c) indicating that both parameters could be reliably used to determine the degree of renal failure.

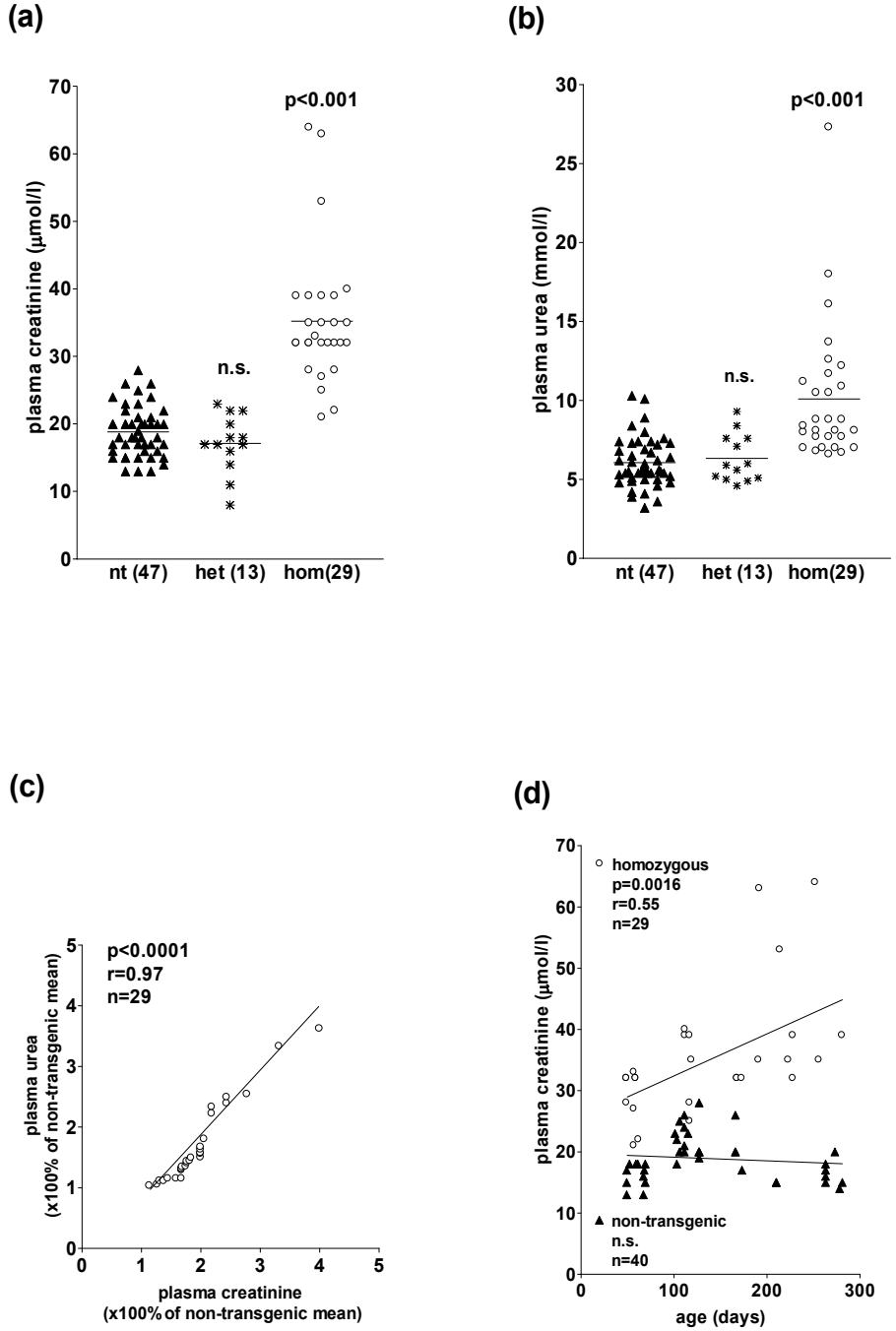


Figure 3. *Reduced renal function in CALC-WT-RET line-49 homozygous transgenic mice.* **(a)**Individual and mean plasma creatinine and **(b)** plasma urea levels in non-transgenic (nt), heterozygous (het) and homozygous (hom) *CALC-WT-RET* line-49 mice. The number of mice analysed is indicated between brackets. Horizontal bar is mean value. Comparison between each group of transgenic mice and the control group was done with the student's T-test. Significant differences are indicated. **(c)** Linear regression analysis between plasma urea and plasma creatinine levels in homozygous *CALC-WT-RET* line-49 mice, as expressed as percentage of the non-transgenic mean. Statistical P and r values and the number of mice (n) are indicated. **(d)** Linear regression analysis between plasma creatinine levels and age in non-transgenic mice and homozygous *CALC-WT-RET* line-49 transgenic mice. Statistical p and r values are indicated, n.s.=not significant, n=number of mice.

We calculated if there was a correlation between plasma creatinine level and age. In the homozygous mice an increase in mean plasma creatinine concentration was already observed at the youngest age investigated (49 days) and a significant increase of the creatinine concentration with increasing age was found ($p=0.0016;r=0.55;n=29$). A mean increase in creatinine concentration of 0.08 +/- 0.03 μmol/day was calculated for the group of homozygous mice, while no age related increase in creatinine concentration was present in non-transgenic mice (Figure 3d).
To investigate if there was progressive increase of plasma creatinine level in individual mice, we determined plasma creatinine concentration for a group of 5 mice at 2 months of age, and another group of 5 mice at 6 months of age and compared these with the concentrations in the same mice 8 weeks later. A mean increase of 6.2 μmol/l was found for the first group, indicating that there was progression of the severity of renal disease in individual mice in time. Also in the older mice a mean increase of 2.2 μmol/l was measured. These analyses indicated that in homozygous line-49 mice renal function had started to decline already at 7 weeks of age and that the severity of the disease increased in time. As a result, 15% of the homozygous *CALC-WT-RET* line-49 mice died before the age of 4 months, 35% died within 8 months and 47% succumbed to severe renal cystic disease before the age of one year.

Growth characteristics of *CALC-WT-RET* line-49 transgenic mice

The homozygous offspring from line-49 displayed a phenotype characterized by growth retardation. Reduced growth was not observed in homozygous offspring from lines-04, 09 and 33. We calculated a mean growth curve for homozygous and heterozygous transgenic mice from line-49, and from their non-transgenic littermates (Figure 4). Linear growth was observed during the first six weeks for all groups with no significant differences between male and female mice. For non-transgenic males and females the mean weight gain was 0.51+/-0.10 g/day and 0.46+/-0.10 g/day respectively. Growth velocity was slightly, but not significantly reduced in heterozygous mice: 0.36+/-0.05 and 0.38+/-0.04 g/day for males and females, respectively. In homozygous mice, however, a significant reduction in growth velocity was observed for both sexes. Mean weight gain was reduced to 0.27+/-0.04 and 0.26+/-0.03 g/day for male and female mice, respectively ($p<0.01$). After 6 weeks growth velocity reduced more rapidly in the homozygous mice and plateau weight was reached at a younger age as compared to non-transgenic and heterozygous mice. Adult weight of the homozygous mice ranged between 25%-81% of the mean weight of non-transgenic mice.

Correlation between growth retardation and renal failure in *CALC-WT-RET* line-49 mice.

Because the RET transgene was also expressed in the thyroid gland and brain, we investigated whether the reduced growth observed may have been caused by failure in the hypothalamus-pituitary gland-thyroid gland axis. Thyroxin (T4) concentrations in blood were determined for non-transgenic (72.0+/-20.5 nmol/l; n=4), heterozygous (54.8+/-16.0 nmol/l; n=9) and homozygous (56.5+/-10.0 nmol/l; n=10) mice. No significant differences were observed between these groups of mice varying in age from 5 to 19 weeks. All values measured were within the normal range, indicating that the reduced growth was not caused by a defect that leads to reduced T4 production.

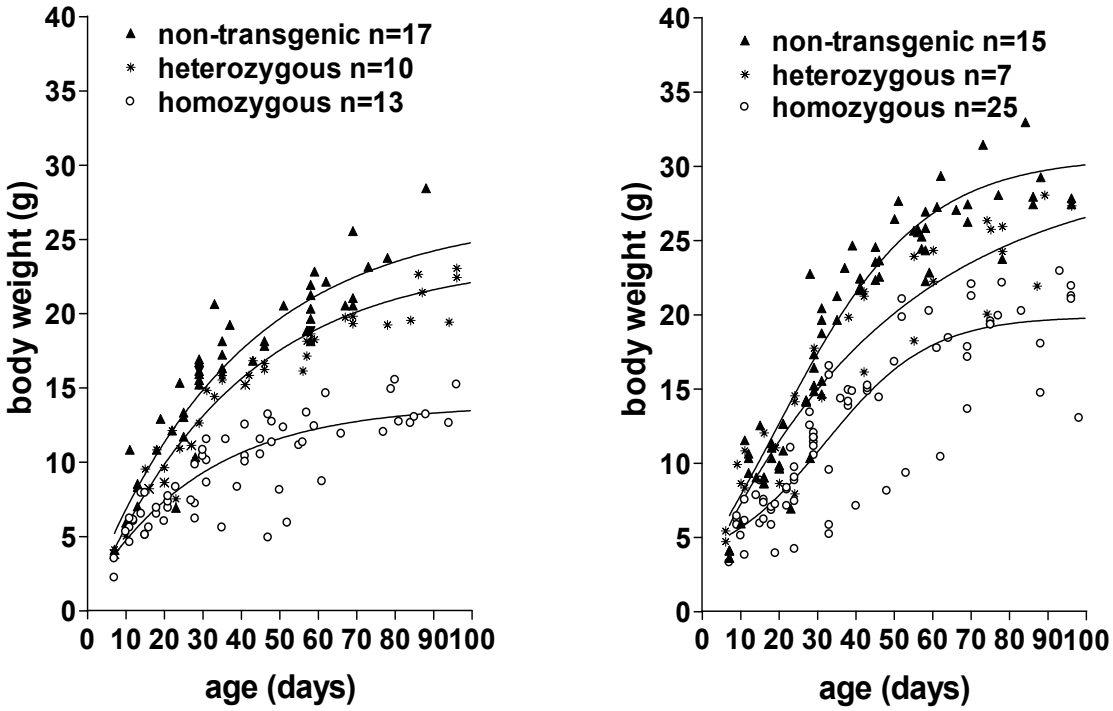


Figure 4. *Growth of female and male homozygous and heterozygous CALC-WT-RET line-49 transgenic mice and non-transgenic mice.*
Mean growth curves, expressed as total body weight, were calculated using non-linear regression analysis with Boltzman Equation, n indicates the number of mice with the indicated genotype used for the calculation. Individual measurement points are indicated.

Because homozygous mice with more severe growth retardation also presented with more severe renal cystic disease, a correlation between the degree growth retardation and the degree of renal failure was suspected. For each individual homozygous line-49 mouse we calculated both the creatinine levels and the body weight, expressed as percentage of the mean non-transgenic level at the same age. A highly significant negative correlation between the values was found (Figure 5), indicating that the observed growth retardation and the renal cystic disease were associated phenomena.

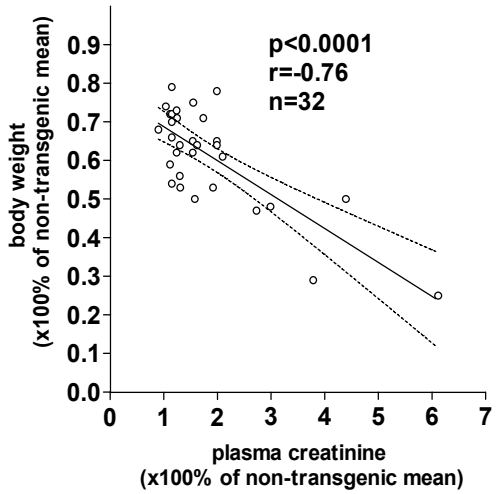


Figure 5. *Correlation analysis between severity of growth retardation and severity of renal failure*.
Body weight and plasma creatinin in homozygous *CALC-WT-RET* line-49 transgenic mice are expressed as percentage of non-transgenic mean. Statistical p and r values and the number of mice analysed (n) are indicated

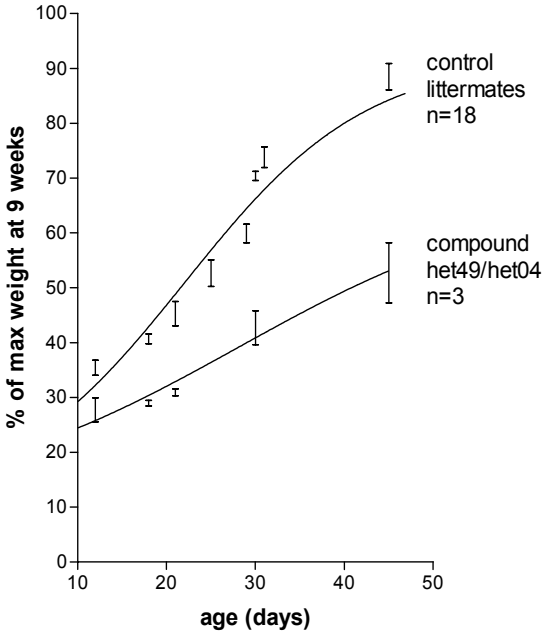


Figure 6. *Growth retardation in compound heterozygous mice from CALC-WT-RET line-49 and line-04.*
Growth is expressed as percentage of the maximal weight measured at 9 weeks of age. The curves were calculated using non-linear regression analysis with Boltzman Equation. n indicates the number of mice with the indicated genotype monitored. The control group consisted of non-transgenic mice (n=12) and heterozygous mice of each transgenic line (n=6).

Growth retardation and renal cystic disease in compound heterozygous *CALC-WT-RET* transgenic mice

The fact that the growth retardation phenotype was observed only in the homozygous offspring from transgenic line-49 and not in homozygous offspring from lines 04, 09 and 33 could be an indication that this phenotypic feature was caused by homozygous inactivation of a gene due to the transgene integration, rather than too high transgenic RET expression. We therefore increased RET expression in line-49 by mating heterozygous line-49 transgenic females with heterozygous line-04 transgenic males and we monitored the offspring for growth abnormalities. Three compound heterozygous mice were obtained which presented with significant growth retardation compared to heterozygous and non-transgenic littermates and to age-matched controls from other nests (Figure 6). One of the compound heterozygous mice had to be sacrificed at the age of 10 weeks. Pathological analysis disclosed severe renal cystic disease. This indicated that the growth retardation phenotype and the associated renal cystic disease were not caused by homozygous inactivation of another gene, but were most likely caused by the high and postnatally sustained expression level of transgenic RET in the kidneys

DISCUSSION

We have generated *CALC-WT-RET* transgenic mice that express the human wildtype *RET* proto-oncogene under control of a human calcitonin gene promoter. In addition to the anticipated expression of the transgene in the thyroid gland C-cells, we observed aberrant postnatal RET expression in the kidney in all transgenic lines and founders examined. The level of transgenic RET protein expression varied between the different transgenic lines obtained. In normal mice, endogenous RET expression in the kidney is observed between day 8 and 17.5 of embryonic development (Pachnis *et al.,* 1993). During this stage RET is essential for kidney development.
The *CALC-WT-RET* transgenic mice presented with renal cystic disease. A correlation was observed between the severity of the kidney disease and the level of RET expression in the kidneys from the founders and mice from each of the established transgenic lines. Homozygous mice from line-49 presented with the highest level of renal RET

expression and with the most severe phenotype. However, variability in the severity of the renal cystic disease was observed between individual mice. Approximately 15% of these mice succumbed to renal cystic disease within 4 months. The majority of the homozygous *CALC-WT-RET* line-49 mice, presented with histologically normal kidneys at birth. Variation in the severity of renal abnormalities has also been reported to occur within lines of Hoxb7-RET transgenic mice, with kidneys ranging from highly dysplastic to normal (Srinivas *et al.,* 1999). The reason for this variability is not clear. In homozygous *CALC-WT-RET* line-49 mice, increased concentrations of plasma creatinine and urea at young age pointed to an early onset of reduced renal function. With increasing age the gradual progression of the renal disease was also reflected by increasing concentrations of these biochemical parameters as well as by histopathological examination. As a result, almost 50% of the homozygous *CALC-WT-RET* line-49 mice had succumbed to severe renal disease before the age of one year.

With respect to the pathogenic mechanism of the observed renal disease, much information could be obtained from the earliest lesions. In kidneys from new-born and adult transgenic mice, ahigh level of RET expression was seen in the more distal parts of the tubular system. Associated with this finding we observed that the earliest cysts arose in the medulla of the kidney, originating from the more distal part of the nephrons. This suggested that the tubular structures with the highest RET expression were first prone to cyst development. At later stages, also cystic involvement of the more proximal tubules became apparent. At more advanced stages the pathology was characterised by atrophy of renal tissue and further progression of cyst formation, with involvement of all nephron areas including the glomeruli. These data support a model in which early, distal nephron cyst formation played an important role in triggering further cyst development in other parts of the kidney. Associated with the early medullary cyst formation, the mice presented with periglomerular sclerosis and mononuclear cell infiltrations. Whether these lesions were secondary to the medullary cysts or played an important causative role in the cyst formation is not clear. However, the occurrence of these lesions early in the pathogenesis in the *CALC-WT-RET* mouse model suggested a relationship with cyst formation.

The *CALC-WT-RET* line-49 transgenic mice presented with a phenotype of growth retardation. Serum T4 concentrations in homozygous *CALC-WT-RET* transgenic mice were within the normal range. This indicated that the growth abnormalities were not caused by disturbance of the hypothalamus-pituitary gland-thyroid gland axis as observed, for instance, in the Snell dwarf mouse model, in which serum T4 levels are significantly reduced (Van Buul-Offers *et al.,* 1981). The highly significant correlation between the severity of growth retardation and the severity of renal failure in individual mice suggested that these phenotypes might be causally related. Since most conditions that impair renal function at young age can impair growth, we hypothesise that aberrant expression of RET leads to early disturbance of kidney function, which then results in reduced growth.

A potential role for RET in kidney pathology in man has not yet been clearly established, but it is of interest that increased plasma levels of GDNF, one of the ligand for RET have been reported in patients with chronic renal disease (Onodera *et al.,* 1999). Furthermore, a family with a germline *RET* missense mutation was recently described in which

individual affected members presented with renal agenesis (Lore *et al.,* 2000). An association between medullary sponge kidney disease and MEN 2 (Diouf et al., 2000) and between renal dysplasia and MEN 2 (McEntire et al., 2003) were reported, raising the question of a causal relationship for RET. Furthermore, increased RET and GDNF expression in collecting duct cysts of polycystic kidney patients was reported and it was suggested that deregulated RET signalling may have contributed to proliferation of the collecting duct epithelium in an autocrine/paracrine manner (Lee et al., 2002). Further experimental analys of the *CALC-WT-RET* transgenic mice may provide more information for understanding the links between RET, renal disease and disturbed growth.

MATERIALS AND METHODS

Transgene construct

The *CALC-WT-RET* transgene construct was described before (Acton *et al.,* 2000). In short, a human *RET* cDNA fragment encoding the short (RETp9) isoform was coupled to a promoter fragment containing the first 24 bp of the non-coding exon 1 and 1.5 kb of upstream sequences from the human calcitonin (*CALC-1*) gene. Downstream from the RET cDNA we introduced a 0.9 kb fragment from the SV40 T antigen, containing consensus polyadenylation signals and a small intron to ensure proper processing of pre-mRNA produced from the transgene.

Transgenic mice

Transgenic mice were generated at the transgenic mouse facility of the Central Animal Laboratory (GDL) in Utrecht. The transgene construct was injected in fertilised oocytes from (C57BL6xDBA2)F1 mice. Transgenic founders were mated with C57BL6 mice to generate transgenic lines, which were further bred to the C57BL6 background. Transgenic offspring were intercrossed to obtain homozygous or compound heterozygous transgenic mice. The transgene was detected using quantitative Southern spot blot analysis. In short, 500 ng of tail tip DNA was spotted on a nitro-cellulose filter and hybridised with a transgene-specific SV40 probe labelled with ^{32}P dCTP by random priming. Hybridisation signals were visualised on Phosphor Imager screens. To discriminate between heterozygous and homozygous transgenic mice, the positive signals were quantified using Image Quant software. Mice were kept under standard laboratory conditions.

Histopathology

Mice showing severe signs of general illness were sacrificed when moribund. For histopathology and immuno-histochemistry, fresh tissues were fixed in 3.7% paraformaldehyde solution, dehydrated and embedded in paraffin. Sections of 1-5 μm were cut. Deparaffinized sections were routinely stained with hematoxylin and eosin (HE). For evaluation of kidney pathology additional staining with the Periodic Acid Shiff (PAS), Periodic Acid Methenamine Silver (PAMS), Elastica van Gieson (EvG) and Fibrine methods were performed according to standard operating procedures. For immuno-histochemical detection of RET a polyclonal antiserum, Ret44, raised in rabbits was used (Van Weering *et al.,* 1995). After blocking of endogenous peroxidase (PO) with 1.5 % H_2O_2, the slides were incubated with the antiserum at a dilution of 1:500 for 1 hour at room temperature (RT). After washing, swine anti rabbit PO antibody (DAKO) was applied, followed by an incubation with rabbit anti swine PO antibodies (DAKO) for 30 minutes each at RT. Peroxidase activity was detected with Di-amino-benzidine tetra-hydrochloride (DAB, Sigma). Slides were

counterstained with Mayer's hematoxylin, dehydrated and mounted in Pertex. Slides incubated without primary antiserum served as negative control.

RNA expression analysis using RT-PCR

For RT-PCR, fresh tissue was isolated and snapfrozen in liquid nitrogen and stored at –80 ºC. Total RNA was isolated from the tissues using the trizol method and cDNA was synthesised using oligo-dT and random hexamers. Proper cDNA synthesis was routinely checked with primers for beta actin. For the detection of transgenic (human) *RET* transcripts a human-specific forward primer 5' TGGAGACCCAAGACATCAAC 3' and reverse primer 5' GTGGGCAAACTTGTGGTAG 3' were used. For detection of endogenous mouse *RET* mRNA we used degenerate primers recognising both the human and the mouse RET gene. The forward degenerate primers 5'CATTGTTGG(G/**A**)G(A/**G**)ACACGAGC 3' and reverse degenerate primers 5' GCTTGTG(G/**C**)(G/**C**)CA(A/**T**)(A/**G**)CTTG TGG 3' were used. Nucleotides that differ between human and mouse *RET* are indicated between brackets and the mouse nucleotide is indicated in bold. For GFR α-1 mRNA detection the forward primer 5'CATGTTCCTAGCCACTCTGT 3' and reverse primer 5'TCCAGTAGGTCATTTCCCTG 3' were applied as described (Buj-Bello *et al.,* 1997)

Apoptotic DNA fragmentation analysis.

For investigation of apoptosis, total DNA was electrophoresed immediately from freshly frozen tissue after incubation in lysis buffer as described previously (Van Hoffen *et al.,* 1998).

Plasma creatinine, urea, and thyroxin (T4)

Mouse blood was collected from the retrobulbar, intraorbital, capillary plexus after ether anaesthesia and stored at 4º C prior to centrifugation at 1500 rpm for 15 minutes. Plasma was collected and stored at –20° C. The creatinine concentration was measured with a Vitros 250 System apparatus (Ortho Clinical Diagnostics, Rochester) using an enzymatic two point rate method, and urea was determined colorimetric with the Vitros 250 System using the urease method according to the instructions of the manufacturer. Thyroxin (T4) concentrations were determined using the Chiron Diagnostics ACS:180 T4 assay according to the instructions of the manufacturer.

Growth

Growth of the mice was monitored by periodic measurement of total body weight. Mean growth curves were calculated by non-linear regression using the Boltzman equation with Graph Pad Prism software.

Statistics

Statistical analysis, linear regression analysis and correlation analysis were performed with Graph Pad Prism and Graph Pad Stat Mate software.

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Chapter 3

Multiple Endocrine Neoplasia type 2B mutation in human *RET* oncogene induces medullary thyroid carcinoma in transgenic mice.

Dennis S. Acton[1,] Danny Velthuyzen[1], Cornelis J.M. Lips[1] and Jo W.M. Höppener [2,1]

Department of Clinical Endocrinology, Division of Internal Medicine and Dermatology[1], department of Metabolic and Endocrine Diseases, Division of Biomedical genetics[2], University Medical Center Utrecht, The Netherlands

Abstract

Multiple Endocrine Neoplasia type 2B (MEN 2B) is a familial cancer syndrome, in which the cardinal feature is medullary thyroid carcinoma (MTC), a malignant tumor arising from the calcitonin producing thyroid C-cells. MEN 2B is associated with a germline point mutation in the *RET* proto-oncogene, leading to a Met→Thr substitution at codon 918 in the kinase domain, which alters the substrate specificity of the protein. We used the human calcitonin gene (*CALC*-I) promoter to generate transgenic mice expressing either the human *RET* oncogene with the MEN 2B-specific 918 Met→Thr mutation (*CALC-MEN2B-RET*) or the human non-mutated *RET* proto-oncogene (*CALC-WT-RET*) in the C-cells. At the age of 20-22 months three out of eight *CALC-MEN2B-RET* transgenic founders presented with macroscopic bilateral MTC. In two founders nodular C-cell hyperplasia (CCH) was observed. Thyroid abnormalities were never observed in *CALC-WT-RET* transgenic mice or control non-transgenic mice analyzed at this age. In some mice from established *CALC-MEN2B-RET* transgenic lines nodular CCH was observed from eight months on whereas MTC was detected in 13% of mice from one *CALC-MEN2B-RET* line, from the age of 11 months on. These result show for the first time that the MEN 2B mutation in the *RET* oncogene predisposes mice for MTC.

Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor that originates from the calcitonin producing and secreting C-cells in the thyroid gland (Horn, 1951). MTC is the most important clinical manifestation of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome (Sipple *et al*., 1961). MEN 2 is a familial cancer syndrome, inherited as a monogenic autosomal dominant trait (Steiner *et al*., 1968). Apart from MTC, this syndrome is characterized by the occurrence of tumors originating from the chromaffin cells of the adrenal medulla (pheochromocytoma). In addition to these tumors, parathyroid hyperplasia is observed in MEN 2A (Sipple *et al.,* 1961, Steiner *et al*., 1968) while in MEN 2B ganglioneuromas of the intestinal tract and mucosal neuromas are found, as well as musculoskeletal anomalies (Carney *et al*., 1976). Mortality in MEN 2 patients is mainly caused by MTC metastases.

MEN 2 is associated with germline mutations in the *RET* proto-oncogene leading to aminoacid substitutions in its encoded transmembrane receptor tyrosine kinase. The RET receptor

tyrosine kinase (Takahashi *et al*., 1988) serves in conjunction with the GFRα co-receptor, as the receptor for the GDNF family of neurothrophic factors (reviewed in Rosental, 1999). Three RET isoforms that differ by 9, 43 and 51 aminoacids in the carboxyterminal domain are encoded due to alternative splicing of the pre-mRNA (Tahira *et al*., 1990, Meyers *et al*., 1996). In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein, which results in constitutive kinase activity (Mulligan *et al*., 1993, Donis-Keller *et al*., 1993). Mutations associated with MEN 2A cause aberrant dimerization of RET via the formation of disulfate bonded homodimers (Assai *et al*., 1995, Santoro *et al*., 1995). In MEN 2B the most common mutation results in a Met→Thr substitution at codon 918 in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40 %) of sporadic MTC cases (Hofstra *et al*., 1994, Eng *et al*., 1994, Carlson *et al*., 1994). The MEN 2B mutation appears to modify the substrate specificity of the tyrosine kinase (Santoro *et al*., 1995). All MEN 2 mutations convert the *RET* proto-oncogene into a dominantly acting transforming gene in humans as expression of the non mutated allele is usually retained in tumor tissue (Landsvater *et al*., 1996), although somatic MEN 2B mutations have been described in tumors from MEN 2A patients (Marsh *et al*., 1996).

To study biological effects of the MEN 2B mutation in the *RET* gene on the thyroid C-cells, we applied transgenic mouse technology to generate mice with C-cell specific expression of a human *RET* transgene with the MEN 2B-specific 918 Met→Thr mutation. As a control we generated mice with a non-mutated human *RET* transgene.

Results

A transgene construct was made with a human *RET* oncogene cDNA encoding the RET9 isoform in which the MEN 2B-specific codon 918 Met→Thr substitution was introduced by site directed mutagenesis. A 1.5 kb human calcitonin gene (CALC-I) promoter fragment was used to direct its expression to the thyroid gland C-cells and an SV40 polyA signal and intron were introduced to obtain good expression of the transgene: *CALC-MEN2B-RET*. As a control a similar transgene construct, but with a non-mutated (wild type) human *RET* proto-oncogene was generated: *CALC-WT-RET*. This enabled discrimination between effects caused by the point mutation and effects caused by the CALC promoter driven expression. Transgenic mice were generated by injection of pro-nuclei from fertilized oocytes from (C57BL6xDBA2) F1 mice and eight founder mice were obtained for each of the two constructs. From these founders, five *CALC-MEN2B-RET* and four *CALC-WT-RET* transgenic lines were established by backcrossing to the C57BL6 strain. The numbers of (transgenic) offspring from each founder is indicated in Table 1. The fact that not all founders established a transgenic line was either due to low reproduction capacity or lack of germline transmission. To test whether the transgene was expressed we isolated RNA from several organs from mice from all transgenic lines established and applied RT-PCR using human *RET*-specific primers. The transgene specific transcript was detected in the thyroid glands in all transgenic lines obtained. In addition, transgene expression was observed in brain, lung and kidney. This method did not allow a quantitative comparison of the expression level between the different transgenic lines. The presence of the MEN 2B-specific point mutation in the transgenic mRNA was detected by

diagnostic restriction enzyme digestion with *Fok1* (Hofstra *et al*., 1994) and was confirmed by direct sequencing of the RT-PCR products obtained.

Mice from all transgenic lines showed normal growth and reproduction and no gross abnormalities became apparent during the first ten months. We therefore sacrificed two transgenic mice and two non-transgenic littermates from each line at the age of 4, 8 and 12 months for histological analyses of the thyroid gland. The thyroid gland was examined by systematically making microscopic slides from the whole thyroid gland and performing immuno-histochemistry for calcitonin to identify the C-cells. In humans, diffuse C-cell hyperplasia (CCH) is characterized by both an increase in the numbers of C-cells per follicle and by an increase in the number of follicles with C-cells and strict criteria have been postulated (Lips *et al*., 1994). In mice, however the anatomical distribution of C-cells in the thyroid gland did not allow a clear distinction of diffuse CCH from normal distribution, as also in non-transgenic mice the C-cells were often clustered in high numbers around individual follicles. A clear distinction however could be made between normal (diffuse) distribution and nodular CCH. Figure 1A shows calcitonin immuno-histochemistry on the thyroid gland from an 8 months old mouse from the *CALC-MEN2B-RET* transgenic line-06. The right hand lobe of the thyroid gland from this mouse shows normal diffuse distribution of calcitonin positive C-cells. In the left lobe, besides diffuse distribution, a nodule of C-cells was observed (arrow). In this lobe a parathyroid gland (P), not staining for calcitonin, was visible. At larger magnification (Figure 1B), abnormal accumulation of C-cells and growth into the follicle lumen, leading to disruption of normal follicular architecture, was observed. These findings are hallmarks for nodular CCH. The nodule of C-cells comprised an area with a crossectional size of ±0.6 mm.

At the age of twenty months the first transgenic *CALC-MEN2B-RET* founder became ill and was sacrificed. In this mouse large bilateral thyroid tumors had developed. The tumors had sizes of 12 mm and 7 mm in diameter. Histopathology of these tumors revealed areas of large rounded epithelial cells, divided by fibrous septa. Tumor cells showed nuclear pleiomorphism and frequent mitosis indicative for malignancy. Amyloid deposits with necrotic cell remnants and cyst formation and capsularisation of the tumor were observed (Figure 1C). In addition to the large tumors, multiple microscopic foci of MTC were observed in the normal thyroid tissue. Figure 1D shows calcitonin immuno-histochemistry on normal parafollicular C-cells (arrows) and micro-MTC's (M). The fields of tumor cells stained positive for calcitonin, unambiguously demonstrating that these were MTC's. Heterogeneity in calcitonin staining of tumor cells was observed in the micro-MTC's as well as in the large tumors in this mouse (Figure 1D and 1E), a feature that is also seen in human MTC. Analyses of the other *CALC-MEN2B-RET* founders at this age revealed two more founders with large bilateral MTC. In Figure 1F the tumors from the founder of *CALC-MEN2B-RET* line 42 are shown, presenting with extensive capsular invasion and vascularisation with associated hemorrhage, in addition to the MTC- features described above. In two other *CALC-MEN2B-RET* founders nodular CCH was observed while three *CALC-MEN2B-RET* founders did not show clear thyroid abnormalities. Additional pathological lesions in these mice included lung carcinoma and lymphoma.

Figure 1. *Thyroid pathology in CALC-MEN2B-RET transgenic mice.*
A and B: thyroid gland from an 8 months old *CALC-MEN2B-RET* line-06 transgenic mouse. C, D and E: MTC and thyroid gland from *CALC-MEN2B-RET* founder-50. F: MTC from *CALC-MEN2B-RET* founder-42. Bar indicates 100μm. P= parathyroid gland, M=micro-MTC. Method: Fresh tissues were fixed in 3.7 % paraformaldehyde, dehydrated and embedded in paraffin. Serial sections of 5 μm were cut. Deparaffinized sections were stained with hematoxylin and eosin (C and F) or used for immuno-histochistry (A, B, D and E).
After blocking endogenous peroxidase (PO) with 1.5% H_2O_2, a rabbit polyclonal anti-calcitonin antibody (DAKO) at 1:8000 dilution was applied at room temperature (RT) for one hour. The second swine anti-rabbit PO (DAKO) and third rabbit anti-swine PO (DAKO) were both used at 1:50 dilutions for 30 minutes at RT. Diaminobenzidine tetrahydrochloride (DAB, Sigma) was used as substrate for PO activity. Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted with DePex. Samples without the use of primary antibody served as negative controls.

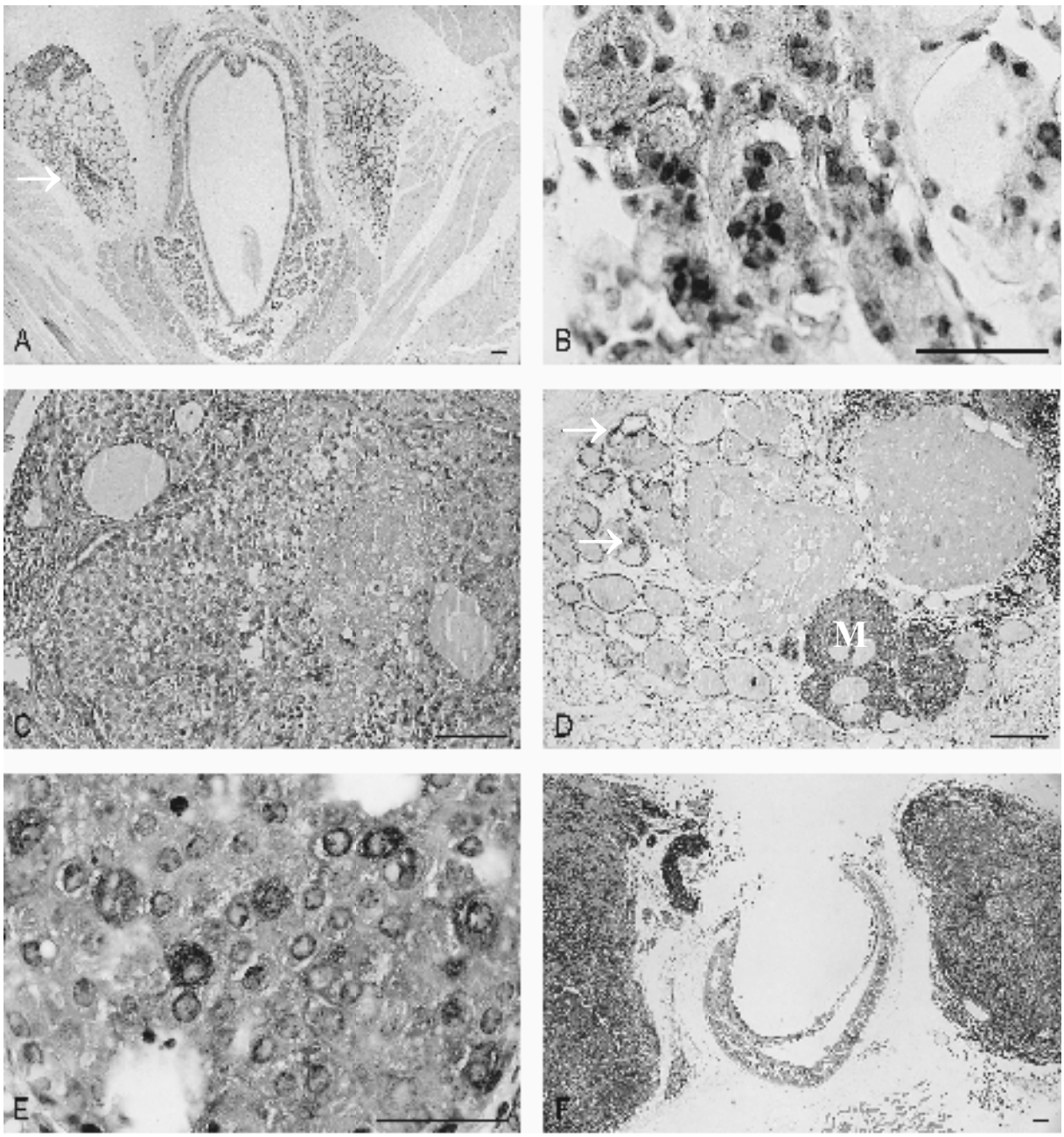

Table 1.
Thyroid pathology in CALC-MEN2B-RET transgenic founders

Founder	sex	Trans mission	Thyroid	Age (mnths)	Plasma calcitonin
2B06	F	8/21	CCH	22	3 ng/L
2B08	F	13/25	Normal	22	2 ng/L
2B27	M	0/47	MTC	20	1140 ng/L
2B28	M	5/44	CCH	22	3 ng/L
2B36	M	19/36	Normal	20	< 1 ng/L
2B41	F	0/0	Normal	22	2 ng/L
2B42	F	1/27	MTC	20	2300 ng/L
2B50	M	0/0	MTC	20	3900 ng/L

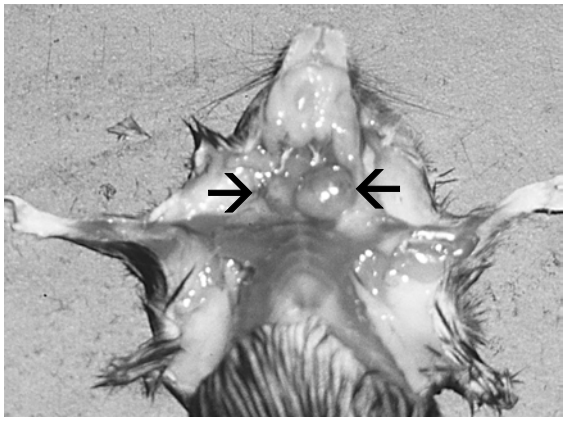

Figure 3
Macroscopic bilateral medullary thyroid carcinoma in a *CALC-MEN2B-RET* line 42 mouse, diagnosed with MTC at the age of 11 months by basal plasma calcitonin concentration of 770 ng/L.

None of the four *CALC-WT-RET* founders or three age-matched non-transgenic control mice analyzed at this age presented with thyroid gland abnormalities. In these mice lymphomas were also observed, while the other *CALC-WT-RET* founders died at earlier age from lymphoma or renal cystic disease (Chapter 2).

Increased basal plasma concentrations of calcitonin are a biochemical indication for MTC. We therefore measured basal calcitonin levels in plasma from the transgenic founders and control non-transgenic age-matched mice using a chemo-luminiscence assay (Nichols Diagnostics). The detection limit of the assay was 1 ng/L. Of the four *CALC-WT-RET* founders or the three non-transgenic mice none had a detectable level of calcitonin. The three tumor-bearing mice had a tremendous increase in plasma calcitonin concentration (1140-3900 ng/L). The mice with nodular CCH had a low but detectable basal plasma calcitonin level of 3ng/L and also in two founders in which no clear nodular CCH was found by histology a detectable plasma calcitonin level of 2ng/L was observed. In one *CALC-MEN2B-RET* founder plasma calcitonin could not be detected (Table 1).
Since the measurement of basal plasma calcitonin levels may give an indication for the presence of MTC or nodular or diffuse CCH, we monitored the *CALC-MEN2B-RET* transgenic mice and a group of non-transgenic littermates from 9-24 months of age by this method. In the majority of non-transgenic littermates (86%) plasma calcitonin could not be detected. However, 5 out of 37 (14%)

presented with basal plasma calcitonin levels of 1-2ng/L, which may be a reflection of diffuse CCH also observed with histological analysis in some non-transgenic mice. Similar percentages were found for three *CALC-MEN2B-RET* transgenic lines of which the founder had not developed MTC. In *CALC-MEN2B-RET* line-06 a significantly larger percentage of mice (33%, $P<0.05$) presented with detectable basal plasma calcitonin levels. In 77 % of the mice from *CALC-MEN2B-RET* line-42 a plasma level of 1-7 ng/L could be detected, while only three mice presented with undetectable levels (figure 2).
In this line-42, in addition to the founder, a MTC was diagnosed in two other mice out of a total of 23 (13%). One presented with a plasma calcitonin level of 29 ng/L at the age of 17 months, while the other had a plasma calcitonin level of 770 ng/L at the age of 11 months. This latter mouse was sacrificed and presented with large bilateral MTC (Figure 3). These data indicate that in *CALC-MEN2B-RET* line-42 there is predisposition for CCH and subsequent progression to development of MTC.

For establishment of primary cell cultures from the *CALC-MEN2B-RET*-induced murine MTC small pieces from a tumor from a *CALC-MEN2B-RET* line-42 transgenic mouse were removed aseptically and placed in sterile PBS. The tissue was cut with two scalpel blades into very small pieces. After washing with PBS, the tissue was used directly for culturing in DMEM supplemented with 10% FCS, After 48 hours non-adherent cells and tissue clumps were removed and fresh medium was added. After two weeks of culturing medium was refreshed. Inspection of the cultures revealed fibroblasts and epithelial cells growing as colonies. After four weeks of culturing approximately 50% of confluency was reached and all medium was refreshed. To determine whether these tumor cells produce and secrete calcitonin, the culture medium was collected after a two weeks culture period and calcitonin concentration in the culture medium was measured for two separate cultures. Whereas in non-conditioned culture medium a calcitonin concentration of 3 ng/L was measured, the media conditioned for two weeks had calcitonin levels of 1000 and 550 ng/L, respectively, indicating that the cultured tumor cells were still highly differentiated and capable of producing and secreting calcitonin. Upon further passage however the growth rate of the epithelial cells decreased and the cultures stopped growing after 3-4 months.

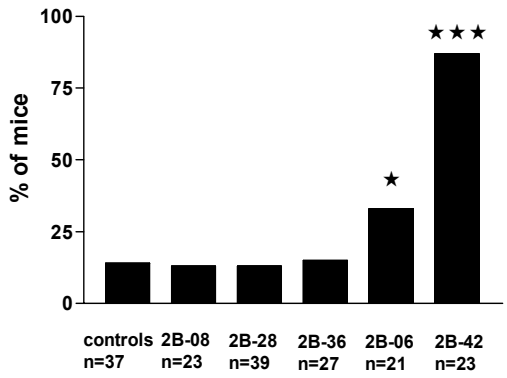

Figure 2. *Percentage of mice with CCH in CALC-MEN2B-RET transgenic mice.*
Method: Basal plasma calcitonin concentration was determined in 200μl plasma samples from mice from each indicated *CALC-MEN2B-RET* line and control non-transgenic littermates, using a chemi-luminescence assay (Nichols Diagnostics). Detection limit of the assay was 1 ng/L. The percentage of mice with detectable basal plasma calcitonin levels (bars) and the total number of mice for each group is indicated (n). Comparison between each group of transgenic mice and the control group was done with the student's T-test. Significant differences are indicated * $P<0.05$ and *** $P<0.0001$.

Discussion

Using transgenic mouse technology we generated a murine model for MTC. The *CALC-MEN2B-RET* transgene induced MTC in three of eight founders. From one of these a transgenic line-42 with predisposition for MTC development was established. One transgenic line-06 presented with a significantly increased number of mice with nodular or diffuse CCH. The fact that not all transgenic lines show predisposition for MTC development may be explained by a difference in expression level of the transgene in the thyroid gland C-cells. Development of a MEN 2B phenotype was already before postulated to be sensitive to gene dosage. Both for transgenic mice in which a *MEN2B-RET* transgene was expressed in the developing sympathetic and enteric nervous system (Sweetser *et al*., 1999) and for a MEN 2B RET mouse strain generated by homologous recombination (Smith-Hicks *et al*., 2000). In this latter mouse strain, surprisingly, MTC has not yet been observed. In contrast to these mice, the *CALC-MEN2B-RET* mice harbor a multicopy transgene under the control of a heterologous promoter, which may account for the difference in development of MTC. Also in transgenic mice with C-cell expression of a *MEN2A-RET* transgene variability in penetrance of the MTC-phenotype between transgenic lines was observed (Michiels *et al*., 1997). In addition to the results obtained with the MEN 2A transgenic mice, involvement of MEN 2A mutations in MTC-induction in mice has been demonstrated in RB +/- and P53 +/- double mutant mice. In MTC from these mice somatic *MEN 2A RET* mutations were detected. The *MEN 2B RET* mutation, however, was not found (Coxon *et al*., 1998). The data presented here are the first demonstrating the involvement of the *MEN 2B RET* mutation in MTC development in mice.

In human MEN 2 patients development of MTC is believed to proceed in several steps. Diffuse C-cell hyperplasia is regarded as a preneoplastic state from which, upon acquirement of additional oncogenic hits and further progression, first hyperplastic nodular foci and finally multicentric and bilateral MTC's develops. A similar phenotypical sequence is observed in the *CALC-MEN2B-RET* transgenic mice. The tumors that finally developed in these mice were morphologically similar to human MTC. The tumor cells produced and secreted calcitonin, which results in highly elevated basal plasma calcitonin levels. High levels of calcitonin production and secretion were maintained when the tumor cells were grown in culture.

The incomplete penetrance and the variable latency period for MTC development in the *CALC-MEN2B-RET* transgenic mice suggest that there is a requirement for additional oncogenic events. Analyses of the MEN 2B RET-induced MTC may provide clues for the identification of these additional hits. By crossing the *CALC-MEN2B-RET* mice with mice harboring mutated tumor suppressor genes or activated oncogenes implicated in MTC development or progression we can further address this issue.

In addition, the *CALC-MEN2B-RET* mice should constitute a useful model for the evaluation of new diagnostic methods and to test novel forms of therapy, as no curative therapy is currently available for MTC.

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Chapter 4

Involvement of P53 inactivation in RET-induced medullary thyroid carcinoma development in human patients and transgenic mice.

Dennis S. Acton[1], Roel de Heus[1], Janine F.M. Roijers[1], Cornelis J.M. Lips[1] and Jo W.M. Höppener [2,1]

Department of Clinical Endocrinology, Division of Internal Medicine and Dermatology[1], department of Metabolic and Endocrine Diseases, Division of Biomedical Genetics[2], University Medical Center Utrecht, The Netherlands

Abstract

Medullary thyroid carcinoma (MTC) develops sporadically or in patients with the hereditary cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN 2), caused by germline mutations in the *RET* proto-oncogene. Previously, we generated *CALC-MEN2B-RET* mice that developed MTC, preceded by C-cell hyperplasia (CCH), with low incidence and a variable latency period, suggesting the involvement of additional oncogenic events. We studied a potential role for the P53 tumor suppressor in human and mouse MTC development. In a panel of human MEN 2 and sporadic MTC samples immuno-histochemistry revealed P53 staining, which is indicative for mutated P53, in three of 15 tumors analysed. In MTC samples from four *CALC-MEN2B-RET* transgenic mice nuclear P53 staining was observed in two cases. A possible causal relation between *P53* gene inactivation and MTC development was studied by monitoring *CALC-MEN2B-RET* transgenic mice lacking one or both alleles of the *P53* gene. Four of six composite *CALC-MEN2B-RET* ;*P53-/-* mice analysed presented with MTC at the age of 3-5 months, while the other two had CCH. At this age MTC was not detected in five *P53-/-* or 14 *CALC-MEN2B-RET* transgenic control mice. A significantly increased incidence of RET-induced MTC development was also observed at later age in composite *CALC-MEN2B-RET;P53+/-* mice compared to controls, in the second year of live. These data indicate that inactivation of P53 contributes to Ret-induced MTC development

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Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor that originates from the calcitonin (CT) producing and secreting C-cells in the thyroid gland (Horn, 1951). MTC is mainly found as a sporadic and solitary tumor. In addition, MTC is the most important clinical manifestation of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome (Sipple *et al*., 1961). MEN 2 is a familial cancer syndrome, inherited as a monogenic autosomal dominant trait (Steiner *et al*., 1968). Apart from MTC, this syndrome is characterized by the occurrence of tumors originating from the chromaffin cells of the adrenal medulla (pheochromocytoma). In addition to these tumors, parathyroid hyperplasia is observed in MEN 2A (Sipple *et al*., 1961, Steiner *et al*., 1968) while in MEN 2B ganglioneuromas of the intestinal tract and mucosal neuromas are found, as well as musculoskeletal anomalies (Carney *et al*., 1976). MEN 2 is associated with

germline mutations in the *RET* proto-oncogene, leading to aminoacid substitutions in its encoded transmembrane receptor tyrosine kinase. In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein, which results in constitutive kinase activity (Mulligan *et al*., 1993, Donis-Keller *et al*., 1993). Mutations associated with MEN 2A cause aberrant, ligand-independent dimerization of RET via the formation of disulphate bonds (Asai *et al*., 1995, Santoro *et al*., 1995). In MEN 2B the most common mutation results in a Met→Thr substitution at amino acid position 918 in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40 %) of sporadic MTC cases (Hofstra *et al*., 1994, Eng *et al*., 1994, Carlson *et al*., 1994). The MEN 2B mutation appears to modify the substrate specificity of the tyrosine kinase (Santoro *et al*., 1995). All MEN 2 mutations convert the c-ret proto-oncogene into a dominantly acting transforming gene in humans, as expression of the non-mutated allele is usually retained in tumor tissue (Landsvater *et al*., 1996). However, somatic MEN 2B mutations have been described in tumors from MEN 2A patients (Marsh *et al*., 1996).

The dominantly acting nature of these *RET* mutations allowed the use of transgenic mouse technology to generate animal models for MTC. We generated transgenic mice with thyroid gland C-cell-specific expression of a human RET transgene in which the MEN 2B mutation was introduced. Three out of eight *CALC-MEN2B-RET* transgenic founders presented with macroscopic, bilateral MTC and two founders presented with preneoplastic C-cell hyperplasia (CCH) at the age of 20-22 months. In transgenic line-42, CCH was detected in 77% of the mice in the second year of life and MTC was diagnosed in 13% of these mice (Acton *et al*., 2000).

The variable latency periods for MTC development in MEN 2 patients and in the transgenic mouse model indicate that besides the presence of the mutated *RET* gene, additional oncogenic events are needed for MTC development. Analyses of human and mouse MTC may identify additional oncogenic events that play an important role in MTC development.

The *P53* tumor suppressor gene protein is involved in the protection against the development of many types of cancer. The P53 protein is responsible for temporarily arresting cell division in response to certain types of DNA damage, allowing such damage to be repaired. In addition, P53 can activate genes that induce apoptosis to eliminate damaged cells. Loss of function of P53 can result in uncontrolled cell division and progressive genomic instability that enhances cell transformation and tumorigenesis. *P53* is the most commonly mutated gene in human cancers (Levine, 1997). Cells expressing mutant P53 can be more tumorigenic than cells lacking endogenous P53 (Dittmer *et al*., 1993). Most mutated P53 proteins are able to drive co-translated wildtype P53 into a mutant conformation, resulting in tetramers that are inactive in DNA binding (Milner and Medcalf, 1991). Mutant P53 has a higher stability than wild-type P53, which allows its accumulation to levels detectable by immuno-histochemistry.

Results

A panel of paraffin embedded MTC tissue samples from 12 MEN 2A or MEN 2B patients and three sporadic MTC patients was used (table 1).

In thyroid gland tissue from the 10 familial patients that had undergone prophylactic surgery, several areas of micro-MTC were observed (photo 1d) as well as C-cells with a normal appearance (Arts *et al*., 1999). From two familial patients

lymphnode metastases were analysed. Of the three sporadic patients one presented with a large primary tumor and from two patients local lymph node metastases from MTC were obtained. The age of the patients ranged from 4 to 60 years. C-cells, small microscopic MTC tumors and metastatic tumor cells were all identified by staining for calcitonin (CT) (photo 1d) and consecutive sections from the corresponding areas were used for immuno-histochemistry for P53. A human specific monoclonal antibody against P53 was used. In normal human C-cells P53 staining was never detected.

Table 1. *Panel of human RET-induced MTC tissue samples, analysed for nuclear P53 staining.*
The asterics indicates a somatic *RET* mutation in the tumor of the sporadic patients.

Tumor number	Surgery	Age years	RET mutation	p53
T97 11123	Prophylactic	4	Cys634Trp	
T97 6270	Prophylactic	5	Cys634Arg	
T95 212	Prophylactic	7	Cys634Arg	
T96 522	Prophylactic	8	Cys634Arg	
T95 2301	Prophylactic	9	Cys634Arg	
T96 20402	Prophylactic	13	Cys634Arg	
T95 2300	Prophylactic	14	Cys634Arg	
T83 2147	Prophylactic	23	Met918Thr	
T86 5369	Prophylactic	36	Cys634Trp	**Nuclear**
T94 7784	Prophylactic	37	Cys634arg	
T95 11029	Primary	29	Met918Thr *	**Nuclear**
T86 9051	Metastasis	26	Met918Thr	
T80 8734	Metastasis	35	Met918Thr *	
T96 4324	Metastasis	37	Cys618Ser	**Nuclear**
T88 4694	Metastasis	60	Met918Thr *	

In 3 out of 15 MTC samples, nuclear P53 staining in MTC tumor cells was observed. One of these samples was from a non-familial, solitary tumor, while another was from a lymph node metastasis of MTC. In the third P53 positive sample which was derived from a MEN 2A patient, there was staining of a micro-MTC (photo 1f), whereas the surrounding. normal parafollicular C-cells did not stain for P53, indicating tumor specificity of nuclear P53 staining

We next studied P53 expression in MTC from four *CALC-MEN2B-RET* transgenic mice. As a control, normal parafollicular C-cells in non-transgenic and in *CALC-MEN2B-RET* transgenic thyroid glands were used. The C-cells in these tissues were identified by staining for CT and consecutive sections from the corresponding areas were used for immuno-histochemistry for P53. A polyclonal antibody specific for the murine P53 protein was used. Irradiated murine testis material served as positive control (Beumer *et al*., 1998). In irradiated testis, cells showing nuclear expression of P53 could easily be identified (photo 1A). C-cells from non-transgenic mice did not show staining for P53. Also in non-tumor C-cells from *CALC-MEN2B-RET* transgenic mice, staining for P53 protein was not observed. However, we did observe nuclear P53 staining in tumor cells from 2 out of 4 *CALC-MEN2B-RET* MTCs analysed (photo 1B). This indicates that in these MTC's a functional inactivation of P53 may has occurred as an additional oncogenic event during MTC development.

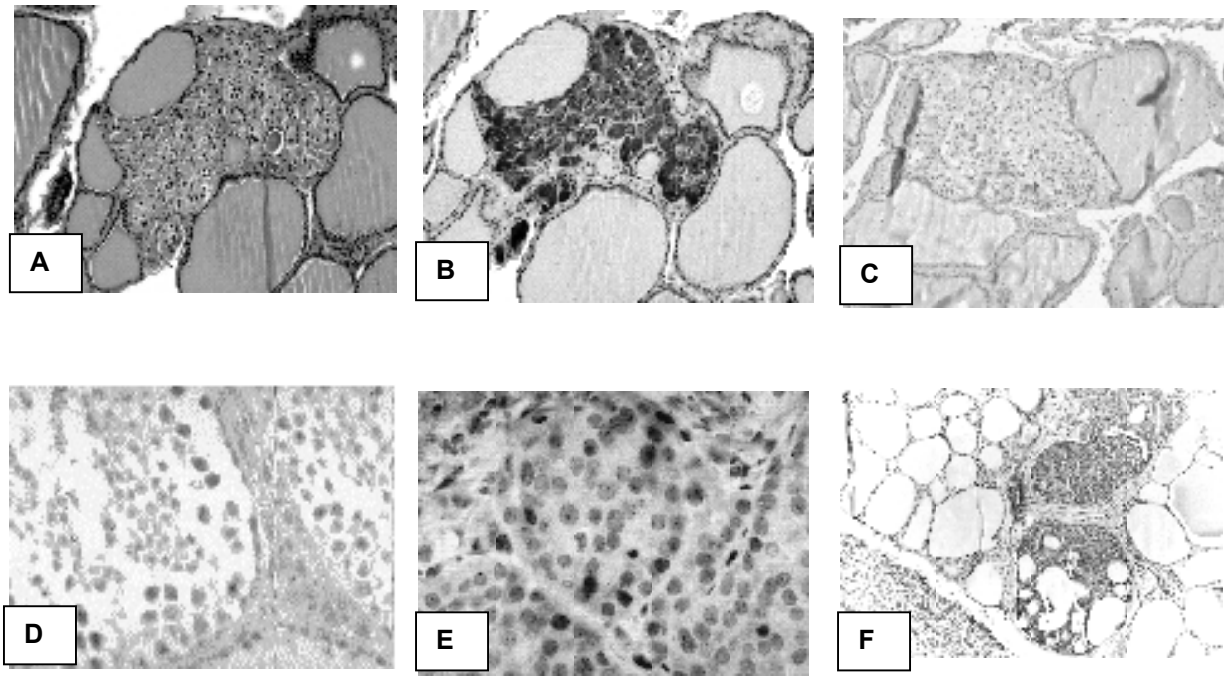

Photo 1
(a) Thyroid gland with small mass of abnormal parafollicular cells from a MEN 2A patient (86 5367) stained with hematoxilin and eosin. **(b)** Same piece of tissue as in (a) stained with CT antiserum indicating microscopic MTC. **(c)** Immuno-histochemistry with monoclonal antibody against human P53 on the same micro MTC as in (a) and (b), showing nuclear P53 staining.
(d) Immuno-histochemistry with a polyclonal rabbit antiserum against murine P53 on MTC from the founder of *CALC-MEN2B-RET* line-42 showing nuclear staining. **(e)** Irradiated testis tissue, stained with the same antiserum, served as positive control for P53 staining. **(f)** CT immuno-histochemistry of a micro MTC in the thyroid gland from a *CALC-MEN2B-RET*;*P53*-/- mouse.
Method: Fresh tissues were fixed in 3.7 % paraformaldehyde, dehydrated and embedded in paraffin. Serial sections of 5 μm were cut. Deparaffinized sections were stained with hematoxilin and eosin (a) or used for immuno-histochemistry. Antigen retrieval was achieved by 20 minutes of boiling in citrate buffer. After blocking of endogenous peroxidase (PO) with 1.5% H_2O_2, a polyclonal rabbit anti-mouse P53 antiserum (de Rooij *et al*, 1998, d and a), a polyclonal rabbit anti-calcitonin antibody (DAKO, b and f) or a monoclonal anti-human P53 antibody (Biogenex, c) were applied at room temperature (RT) for one hour. The second and third PO-coupled antisera (DAKO) were both used at 1:50 dilutions for 30 minutes at RT. Di-amino-benzidine tetra-hydrochloride (DAB, Sigma) was used as substrate for PO activity. Immuno-stained sections were counterstained with Mayer's hematoxilin, dehydrated and mounted with Pertex. Sections without the use of primary antibody served as negative controls.

A potential role for loss of P53 function in MEN2B-RET-induced MTC development *in vivo* was studied by crossbreeding the *CALC-MEN2B-RET* transgenic mice with *P53* knockout mice (Jacks *et al*., 1994). *CALC-MEN2B-RET* transgenic mice from line-42 (C57BL6/DBA2, Acton *et al*., 2000) were crossed with P53 +/- mice (C57BL6/SKH1, Rebel *et al*., 2001), and the offspring was intercrossed. We monitored mice that carried the *CALC-MEN2B-RET* line-42 transgene and in which, in addition, both alleles of the *P53* gene were inactivated (*CALC-MEN2B-RET*;*P53*-/-). These mice were compared with *P53*-/- mice, *CALC-MEN2B-RET* mice and wildtype mice with respect to MTC development. All *P53* -/- mice

monitored died from lymphomas at a mean age of112 days (n=8). Also the vast majority of *CALC-MEN2B-RET*;*P53*-/- mice succumbed to lymphomas with a similar mean latency period of 104 days (n=10). This indicates that the *CALC-MEN2B-RET* transgene does not influence the detrimental effect of loss of P53 function on the lymphoid compartment. Macroscopic MTC was not observed in these mice upon autopsy. We therefore performed histo-pathological analysis of the thyroid glands from five *P53*-/- and six *CALC-MEN2B-RET*;*P53*-/- transgenic mice. The thyroid glands were examined by systematically making microtomic sections from the entire thyroid gland and performing immuno-histochemistry for CT to identify the C-cells. In none of the five *P53*-/- mice increased C-cell numbers were observed as compared to 10 non-transgenic control mice, analysed at the same age. In contrast, in four out of six *CALC-MEN2B-RET*;*P53*-/- transgenic mice microscopic-MTC was obser ved (67%, P<0.001)(figure1, photo 1c), while two presented with increased C-cell number. To further study this, we determined plasma CT levels in these mice. Previously we have shown, by using a chemo-luminescence assay (Nichols Institute Diagnostics), that in non-transgenic mice with normal C-cell numbers plasma CT levels are not detectable (CT<1ng/L). A detectable basal plasma CT level (CT > 1 ng/L) is indicative for CCH, whereas microscopic MTC is associated with basal plasma CT levels>3 ng/L) and macroscopic MTC by plasma CT levels of up to 3000ng/L (Acton *et al*., 2000). All six *CALC-MEN2B-RET;P53*-/- mice presented with detectable basal plasma CT levels. The four mice with microscopic MTC had CT levels of 4-9 ng/L, whereas CCH was confirmed in the two other mice by CT levels of 2 and 3ng/L respectively. In agreement with the histo-pathology, none of the five *P53*-/- mice had a detectable basal plasma CT level.

As a control, plasma CT levels were measured in mice at an age of 6-9 months. Three out of 19 *CALC-MEN2B-RET* transgenic mice (16%) presented with CCH but MTC was not detected in these mice at this age.

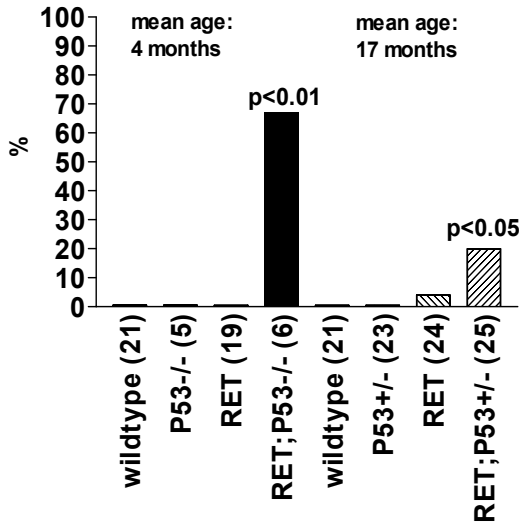

Figure 1

Effect of *P53* knockout on MTC development in *CALC-MEN2B-RET* transgenic mice.

The percentage of mice with MTC, as detected by elevated basal plasma calcitonin concentration (CT>3ng/L) and confirmed by histopathology. Genotype and the mean age of the mice are indicated.

Method: Basal plasma calcitonin concentration was determined in 200μl plasma samples from animals from the indicated groups of *CALC-MEN2B-RET* (RET) and *P53* homozygous (P53-/-) or heterozygous (P53 +/-) knockout mice and control non-transgenic littermates (wildtype), using a chemi-luminescence immuno-assay (Nichols Institute Diagnostics). Detection limit of the assay was 1 ng/L. The percentage of mice with MTC (bars), the genotype, the number of mice (n) and the mean age of the mice are indicated. Comparison between each group of transgenic mice and the control wildtype group was done with the student's T-test. Significant differences are indicated

MTC was never detected in 21 wildtype mice analysed up to an age of 17 months (figure 1).
These data indicate that loss of P53 function increases the incidence of RET-induced MTC development and that it also shortens the latency period for MTC development.
From the MTC from a *CALC-MEN2B-RET*:*P53*+/- mice, a primary culture was established. From this culture a continuously growing cell line (MTC9817) was derived that has been in culture for over two year now. Preliminary analysis revealed that these cells have RNA expression of CT, and of the gastrin/cholecystokinin B receptor which is expressed in thyroid C-cells (Reubi and Wasser, 1996). Furthermore these cells show release of CT in the culture medium, which is increased upon stimulation with pentagastrin, further indicating that these cells have retained some functional characteristics of C-cells.

Discussion

Development of MTC, particularly in the hereditary setting of MEN 2, has been shown to be a multistage event. Analysis of the age of onset data for MTC (Ponder *et al*., 1988) indicates that in addition to the mutated RET gene, further tumorigenic events must accrue for carcinoma development: first from normal C-cells to diffuse C-cell hyperplasia, subsequently to several independent nodular lesions (micro MTC) and finally to the full blown carcinoma stage. These specific progression steps in MTC tumorigenesis probably reflect successive genetic or epigenetic changes that are accompanied, for instance, by loss of differentiation of the C-cells, by the induction of neo-vascularization or by alterations in the balance between apoptosis and proliferation. The consecutive genetic lesions underlying MTC development and progression are still largely unknown.
Clues for potential involvement of several oncogenes and tumor suppressor genes in MTC tumorigenesis has come from studies with transgenic mouse strains. Mice carrying a c-mos transgene linked to an MSV-LTR displayed MTC (Schulz *et al*., 1992). Expression of additional c-mos mRNA species was reported previously in human MTC, however, rearrangements of the gene were not detected (Parkar *et al*., 1988). In addition mutations of c-mos have not been identified so far in sporadic MTC (Eng *et al*., 1996). In a transgenic mouse model with expression of a v-Ha-ras gene driven by the rat CT promoter MTC development was observed (Johnston *et al*., 1998). In human MTC, however, activating mutations in N-ras, K-ras or Ha-ras DNA were not associated with MTC (Moley *et al*., 1991). Also in mice heterozygous for a mutation that disrupts the retinoblastoma (*RB*) gene, MTC was detected (Williams *et al*., 1994). In humans, however, no loss or major rearrangements of the *RB1* gene have been detected in MTC samples (Holm and Nesland, 1994). Hence, there appears to be some discrepancy between mice and man with regard to genetic changes involved in MTC development. These discrepancies may be caused by species differences but are more likely to reflect differences in the initiating events in MTC oncogenesis. The existence of a hereditary form of MTC in MEN 2 has allowed the identification of the RET oncogene as such a crucial initiating event, with a dominantly acting nature. Therefore, by generating transgenic mice expressing a human RET oncogene, known to be crucial in MTC initiation and development in man, such discrepancies may be overcome. We have generated *CALC-MEN2B-RET* transgenic mice expressing the human RET gene with a MEN 2B-specific mutation. The variable latency periods for MTC development and

its low penetrance indicate that additional oncogenic events are needed for MTC development.

In this study we identified P53 inactivation as an additional oncogenic event in MTC from human patients and in RET-induced MTC development in the *CALC-MEN2B-RET* transgenic mouse model. With respect to P53 involvement in human MTC, several series of such tumors have been previously studied using immuno-histochemistry. P53 expression was reported in 5-15% of MTC's analysed (e.g. Pollina *et al*., 1996). A role for P53 in MTC development in mice has also been previously reported. In mice that have, in addition to an *RB* gene mutation, also an inactivating *P53* mutation, MTC development was induced (Williams *et al*., 1994). Interestingly, somatic MEN 2A mutations in the murine *RET* gene were detected in these MTC's, indicating that loss of RB- and P53 function collaborates with RET in MTC induction and development in mice (Coxon *et al*., 1998). By inactivating the *P53* alleles in our *CALC-MEN2B-RET* transgenic mice, we observed early induction of MTC, i.e. already before 4 months of age. However, due to the detrimental effect of loss of P53 on the lymphoid compartment, these mice succumb to lymphomas at a very early stage of MTC development. Therefore, the effects of loss of P53 on later stages of MTC development cannot be studied in these mice. To overcome this limitation, we are currently applying cre-recombinase technology to generate mice with C-cell specific inactivation of P53.

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Chapter 5

Mutations and homozygous deletion of cell cycle regulator P18/INK4C/CDKN2C in human medullary thyroid carcinoma and the MTC cell line TT.

Dennis S. Acton [1] , Jo W. M. Höppener [2,1] and Cornelis J. M. Lips [1].

Department of Clinical Endocrinology, Division of Internal Medicine and Dermatology[1], department of Metabolic and Endocrine Diseases, Division of Biomedical Genetics[2], University Medical Center Utrecht, The Netherlands.

Abstract

Medullary thyroid carcinoma (MTC) arises sporadically or as part of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome, which is caused by activating germline mutations in the *RET* gene. A frequent finding in MTC is loss of heterozygosity at chromosome 1p, indicating a potential tumor suppressor gene on this chromosome arm. Therefore, we performed mutation analysis of the *P18/INK4C/CDKN2C (P18)* gene, located on 1p32 and involved in cell cycle regulation, from eight human MTC samples. In one MEN 2 and two sporadic MTC cases mutations in the *P18* gene were detected. Both sporadic MTCs had also acquired a somatic *RET* mutation. Homozygous deletion of the *P18* gene was observed in the *RET* mutation-bearing MTC cell line TT. These data strongly indicate, to our knowledge for the first time, that P18 can act as a tumor suppressor of human MTC and suggest collaboration of loss of P18 function with activation of RET during MTC development.

Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor from the calcitonin producing neuroendocrine C-cells in the thyroid gland. MTC is found as a sporadic tumor and as the most important clinical manifestation of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome (Sipple, 1961). MEN 2 is a familial cancer syndrome, inherited as a monogenic autosomal dominant trait. MEN 2 is caused by activating germline mutations in the *RET* proto-oncogene leading to aminoacid substitutions in its encoded transmembrane receptor tyrosine kinase. In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein (Mulligan *et al*., 1993). In MEN 2B the most common mutation results in a Met→Thr substitution at position 918 in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40%) of sporadic MTC cases (Hofstra *et al*., 1994).

Development of MTC, especially in the hereditary setting of MEN 2, has been shown to be a multistage event. In addition to the mutated *RET* gene further tumorigenic events must accrue for carcinoma development. The consecutive genetic lesions underlying MTC

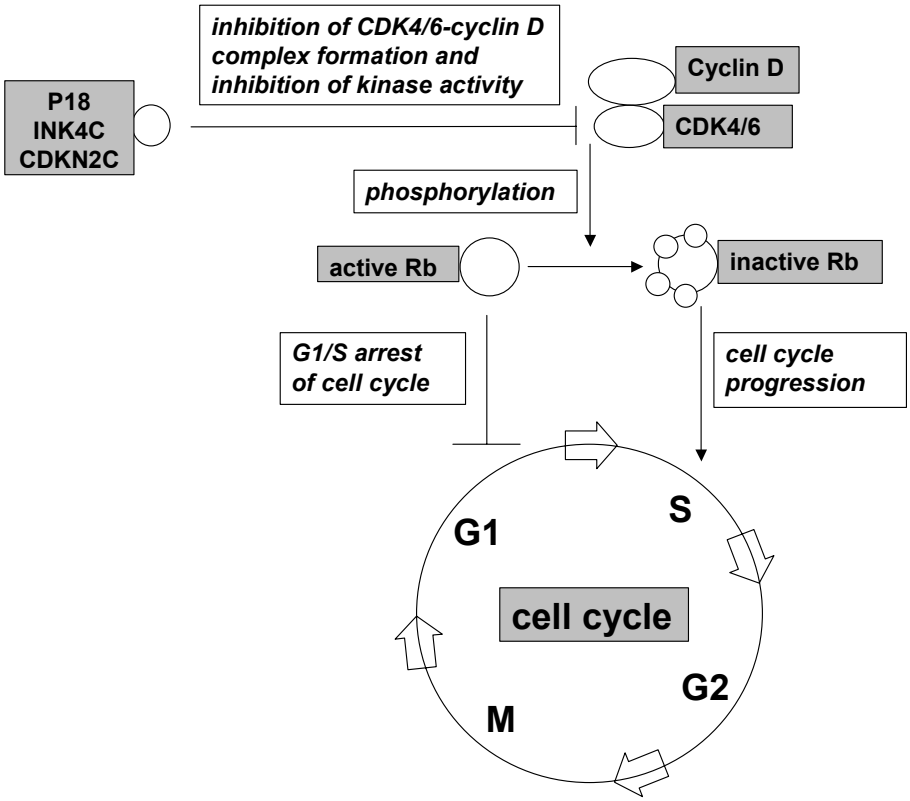

Figure 1. Role of P18 in cell cycle regulation.

development and progression are still largely unknown.

Several reports have described allelic loss of 1p markers, in the region 1p32-1p36, in sporadic and MEN 2 MTCs, MTC cell lines or primary cultures of MTC specimen (Matthew *et al*., 1987; Khosla *et al*., 1991; Moley *et al*., 1992; Cooley *et al* 1995). In a more recent study the genome-scanning technique comparative genomic hybridization (CGH) revealed loss at 1p in 8 of 28 cases of MTC as well as in the MTC cell line TT (Marsh *et al*., 2003).The identity of the putative tumor suppressor gene located on 1p and involved in the tumorigenesis of MTC is not known. A possible candidate is the *P18/INK4C/CDKN2C (P18)* gene, which is located on 1p32. P18 is a member of the family of cyclin dependent kinase (CDK) inhibitors (CDKIs). These CDKIs play an important role in the regulation of cell cycle progression (Figure 1). The CDKIs are subdivided in two classes. One is the class of the kinase inhibitor proteins (KIP). The other class is the inhibitors of CDK4 (INK4), of which P18 is a member. P18 binds to CDK4, but to a lesser extent than to the preferentially bound CDK6, inhibits their association with cyclin D and inactivates their kinase activity (Guan *et al*., 1994). As a consequence the Rb protein remains active in its growth-suppressive state and prevents the G1/S transition of the cell cycle. On the other hand, when P18 is absent or downregulated, cyclin D is able to activate CDK4 and CDK6, which in turn result in phosphorylation and functional inactivation of Rb (Guan *et al*., 1994). This protein can then no longer prevent S-phase entry and therefore cell cycle progression will occur (Figure 1). Deregulation of cell cycle control by loss of function of proteins involved is believed to contribute to the development and

progression of many types of cancers (Hahn and Weinberg, 2002). We studied the potential involvement of loss of P18 function during MTC development.

Results

We performed nucleotide sequence analysis of the *P18* gene and the *RET* gene from eight human MTCs. Four tumor samples were frozen in liquid nitrogen when the patients were operated upon. Tumor 87A-20346 was an MTC from a 30 years old MEN 2A patient with a germline C634W RET mutation. Tumor 90-4318 and tumor 94-4879 were non-familial solitary MTCs obtained at the age of 51 years and 39 years respectively. Tumor 88-9801 was an MTC metastasis from a 43 years old sporadic patient. This tumor was previously shown to harbor a somatic M918T mutation in the *RET* gene (Hofstra *et al*., 1994). Four other MTC samples were archival paraffin embedded tissue material. Tumor 96-4324 was a primary MTC from a 37 years old MEN 2A patient with a C618S germline mutation in the *RET* gene. Tumor 95-11029 was a primary MTC from a 29 years old sporadic patient. Tumor 88-4694 was a MTC metastasis from a 60 years old sporadic patient. Tumor 80-8734 was derived from the sporadic patient mentioned above with the MTC metastasis with a somatic M918T RET mutation. Also this tumor was a metastasis removed eight years earlier from the neck region. As a control, blood DNA from an anonymous healthy person was used.

Genomic DNA was isolated using proteinase-K lysis buffer, phenol-extraction and ethanol precipitation. For DNA isolation from the frozen tumors a small piece of tissue was cut off and for the TT cell line a cell pellet was used. DNA was purified from the paraffin embedded material, after specifically collecting the tumor material, as much as possible free from surrounding normal tissue, by scraping it from microscopic sections.

The PCR primers used for amplification of *P18* exon 2 and exon 3, as well as for amplification of *RET* exon 11 and exon 16 and the size of the fragments obtained are depicted in table I.

The *P18* exon 2 and exon 3 fragments could be amplified from all samples, indicating that in these tumors homozygous deletion of the *P18* gene had not occurred. Nucleotide sequence analysis of the *P18* gene fragments from the sporadic tumors 94-4879 and 90-4318 revealed no sequence differences when compared to normal blood DNA. Analysis of the DNA from the MEN 2A tumor 96-4324 showed a heterozygous C>T polymorphism at coding nucleotide position 342, which did not affect the lysine residue at amino acid position 114. This silent polymorphism was also found heterozygous in the DNA from tumor 80-8734 as well as tumor 88-9801 from the same sporadic patient.

Table 1. Nucleotide sequence of *P18* and *RET* PCR primers and size of the PCR products. The PCR reactions for *P18* and *RET* were performed at an annealing temperature of 58°C and 60°C respectively.

P18E2F	5'CATCATGCAGCCTGGTTAGG3'	237bp
P18E2R	5'TCCCCACCTCTCTTAATACC3'	
P18E3F	5'TGGGTCTTCCGCAAGAACTC3'	535bp
P18E3R	5'AGTAGAGGCAACGTGGGGGA3'	
P18E3AF	5'GTAGCATATGCACTTGAAGG3'	251bp
P18E3AR	5'TGGCAGCCAAGTGCAAGGGC3'	
P18E3BF	5'AGCTGATGTTAACATCGAGG3'	244bp
P18E3BR	5'GGAGCCCTCCCCACGTTTAT3'	
RETE11F	5'CCTCTGCGGTGCCAAGCCTC3'	191bp
RETE11R	5'TCCGGAAGGTCATCTCAGCT3'	
RETE16F	5'AGGGATAGGGCCTGGGCTCC3'	195bp
RETE16R	5'TAACCTCCACCCCAAGAGAG3'	

This indicates that in these tumors both *P18* alleles are retained. No other differences in the coding nucleotide sequences of the P18 gene were found in these five tumors when compared to normal blood DNA. In the DNA from sporadic tumor 88-4694 a missense mutation in the *P18* gene was detected. At coding nucleotide position 151 a G>A substitution was found (Figure 2A). This results in an E51K aminoacid substitution in the P18 protein. The G containing normal allele could not be detected in the nucleotide sequence, which indicates that in this tumor the other allele is lost. Also in the sporadic tumor 95-11029 a missense mutation was observed. At coding nucleotide position 181 a G>A transition had occurred (Figure 2A). This change leads to an A61T aminoacid substitution. The allele containing the normal G nucleotide at this position was retained in the DNA from this tumor. No other mutations were found in the coding sequences. In DNA from the MEN 2A tumor 87A-20346 yet another missense mutation G>A at coding nucleotide position 214, leading to an A72T aminoacid substitution, was detected (Figure 2A). In this DNA sample a non-mutated allele was detected as well.

We next determined if the sporadic MTCs examined had acquired a somatic *RET* mutation. *RET* exon 16 fragments were amplified from the six sporadic tumors and from normal blood. Nucleotide sequence analysis of the *RET* exon 16 fragment revealed the somatic M918T RET mutation in the DNA from the tumors 80-8734 and 88-9801 (Figure 2B), as previously observed. The sporadic tumors 94-4879 and 90-4318 had no mutations in *RET* exon 16 when compared to normal blood DNA. However, the M918T RET mutation was detected in DNA from the two other sporadic tumors, 88-4694 and 95-11029 (Figure 2B). Interestingly, these were the same tumors that had also acquired a mutation in the *P18* gene.

In order to analyze the *P18* gene from the MTC cell line TT, we performed PCR on DNA isolated from this cell line and from normal blood DNA obtained from an anonymous healthy person. With all primer sets we failed to PCR the *P18* gene from TT cell line DNA, even when used in a nested re-PCR setting, whereas the *P18* gene was amplified from normal blood DNA. As a control we also amplified the 195bp *RET* exon 16 fragment from both TT and normal blood DNA (Figure 3a) as well as the 191bp *RET* exon 11 fragments (not shown). These data indicate that there is homozygous deletion of the *P18* gene in the TT cell line. The presence of the C634W RET mutation in the TT cell line (Cooley *et al*., 1995) was confirmed by direct sequencing of the *RET* exon 11 PCR products. The absence of P18 DNA and P18 protein expression in the TT cell line was confirmed by Southern blot (not shown) and by Western blot, respectively (Figure 3b).

Discussion

To our knowledge this is the first report indicating the involvement of P18 in human MTC development. We detected three different missense mutations in the *P18* gene in tumors derived from a MEN 2A patient and two sporadic MTC patients. In the MTC cell line TT homozygous deletion of the *P18* gene was observed. These data strongly indicate that the cell cycle regulator P18 acts as a tumor suppressor of MTC development. Analysis of the *P18* gene has been performed in a large number of different types of tumors. Genetic alterations in the *P18* gene were rarely found. Identification of somatic mutations in the *P18* gene has been described in only five reports previously.

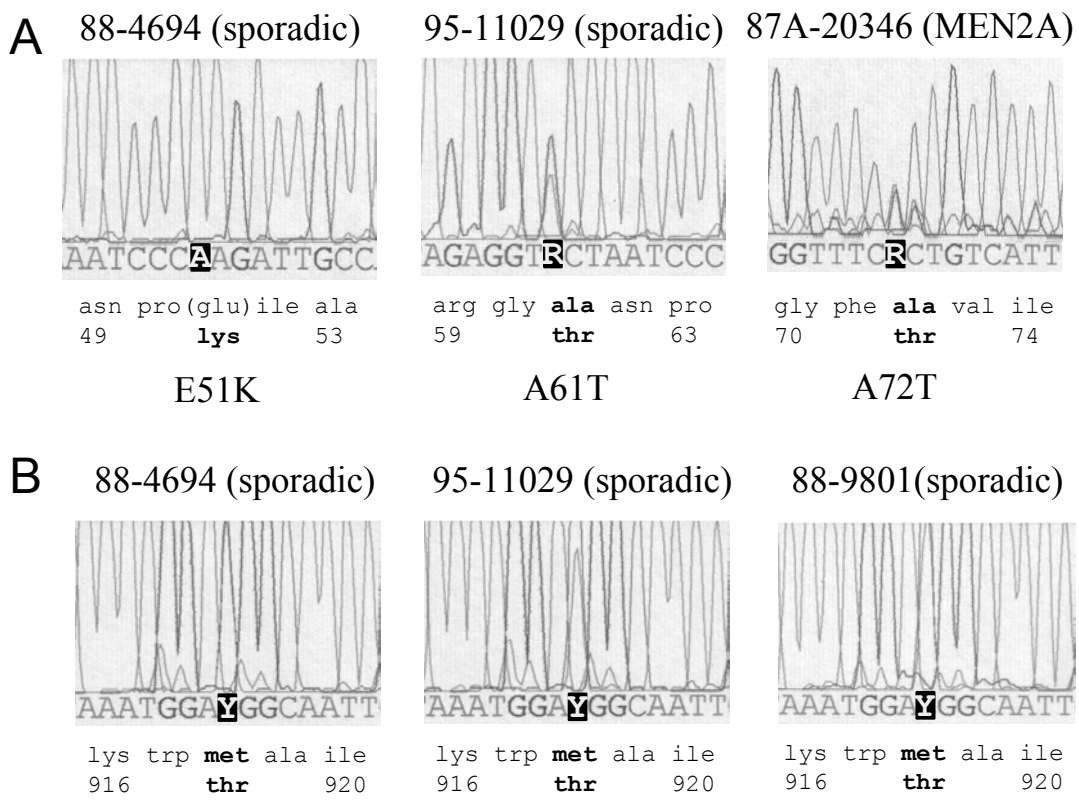

Figure 2. A: Missense mutations in the *P18* gene detected in human MTCs. B: Somatic M918T RET mutations in human MTCs. R indicates A/G and Y indicates C/T. For sequencing, the PCR fragments obtained were gel purified. Sequencing reactions were prepared according to the ABI Prism Big Dye terminator cycle sequencing protocol (PE Biosystems, Warrington, England) with the primers used for the PCR. All fragments were sequenced with a forward and a reverse primer. An ABI prism automated sequencer was used. The sequencing results were confirmed by analysis of a second, independently generated PCR fragment

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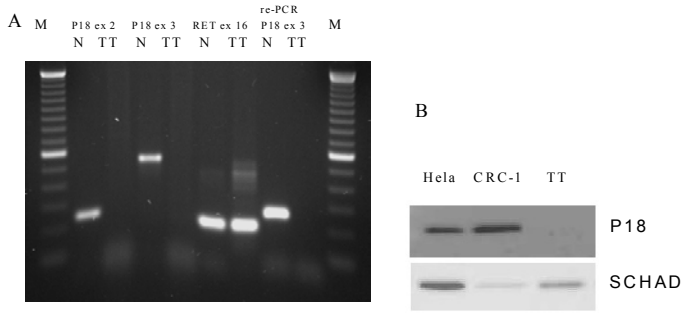

Figure 3. A: Homozygous deletion of the *P18* gene in TT cells. PCR of *P18* exon 2, *P18* exon 3 and *RET* exon 16 and nested re-PCR of *P18* exon 3 on TT cell line (TT) and normal blood (N) DNA, M=100bp size marker. B: Lack of P18 protein expression in TT cells. P18 western blot, Hela cells and CRC-1 (human MTC cell line, Cooley *et al*., 1995) served as control. Schad was used as loading reference.

A missense mutation in the *P18* gene was reported for the breast tumor cell line BT-20 (Lapointe *et al*., 1996). This G→C substitution at coding nucleotide position 214, resulting in an A72P substitution, was also detected in 3 of 35 breast tumors analyzed (Blais *et al*., 1998). In a study of atypical and anaplastic meningiomas a nonsense R68X mutation was observed in 1 of 67 cases analyzed (Bostrom *et al*., 2001). A Glu113X nonsense mutation in the *P18* gene was found in 1 of 35 oligodendrogliomas analyzed (Husemann *et al*., 1999). In a study of 30 oligodendrogliomas a deletion of the glutamine aminoacid residue at position 113 was reported (He *et al*., 2000). Numerous studies have reported the lack of *P18* mutations in: invasive primary breast cancers (Okamoto *et al*., 1995), childhood acute lymphoblastic leukemias (Takeuchi *et al*., 1995), myeloid leukemias and myeloid leukemia cell lines (Nakamaki *et al*., 1995), primary or metastatic lung cancers (Okamoto *et al*., 1995), osteosarcomas and various other sarcomas (Miller *et al*., 1996), non-small-cell lung cancers (Russin *et al*., 1996), primary lymphoid tumors (Otsuki *et al*., 1996), neuroblastomas (Kawamata *et al*., 1996), primary multiple myelomas (Tasaka *et al*., 1997), adult T-cell leukemias, T-cell leukemia cell lines and non-Hodgkin's lymphomas (Hata *et al*., 1997), parathyroid adenomas (Tahara *et al*., 1997), hepatoblastomas (Iolascon *et al*., 1998), oligodendrogliomas (Pohl *et al*., 1999), meningiomas (Santarius *et al*., 2000; Leuraud *et al*., 2000), primary melanomas (Kumar *et al*., 2001), ovarian granulosa cell tumors (Arcellana-Panlillio *et al*., 2002) or multiple myeloma cell lines (Kulkarni *et al*., 2002). *P18* mutations were also not found in a study with 15 normal and 73 tumor-derived cell lines from 23 different tissues, with the exception of the T-47D breast cancer cell line in which 13 nucleotide changes within a region of 111 nucleotides in the last coding part of exon 3 were observed (Zarawali *et al*., 1996), a finding which may be explained by contaminating cells from another species.

Indications for the consequence for P18 function of the aminoacid substitutions in P18 reported in this study: E51K, A61T and A72T, can be obtained from previous studies. The sole previously described missense mutant P18 (Lapointe etal., 1996) also affects the alanin residue at position 72. In this case however this residue is replaced by a prolin. This A72P mutant was shown to be defective in binding CDK6 and in inhibiting cell proliferation when being overexpressed (Lapointe *et al*., 1996). This stresses the importance of the alanin residue at position 72 for normal P18 function and suggests that the A72T mutant may also be defective in performing normal P18 function. Previously, a A61T P18 mutant was selectively produced by oligonucleotide-directed mutagenesis and biochemical characterization of this mutant was performed (Noh *et al*., 1999). The A61T mutant was 16-fold less potent as CDK6 or CDK4 inhibitor and was impaired in its growth inhibitory capacity.

In the MTCs harboring the A61T and the A72T mutations, a wild type *P18* allele seems to be retained. Retention of a non-mutated allele was also described for the BT-20 cell line with the A72P mutation in P18 (Lapointe *et al*., 1996). According to the classical concept of tumor suppressor genes, for these P18 mutations to exert an effect on cell cycle control, the other allele should also be defective in its function. Since no other mutation was detected in the *P18* coding sequence, other means of gene inactivation, e.g. hypermethylation or promoter mutations, may have occurred. In addition, we cannot exclude that the detection of a normal *P18* allele was caused by the presence of "contaminating" DNA from non-tumor tissues. The consequence for normal P18 function of the E51K mutation reported in this study, remains to be elucidated. In the MTC with the E51K P18

mutation the other *P18* allele seems to be lost. Therefore, this tumor most probably lacks a normal functional P18.

Considering its important role in cell cycle control it is intriguing why *P18* mutations have only rarely been found in large numbers of different types of cancers analyzed. This is not simply due to lack of P18 expression as P18 transcripts and protein have been reported in these tumors and most tissues (Kulkarni *et al* 2002; Lapointe *et al*., 1996, Bostrom *et al*., 2001). A possible explanation may be redundancy of P18 function in most cell types due to expression of other CDKIs, which control function of Rb. Since we observed P18 mutations in three of eight MTCs analyzed, this suggests that in thyroid C-cells P18 is the CDKI that plays the major role in suppressing tumorigenesis by controlling Rb function. Whether this also holds for other cells of neuroendocrine origin and the tumors derived thereof (e.g. adrenal chromaffin cells and pheochromocytomas) is currently under investigation.

Another challenging explanation may be that the presence of constitutively active RET makes the C-cells more susceptible to effects of P18 mutation. The mutations in *P18* reported in this study were detected in a familial MTC (with a germline *RET* mutation) and in two sporadic MTCs. Interestingly, both sporadic tumors had also acquired a somatic activating mutation in the *RET* gene. Also in the TT cell line, loss of P18 in combination with RET activation is observed. These data suggest that loss of P18 function and activation of RET collaborate by deregulating pathways which act synergistically in MTC induction and progression. Further study of pathways, which are deregulated in MTCs, may unravel a coherent set of rules and principles for tumorigenesis rather than a phenomenology of unlimited complexity.

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Chapter 6

Deregulation of Wnt/beta-Catenin/Tcf signalling contributes to RET-induced medullary thyroid carcinoma development in transgenic mice and human patients.

Dennis S. Acton[1,2], Wendy van Veelen [1,2], Roel de Heus[1], Krista den Ouden [1], Cornelis J.M. Lips[1] and Jo W.M. Höppener [2,1]

Department of Clinical Endocrinology, Division of Internal Medicine and Dermatology[1], Department of Metabolic and Endocrine Diseases, Division of Biomedical genetics[2], University Medical Center Utrecht, The Netherlands

Abstract

To study the tumorigenesis of medullary thyroid carcinoma (MTC) we previously generated transgenic mice with thyroid C-cell expression of a human *RET* oncogene with a Multiple Endocrine Neoplasia type 2B-specific mutation (*CALC-MEN2B-RET*). From one of the founders a line was derived in which the majority of mice developed focal C-cell hyperplasia (CCH) and a minority presented with macroscopic MTC at 24 months of age. The low tumor incidence and the long latency period for MTC development suggested the need for additional oncogenic events. Deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway has been associated with several forms of cancer. Potential involvement of this pathway in RET-induced MTC development was investigated by immuno-histochemical analysis for beta-catenin expression of thyroid gland C-cells and MTC from *CALC-MEN2B-RET* transgenic mice. While in normal C-cells beta-catenin staining is observed solely associated with the plasma membranes, in MTC tumor cells beta-catenin staining was also observed in the nucleus in 6/7 cases analysed, indicating tumor-specific deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway. The significance of these findings for human MTC was evaluated using tumor samples derived from MEN 2 patients and sporadic MTC cases. Nuclear beta-catenin staining was detected in tumor cells from 6 out of 15 human MTC samples investigated. CCH and microscopic MTC from MEN 2 patients or *CALC-MEN2B-RET* mice were not associated with nuclear beta-catenin staining, suggesting that this is a late event in tumorigenesis. To study the effect of deregulated Wnt/beta-Catenin/Tcf signalling on RET-induced MTC development *in vivo,* we monitored *CALC-MEN2B-RET* transgenic mice also carrying the *APC(min/+)* mutation. MTC development was not observed, but a significant increase in CCH prevalence was detected in these mice. Additional introduction of *tcf-1* knock-out alleles did not result in MTC development or further increase in CCH incidence. The increased CCH incidence in *CALC-MEN2B-RET;APC(min/+*) mice was age dependent and associated with nuclear beta-catenin in hyperplasic C-cell areas in part (1/6) of the mice analysed. These data indicate that deregulation of Wnt/beta-Catenin/Tcf signalling contributes to RET-induced MTC development.

Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor that originates from the calcitonin (CT) producing and secreting C-cells in the thyroid gland (Horn, 1951). MTC is mainly found as a sporadic and solitary tumor. In addition, MTC is the most important clinical manifestation of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome (Sipple *et al*., 1961). MEN 2 is a familial cancer syndrome, inherited as a monogenic autosomal dominant trait (Steiner *et al*., 1968). Apart from MTC, this syndrome is characterized by the occurrence of tumors originating from the chromaffin cells of the adrenal medulla (pheochromocytoma). In addition to these tumors, parathyroid hyperplasia is observed in MEN 2A (Sipple *et al*., 1961, Steiner *et al*., 1968) while in MEN 2B ganglioneuromas of the intestinal tract and mucosal neuromas are found, as well as musculoskeletal anomalies (Carney *et al*., 1976). MEN 2 is associated with germline mutations in the RET proto-oncogene leading to aminoacid substitutions in its encoded transmembrane receptor tyrosine kinase. In MEN 2A the mutations affect cystein residues in the extracellular domain of the protein, which results in constitutive kinase activity (Mulligan *et al*., 1993, Donis-Keller *et al*., 1993). Mutations associated with MEN 2A cause aberrant dimerisation of RET via the formation of disulfate bonds (Assai *et al*., 1995, Santoro *et al*., 1995). In MEN 2B the most common mutation results in a Met→Thr substitution at codon 918 in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40 %) of sporadic MTC cases (Hofstra *et al*., 1994, Eng *et al*., 1994, Carlson *et al*., 1994). The MEN 2B mutation appears to modify the substrate specificity of the tyrosine kinase (Santoro *et al*., 1995). All MEN 2 mutations convert the RET proto-oncogene into a dominantly acting transforming gene in humans as expression of the non-mutated allele is usually retained in tumor tissue (Landsvater *et al*., 1996), although somatic MEN 2B mutations have been described in tumors from MEN 2A patients (Marsh *et al*., 1996).

The dominantly acting nature of these mutations allowed the use of transgenic mouse technology to generate animal models for MTC. We generated transgenic mice with thyroid gland C-cell-specific expression of a human RET transgene in which the MEN 2B mutation was introduced. Three out of eight *CALC-MEN2B-RET* transgenic founders presented with macroscopic, bilateral MTC and two founders presented with preneoplastic C-cell hyperplasia (CCH) at the age of 20-22 months (Acton *et al*., 2000). In transgenic line-42, CCH was detected in 77% of the mice in the second year of life and MTC was diagnosed in 13% of these mice. The long latency periods for MTC development and the low frequency indicates that additional oncogenic events, besides the presence of the *CALC-MEN2B-RET* transgene, are needed for MTC development. Therefore, analyses of the tumors from these mice may identify secondary oncogenic events, which play in important role in MTC development.

Deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway has been associated with several forms of cancer (Bienz end Clevers, 2000). Beta catenin plays a central role in this signal transduction pathway. Besides its role in signal transduction beta-catenin also has a function as cell adhesion mediator, as part of the E-cadherin/beta-catenin complex at cell surfaces.

When Wnt-mediated controlled cell division is required e.g. during embryonic development or in stem cells, the Wnt growth factor binds the frizzle receptor to transduce an inhibiting signal to GSK3β. Therefore, beta-catenin is able to

accumulate and migrates to the cell nucleus, where it prevents inhibitor Groucho from binding to the LEF/TCF transcription complex. The resulting beta-catenin/LEF/TCF complex stimulates transcription, of e.g. c-myc, which results in cell division (Figure 1A). In normal intestinal epithelial cells, regulation of the transcriptional activity of this complex is mainly achieved by controlling the level of free cytoplasmic beta-catenin available for nuclear translocation and binding to TCF4. In the absence of a Wnt signal, GSK3β forms a complex with APC and conductin/axin. This activates the kinase activity of GSK3β, which phosphorylates beta-catenin. Phosphorylated β-catenin is labelled with ubiquitin proteins and will be degraded by proteasomes (Figure 1B).

In several types of tumor cells, such as colon cancers, the beta catenin phosphorylating complex is not able to function properly, due to mutations of APC or other components of this complex, including beta-catenin itself. This results in high levels of cytoplasmic and nuclear beta-catenin. In the nucleus, beta-catenin associates with TCF4 and forms a transcriptionally active complex (Korinek *et al*., 1997) (Figure 1C). TCF1 serves as a negative regulator of this complex (Roose *et al*., 1999).

We studied the potential involvement of deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway in RET-induced MTC development.

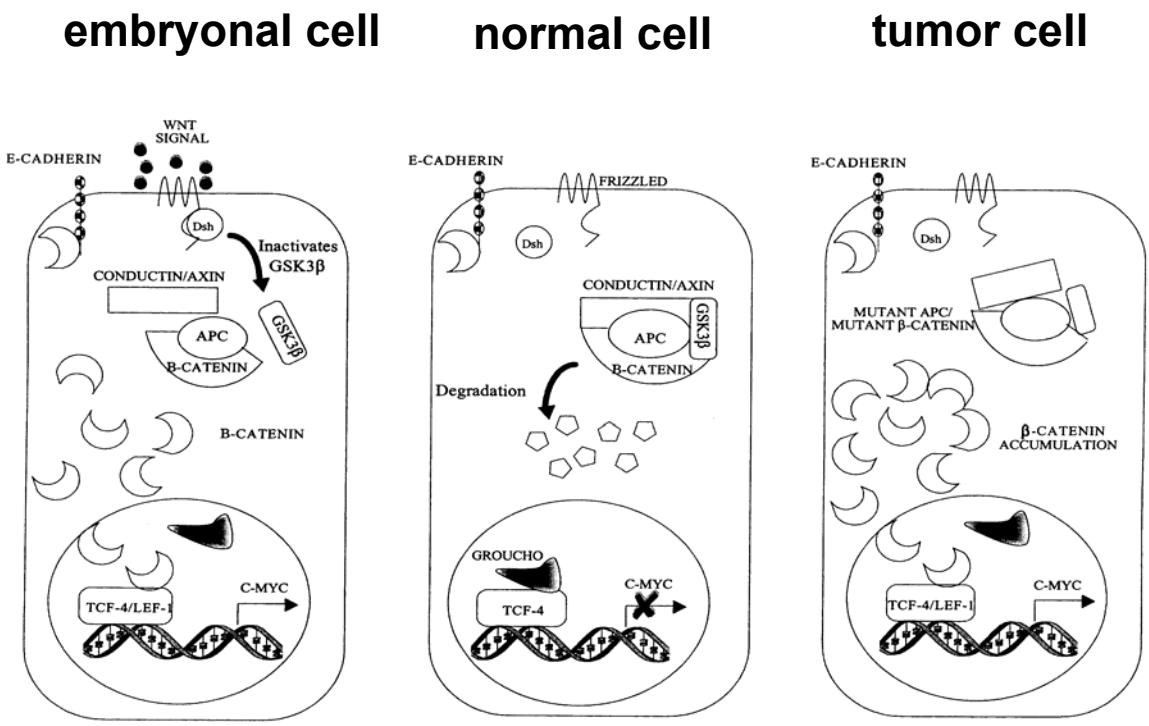

Figure 1. A. Wnt mediated transcriptional activation in embryonic cell. B. In the absence of a Wnt signal cytoplasmic beta-Catenin is degraded. C Deregulation of Wnt signalling in tumor cell

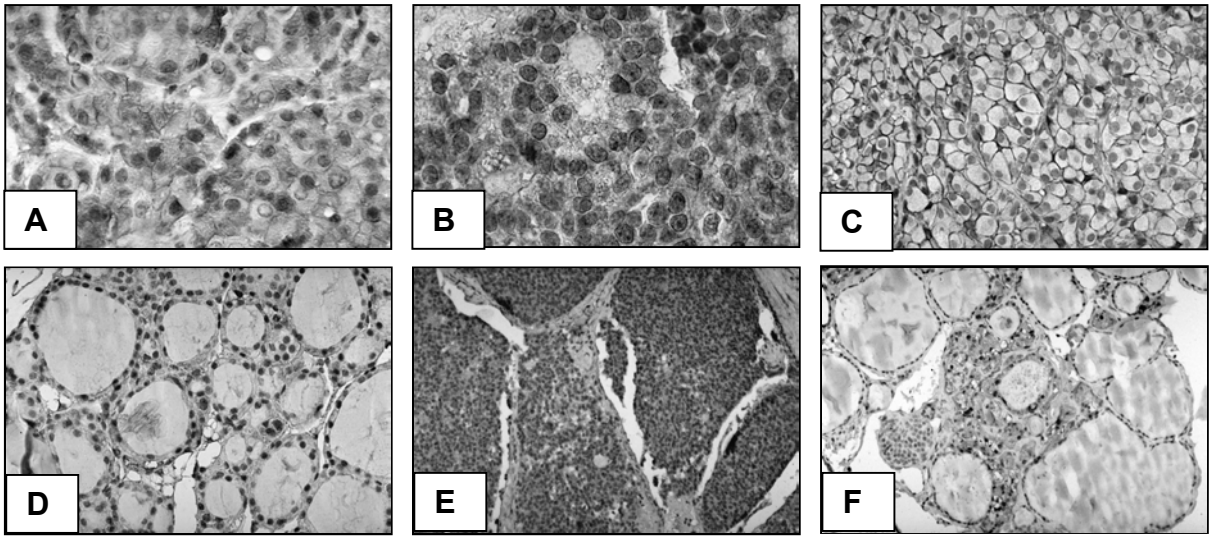

Figure 2.
Immuno-histochemistry for beta-Catenin.on: (A) murine MTC cells from a *CALC-MEN2B-RET* transgenic mouse, showing nuclear and cytoplasmic beta Catenin staining, (B) human MTC metastasis showing nuclear and cytoplasmic beta-Catenin staining and (C) human MTC showing only membrane-associated beta-Catenin expression.
Immunohistochemistry for APC on (D) murine C-cells, (E). human MTC metastasis with nuclear beta Catenin staining and (F) human microscopic-MTC without nuclear beta Catenin staining all showing high expression levels of APC.

Results

Deregulated Wnt/beta-Catenin/Tcf signalling can thus be studied by immuno-histochemical localisation of the beta-Catenin protein. We used paraffin embedded MTC material derived from seven *CALC-MEN2B-RET* transgenic mice. As a control, normal parafollicular C-cells in non-transgenic and in *CALC-MEN2B-RET* transgenic thyroid glands were used. The C-cells in these glands were identified by staining for CT and consecutive tissue sections were used for beta–Catenin immuno-histochemistry. As a positive control murine intestinal tissue was used. In the intestinal epithelium beta-Catenin expression is found associated only with the plasma membrane where it has a function in cell adhesion. In intestinal stem cells nuclear staining of beta-Catenin was observed indicating a role in transcription regulation.

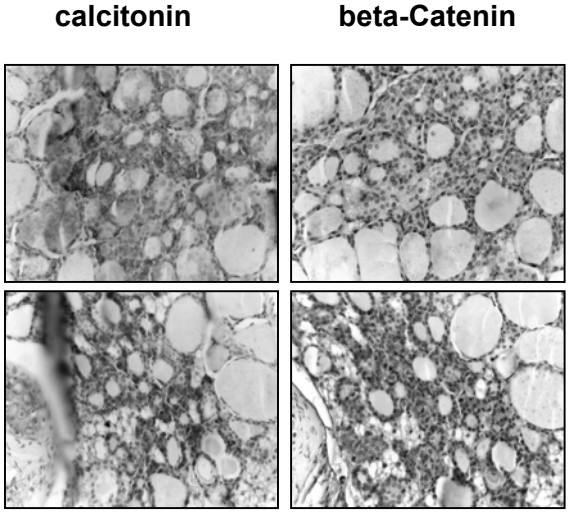

Figure 6. Calciton (left) and beta-Catenin (right) staining in hyperplastic C-Cell areas from a *CALC-MEN2B-RET;APC(min/+)* mouse with CCH not associated with Wnt deregulation (top) and a *CALC-MEN2B-RET;APC(min/+)* mouse with CCH with nuclear beta-Catenin and Wnt deregulation (bottom).

C-cells from non-transgenic mice only show staining for beta Catenin associated with the plasma membrane, indicating a role for beta-Catenin in cell adhesion in these cells. Also in C-cells from *CALC-MEN2B-RET* transgenic mice only membranous staining of beta-Catenin was observed. In the MTC tumor cells however, strong cytoplasmic and nuclear staining for beta-Catenin was seen in 6/7 cases, in addition to the membranous staining (Fugure 2 A). Not all cells in the tumors showed nuclear staining for beta-Catenin and the percentage of cells with nuclear staining varied between areas in the tumors. These data indicates that oncogenic transformation of the murine C-cells was associated with deregulation of the Wnt/beta-Catenin/Tcf signalling pathway. We next performed immuno histochemistry for beta-Catenin on eight thyroid glands with CCH and on four microscopic MTC's from *CALC-MEN2B-RET* and *CALC-MEN2B-RET;P53-/-* (Chapter 4) mice. Only menbrane-associated beta-Catenin expression was observed indicating that deregulation of Wnt/beta-Catenin/Tcf signal transduction is not an early event in murine MTC development. We evaluated the significance of nuclear beta-Catenin staining in MTC from *CALC-MEN2B-RET* transgenic mice with respect to MTC from human patients. A panel of paraffin embedded MTC tissue samples from 15 patients was used. This panel consisted of MTC from MEN 2A, MEN 2B and sporadic patients. In thyroid gland from patients that had undergone prophylactic surgery several areas of micro-MTC were observed. One solitary patient presented with a large primary tumors and from four patients local lymphnode metastases from MTC were obtained. The age of the patients ranged from 4 to 60 years. The presence of germline or somatic RET mutations was determined for all patients (Table 1). C-cells and small microscopic tumors and metastatic tumor cells were identified by positive staining for CT. The material was screened for beta-Catenin protein expression. Para-follicular C-cells and micro-MTC from young patients showed membrane-associated staining of beta-Catenin (Figure 2C). In MTC from 6 out of 15 patients cytoplasmic and nuclear beta-Catenin staining was found in addition to the membranous staining observed in all tumors (Figure 2B). The 6 MTCs were in advanced stage and all derived from older patients, which indicates that deregulation of Wnt/beta-Catenin/Tcf signalling probably is not an early event in tumorigenesis in human patients

No mutations in beta-Catenin hotspot

The main cause for deregulation of the Wnt/beta-Catenin/Tcf signalling pathway is a mutation in one of the components of the inhibiting complex, including beta-Catenin itself. To study the involvement of beta-Catenin mutations, we performed nucleotide sequence analysis for exon 3 of the human beta-Catenin gene. This exon encodes the N-terminal phosphorylation site crucial for beta-Catenin degradation, which was demonstrated to be a hotspot for somatic mutations. DNA was isolated from the paraffin embedded material from four human MTC's with nuclear beta-Catenin staining and four additional MTC's. The PCR amplified exon 3 fragment was sequenced but no mutations were detected. Also in eight murine MTC's, analysed for the homologous murine beta-Catenin exon 2, mutations in the beta-Catenin hotspot were not found

Table 1. Panel of RET-induced human MTC samples.

Patient number	Surgery	Age (years)	RET mutation	MTC	Beta catenin staining
T97 11123	Prophylactic	4	Cys634trp	familial	membranous
T97 6270	Prophylactic	5	Cys634arg	familial	membranous
T95 212	Prophylactic	7	Cys634arg	familial	membranous
T96 522	Prophylactic	8	Cys634arg	familial	membranous
T95 2301	Prophylactic	9	Cys634arg	familial	membranous
T96 20402	Prophylactic	13	Cys634arg	familial	membranous
T95 2300	Prophylactic	14	Cys634arg	familial	membranous
T83 2147	Prophylactic	23	Met918thr	familial	membranous
T86 5369	Prophylactic	36	Cys634trp	familial	membranous
T94 7784	Prophylactic	37	Cys634arg	familial	**nuclear**
T95 11029	Solitary	29	Met918thr	sporadic	**nuclear**
T86 9051	Metastasis	26	Met918thr	familial	**nuclear**
T80 8734	Metastasis	35	Met918thr	sporadic	**nuclear**
T96 4324	Metastasis	37	Cys618ser	familial	**nuclear**
T88 4694	Metastasis	60	Met918thr	sporadic	**nuclear**

No downregulation of APC expression in MTC cells

Deregulation of Wnt/beta-Catenin/Tcf signalling has been reported to be associated with downregulation of APC expression (Roose *et al* 1999) Downregulation of APC expression may be indicative for mutations in this gene. We therefore performed immuno-histochemistry for this protein. Normal C-cells from non-transgenic mice show APC expression, suggesting that beta-Catenin expression is controlled by APC in these cells. In C-cells from *CALC-MEN2B-RET* transgenic mice a similarly high expression level of APC was present (Figure 2D), indicating that expression of the mutated RET gene does not directly affect the expression of the APC component of the Wnt pathway in normal murine C-cells. In the tumor cells from the murine MTC's, that showed nuclear beta-Catenin expression, the APC expression level was not altered compared to that observed in normal C-cells.

Also for the human MTC's immuno-histochemistry for APC did not reveal a difference in expression pattern between normal parafollicular C-cells and microscopic MTC (Figure 2F) as compared to MTC's with nuclear beta-Catenin staining (Figure 2E).

Increased CCH incidence in *CALC-MEN2B-RET;APC(min/+)* mice

Next, the potential involvement of Wnt/beta-Catenin/Tcf signal transduction deregulation in MTC development was studied in a more direct manner. The APC(min/+) mouse strain is a carcinogen induced mutant with predisposition for intestinal tumors (Moser *et al*., 1990) The genetic defect underlying this phenotype is a mutation in the APC gene leading to a truncated APC protein (Moser *et al*., 1995a). Upon loss of the wildtype *APC* allele deregulation of Wnt signalling occurs, which leads to tumor initiation, mainly in intestinal epithelial cells. Homozygous APC(min/min) mice are not viable (Moser *et al*., 1995b).

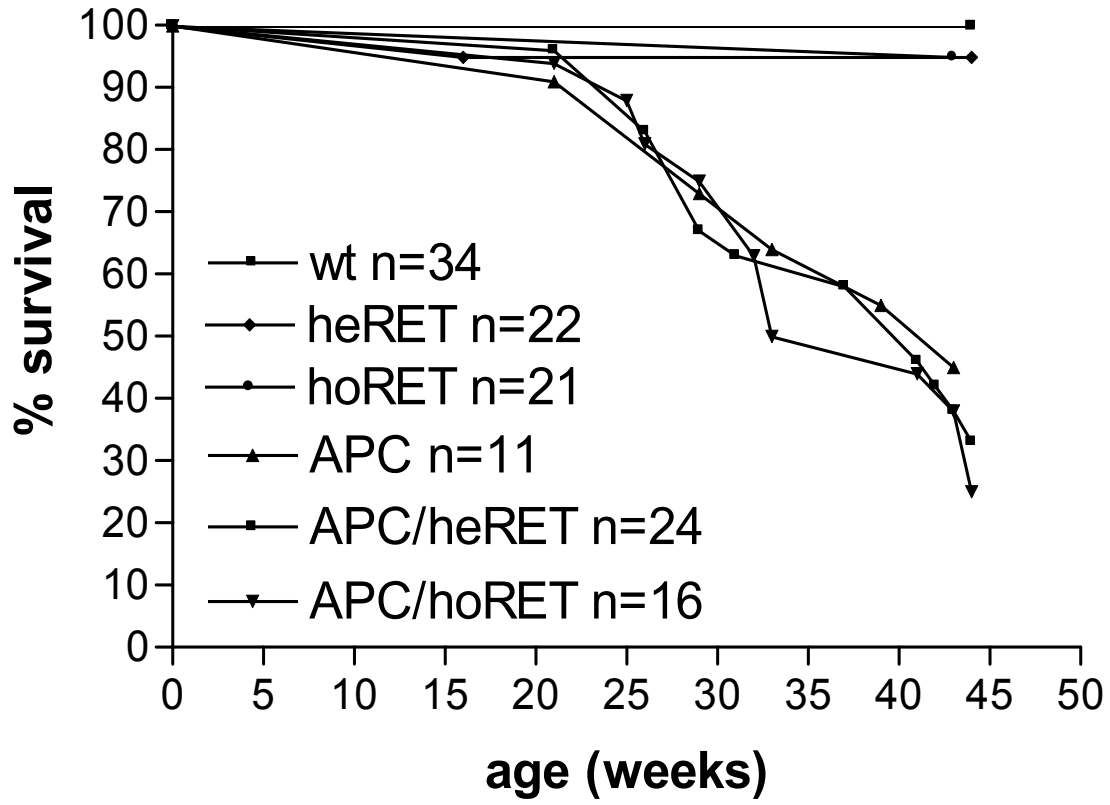

Figure 3. Survival of composite *CALC-MEN2B-RET;APC(min/+)* mice and control mice. Both heterozygous (he) and homozygous (ho) *CALC-MEN2B-RET* (RET) mice with or without the *APC(min/+)* mutation (APC), and non-transgenic (wt) mice were monitored. The genotype and the number of mice monitored are indicated.

We studied the potential role of deregulated Wnt/beta-Catenin/Tcf signalling in RET-induced MTC development *in vivo* by crossbreeding the *CALC-MEN2B-RET* transgenic mice with *APC(min/+)* mice. The latter mice die from intestinal tumors within the first year of life. Due to the breeding scheme both heterozygous and homozygous *CALC-MEN2B-RET* mice were obtained with or without the *APC(min/+)* mutation. An effect of the *RET* transgene on the survival rate of APC(min/+) mice was not observed: at the age of 35 weeks more than 50% of the *CALC-MEN2B-RET;APC(min/+)* mice had succumbed to intestinal tumors. (figure 3)Mice usually suffered from prolaps and were sacrificed when showing clear signs of serious illness. Autopsy of these mice revealed colon carcinomas in the majority of cases. No macroscopic abnormalities were observed upon thorough examination of the thyroid glands of these mice. Histological evaluation of the thyroid was performed by making sections of the entire gland followed by staining with CT antiserum to identify the C-cells. Evaluation of the microscopic slides revealed areas of CCH in the thyroid glands from the majority of *CALC-MEN2B-RET;APC(min/+)* mice. To obtain a quantitative measure of the CCH incidence in these mice, plasma samples were collected when mice were sacrificed at an age ranging from 17 to 45 weeks.

From all mice surviving until 45 weeks of age, plasma samples were collected at that timepoint. Previously we have shown, by using a chemo-luminescence assay (Nichols Institute Diagnostics), that in non-transgenic mice with normal C-cell numbers plasma CT levels are not detectable (CT<1ng/L). A basal plasma CT level of 1-3 ng/L is indicative for CCH, whereas microscopic MTC is associated with basal plasma CT levels>3 ng/L) and macroscopic MTC by plasma CT levels of 100-3000ng/L (Acton *et al*., 2000, Chapter 3).

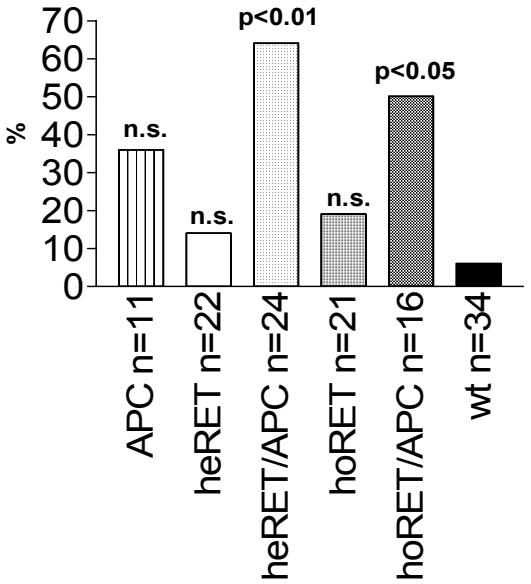

Figure 4. CCH in offspring from of *CALC-MEN2B-RET* (RET) and *APC(min/+)* (APC) mice at an age of up to 45 weeks.. Due to the breeding scheme heterozygous (heRET) and homozygous (hoRET) *CALC-MEN2B-RET* transgenic mice were obtained. Non transgenic mice (wt) served as control. The number of mice monitored for each genotype is indicated. The percentage of mice with CCH was determined by measurement of plasma CT levels. Significant differences compared to non-transgenic controls are indicated.

Measurement of the plasma CT level revealed a slight but not significant increase in the prevalence of CCH in *CALC-MEN2B-RET* heterozygous (14%) and homozygous (16%) mice compared to wt controls (6%). The increase in the percentage of APC(min/+) mice with CCH (present in 36%) was also not significant. However, a significant increase in CCH was observed in the composite *CALC-MEN2B-RET;APC(min/+)* mice, both heterozygous (64%, p<0,01) or homozygous (50%, p<0,05), for the *CALC-MEN2B-RET* transgene, as compared to the wildtype control mice (Figure 4).

Effect of APC(min/+) on CCH incidence is age dependent

We determined if the increase in the percentage of mice with CCH due to the APC(min/+) mutation was age related. Therefore plasma CT levels from heterozygous *CALC-MEN2B-RET* mice were compared to those of heterozygous *CALC-MEN2B-RET;APC(min/+)* mice at an age of 4-6 months. CT levels indicative for CCH, were observed in 25% and 24% of the *CALC-MEN2B-RET* mice (n=16) and the *CALC-MEN2B-RET;APC(min/+)* mice (n=25), respectively, indicating that at this age an effect of the APC(min) mutation is not observed. However, when groups of mice were compared at the age of 8-12 months, a significant difference in prevalence of CCH was observed. Only 16% of the heterozygous *CALC-MEN2B-RET* mice (n=25) had CCH, whereas 79% of the heterozygous *CALC-MEN2B-RET;APC(min/+)* mice (n=16), (p<0.05) analysed presented with CCH. This indicates that the effects of the APC(min/+) mutation on CCH incidence becomes manifest at later age and this suggests that more additional oncogenic events are needed.

No additional effect of loss of TCF1

One such event shown to contribute to the *APC(min/+)* phenotype is loss of TCF1 expression (Roose *et al*., 1999). By crossbreeding we obtained a small group of *CALC-MEN2B-RET;APC(min/+);TCF1-/-* mice (n=8) and a group of *CALC-MEN2B-RET;APC(min/+);TCF1+/-* mice (n=7) and monitored the survival and CCH incidence up to 45 weeks of age. All *CALC-MEN2B-RET* transgenic mice heterozygous for the *TCF1* knockout survived. Homozygous loss of *TCF1* in *CALC-MEN2B-RET* mice resulted in reduced survival, mainly due to the development of lymphomas, a phenomenon noted previously for TCF1-/- mice (Verbeek *et al*., 1995). In *CALC-MEN2B-RET;APC(min/+)* mice homozygous loss of *TCF1* resulted in rapid dead of the mice within 20 weeks and heterozygous loss of *TCF1* resulted in a reduced survival rate as well. MTC development was not observed in these mice and plasma CT levels of the mice indicated that loss of *TCF1* had no significant effect on the incidence of CCH (Figure 5).

Increase in CCH is in part associated with nuclear beta-Catenin

We investigated if the increased incidence of CCH in composite *CALC-MEN2B-RET:APC(min/+)* transgenic mice was associated with deregulation of Wnt/beta-Catenin/Tcf signal transduction. For this purpose we analysed the C-cells in the thyroid glands from 6 *CALC-MEN2B-RET* and 6 *CALC-MEN2B-RET;APC(min/+)* mice with CCH. CT immuno-histchemistry was performed to identify the expanded areas of C-cells, and consecutive tissue sections were stained for beta-Catenin expression. In none of the thyroid glands from the *CALC-MEN2B-RET* transgenic mice nuclear or cytoplasmic staining of beta-Catenin in the C-cells was observed. In the thyroid gland from one out of six composite *CALC-MEN2B-RET;APC(min/+)* mouse the hyperplastic C-cells showed nuclear and cytoplasmic staining of beta-Catenin. This indicates that CCH in this mouse was associated with deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway. In the other five glands, only membranous beta-Catenin was found in the C-cells. This indicates that the increased incidence of CCH in the composite mice is not generally associated with nuclear beta-Catenin accumulation.

Discussion

Development of MTC, especially in the hereditary setting of MEN 2, has been shown to be a multistage event. Analysis of the age of onset data for MTC (Ponder *et al*., 1988) indicates that in addition to the mutated RET gene further tumorigenic events must accrue for carcinoma development: first from normal C-cells to diffuse CCH, subsequently to several independent nodular lesions and finally to the carcinoma stage. These specific progression steps in MTC tumorigenesis probably reflect additional genetic or epigenetic changes that are accompanied, for instance, by loss of differentiation of the neoplastic C-cells, by the induction of neo-vascularization or by alterations in the balance between apoptosis and proliferation. The subsequent genetic lesions underlying MTC development and progression are still largely unknown.

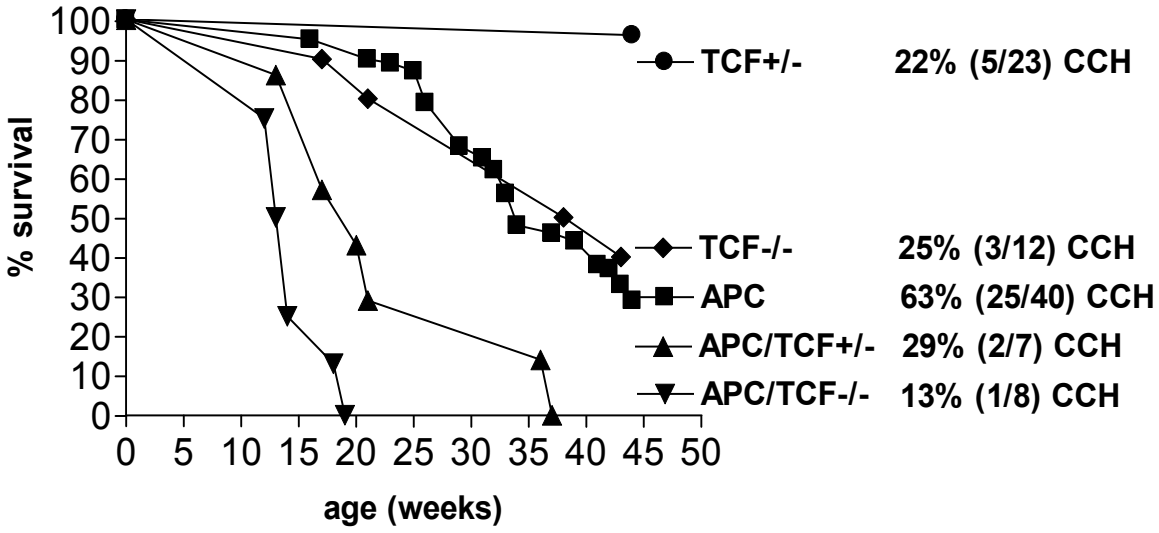

Figure 5. Survival and CCH incidence of *CALC-MEN2B-RET* transgenic mice having the *APC(min/+)* mutation (APC) and/or one (TCF+/-) or two (TCF-/-) knockout alleles of *TCF1*. The percentage and number of mice with CCH and total amount of mice (monitored for 45 weeks) is indicated.

In this paper we report the identification of deregulation of Wnt/beta-Catenin/Tcf signal transduction as an oncogenic event in MTC induced in the *CALC-MEN2B-RET* transgenic mouse model, which seems to be crucial for MTC development in human patients as well. In both murine and human RET-induced MTC, nuclear accumulation of beta-Catenin was detected. In contrast, in normal C-cells, CCH and microscopic MTC from *CALC-MEN2B-RET* transgenic mice and MEN 2 patients, nuclear beta-Catenin staining is not observed. This suggests that Wnt/beta-Catenin/Tcf signalling deregulation occurs through the acquirement of somatic mutations and that it is not an early event in MTC development. After crossbreeding *CALC-MEN2B-RET* transgenic mice with the *APC(min/+)* strain we observed an increase in the percentage of mice with CCH. MTC was not observed in these mice. This can be explained by the limited survival of these mice due to the detrimental effect of the *APC(min)* mutation, leading to premature death from colon carcinomas. The absence of MTC development in these mice suggests that besides the expression of mutated RET and the presence of the mutated *APC(mi*n) allele, additional oncogenic events have to occur. Introduction of TCF-1 knockout alleles, as an additional oncogenic event, further limited the survival of these mice but no MTC development or CCH was observed in this short lifespan. To overcome these undesired effects we are currently applying Cre recombinase technology to generate mice in which inactivating mutations can be introduced specifically in the thyroid gland C-cells.

The increased incidence of CCH, observed in composite *CALC-MEN2B-RET;APC(min/+)* mice was age-dependent, which indicates the contribution of additional oncogeneic events. For the colon carcinomas which develop in APC(min/+) mice, it has been shown that during pre-neoplastic stages the accumulation of additional oncogenic events, one of which is loss of the wildtype *APC* allele, eventually leads to

deregulation of Wnt/beta-Catenin/Tcf signal transduction as indicated by nuclear beta-Catenin staining. Analyses of areas of CCH in composite *CALC-MEN2B-RET;APC(min/+)* mice indicated that this pre-neoplastic stage was, in part, associated with deregulation of Wnt/beta-Catenin/Tcf signal transduction, as indicated by nuclear and cytoplasmic beta-Catenin staining in one out of six mice investigated. This finding indicates that in these particular C-cells additional events, leading to deregulated Wnt/beta-Catenin/Tcf signalling, contributed to the hyperplasia. However, in the other five mice, the hyperplasic C-cells had only membrane-associated beta-Catenin staining. This suggests that nuclear beta-Catenin accumulation is not a prerequisite for increased CCH development in these mice. Since there is contribution of the *APC(min)* allele to an increased CCH prevalence, there may be an (gene dosage) effect of APC, in the presence of mutated RET, in the absence of additional oncogenic events. The effect of APC(min) allele on CCH, however, was age dependent which does suggest the need for additional oncogenic events. These additional oncogenic events would contribute to the increased CCH, in the absence of nuclear beta-Catenin accumulation. In line with this notion, it has been suggested that for colon carcinoma development in APC(min/+) mice several oncogenic events must occur before nuclear accumulation of beta Catenin is observed (Baker *et al.,* 1998). Further analysis of hyperplasic C-cells from composite *CALC-MEN2B-RET;APC(min/+)* mice using micro-dissection and micro-array techniques will be needed to obtain more insight in the molecular events contributing to the increased CCH prevalence in these mice.
Deregulation of the Wnt/beta-Catenin/Tcf pathway has not previously been associated with MTC induction or development. Other types of thyroid tumors have been associated with Wnt/beta-Catenin/Tcf signalling. In anaplastic thyroid tumors nuclear beta-Catenin staining was observed (Carcia-Rostan *et al.,* 2001) but no association with RET has yet been described. In patients with familial adenomatous polyposis coli (FAP), the papillary type of thyroid gland tumor can occur. In some of these papillary thyroid tumors RET translocations have been described (Cetta *et al.,* 2001). Although the mechanism of RET deregulation in the papillary type of thyroid tumors (chromosomal translocations) differs from that observed in MTC (activating point mutations) it is interesting that RET and Wnt/beta-Catenin/Tcf deregulation seem to collaborate in development of both types of tumor. Deregulation of Wnt/beta-Catenin/Tcf signal transduction seems to occur rather late in MTC tumorigenesis. Further analysis of early stages of MTC development in the CALC-MEN2B-RET transgenic mice and MEN 2 patients may result in the identification of oncogenic events crucial for the initiating stages of MTC development.

Materials and methods

Mouse strains

All experiments were performed with the approval of the animal experimental and ethic committee of the local university.
The generation and characterisation of the *CALC-MEN2B-RET* mice (Acton *et al*., 2000), the *APC(min/+)* mice (Moser *et al*., 1995) and the *TCF1 -/-* mice (Verbeek *et al*., 1995) were described before. For genotyping of the strains, PCR on tail DNA was performed using the primers indicated in table 1.

Table 1. PCR primers for mouse genotyping

primer	Nucleotide sequence	Position
RET-P2-f	5'-TGGAGACCCAAGACATCAAC-3'	nt1718-1737 RET cDNA
RET-2583-r	5'-GGAGAAGAGGACAGCGGCT-3'	nt1935-1917 RET cDNA
APC9-f	5'-GCCATCCCTTCACGTTAG-3'	exon 9 APC
APC15-r	5'-TTCCACTTTGGCATAAGGC-3'	exon 15 APC
APCmt-f	5'-TTCTGAGAAAGACAGAAGTTA-3'	Codon 850
TCFE6-f	5'-GGAGATGAGAGCCAAGGTCA-3'	exon 6 TCF1
TCFE7-r	5'-CGTAGTTATCCCGCGCGGAC-3'	exon 7 TCF1
Neo-f	5'-CATTCAGGACATAGCGTTGG-3'	Neomycin gene

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue. Calcitonin staining was performed as previously described (Acton *et al.,* 2000). For beta-Catenin and APC staining the slides were dewaxed, rehydrated and treated with 1.5 % hydrogen peroxide in blocking buffer to inhibit endogenous peroxidase activity. For antigen retrieval the slides were immersed in boiling citrate buffer in a high-pressure-cooker for one sterilizing cycle (20 minutes 120°C) and slowly cooled down to room temperature (RT). After washing with tap water we incubated with PBS / 5% normal human serum / 5% swine serum for 5 minutes. Subsequently the first step antibody was incubated for 60 minutes at RT. For beta-catenin staining the C19220, (Transduction labs, Lexington) and for APC the C-20 (Santa Cruz Biotechnology, Santa Cruz) antibodies were used. Then we incubated with a 1:50 dilution of peroxidase-labeled rabbit antiserum to mouse immunoglobulin (Dako, Denmark) for 30 minutes at RT. The third step antibody was a 1:50 dilution, peroxidase-labeled swine antiserum to rabbit immunoglobulin (Dako, Denmark) for 30 minutes at RT. Finally sections were incubated with DAB and counterstained with Mayer's haematoxylin. Negative control experiments were performed by omitting the first step antibody.

DNA mutation analysis of the beta-Catenin gene

For sequence analysis DNA was isolated from from paraffin-embedded material or frozen tissue. We performed PCR with a forward and reverse primer for exon 3 of the human beta-Catenin gene hotspot, encoding the GSK-3ß targeted phosphorylation residues. The nucleotide sequences of the PCR primers were: PH1 TACAGCTACTTGTTCTTGAGTG-forward and PH2: CTGATTTGATGGATGGAGTTGGAC-reverse. For the murine beta-Catenin hotspot we performed PCR with a forward and reverse primer for exon 2 of the mouse beta-Catenin gene.: PM1 TACAGGTAGCATTTTCAGTTCAC-forward and PM2 TAGCTTCCAAACACAAATGC-reverse. The nucleotide sequence analyses were performed by Eurogentech (Belgium).

Plasma CT measurement

Basal plasma calcitonin concentration was determined in 200µl plasma samples from mice from each indicated *CALC-MEN2B-RET* line and control non-transgenic littermates, using a chemi-

luminescence assay (Nichols Diagnostics). Detection limit of the assay was 1 ng/L.

Statistics
Comparison between each group of transgenic mice and the control group was done with the student's T-test. Significant differences are indicated * $P<0.05$ and *** $P<0.0001$.

Aknowledgement

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Chapter 7

Discussion and future research

Dennis S. Acton
Department of Clinical Endocrinology, Division of Internal Medicine, University Medical Center Utrecht

1. RET transgenic mouse models to study tumorigenesis of MTC

The identification of RET mutation as a crucial initiating event in MTC development, with a dominantly acting nature, allowed the use of transgenic technology to generate mouse models to study the tumorigenesis. There are three isoforms of RET: RET9, RET43 and RET51, and although *in vitro* evidence suggests that they vary in cellular transformation activities, little is known about their function in tumorigenesis *in vivo*. Several RET transgenic mouse models for MTC have been generated. Transgenic mice with C-cell expression of a human RET9 oncogene with a MEN 2A-specific mutation were generated by Michiels *et al*., (1997) . Animals of three independent transgenic mouse lines, which expressed the transgene in the thyroid, displayed overt bilateral C cell hyperplasia as early as 3 weeks of age and subsequently developed multifocal and bilateral MTC. Moreover, these tumors were morphologically and biologically similar to human MTC, which afflicts MEN 2 individuals. These findings provided evidence that the MEN 2A mutant form of RET is oncogenic in parafollicular C cells and suggest that these transgenic mice should prove a valuable animal model for hereditary MTC (Michiels *et al*., 1997). A RET51 cDNA was used to generate mice in which a MEN 2A mutation, Cys-634-Arg, was expressed under the control of a human calcitonin gene promoter (CT-2A mice, Reynolds *et al*., 2001). These mice developed C-cell tumors resembling human MTC and follicular tumors resembling human papillary thyroid carcinoma (PTC) depending on the transgenic line examined. One line developed compound MTC/PTC at low frequency (8%) and pancreatic cystadenocarcinoma. CT-2A mice also displayed a developmental defect in thyroid follicular structure, in which much of the thyroid was occupied by large irregular cystic follicles thought to be derived from the ultimobranchial body, a developmental precursor of the thyroid gland (Reynolds *et al*., 2001). When this CT-2A transgene was introduced onto four different genetic backgrounds, between 0 and 98% of transgenic progeny developed thyroid tumors by 10 months of age, indicating that tumor penetrance could be modulated by genetic background. Furthermore, tumors on the CBA/ca and C57BL/6J backgrounds were significantly larger than those arising in BALB/c transgenic mice. These results are relevant to human MEN 2 disease, because this model system may be used to study genes modifying thyroid tumor penetrance in the dominantly inherited human cancer syndrome (Cranston and Ponder, 2003). Others reported the production of a mouse model of MEN 2B by introduction of the corresponding M918T mutation into the murine *RET* gene by homologous recombination.

Mutant mice displayed chromaffin cell hyperplasia progressing to phaeochromocytoma. Homozygotes did not develop gastrointestinal ganglioneuromas, but displayed ganglioneuromas of the adrenal medulla, enlargement of the associated sympathetic ganglia and a male reproductive defect. Surprisingly, in these mice with a MEN 2B mutation in the murine *RET* gene, generated by homologous recombination, only C-cell hyperplasia, but not 'full blown' MTC development was reported (Smith-Hicks *et al*., 2000). We generated transgenic mice using the human RET9 gene. We used a human calcitonin gene (*CALC-I*) promoter to generate mice expressing either the human RET oncogene with the most common MEN 2B mutation M918T (*CALC-MEN2B-RET*) or the human non-mutated RET proto-oncogene (*CALC-WT-RET*) in the C-cells. At the age of 20-22 months three out of eight *CALC-MEN2B-RET* transgenic founders presented with macroscopic bilateral MTC. In two founders nodular C-cell hyperplasia (CCH) was observed. Thyroid abnormalities were never observed in *CALC-WT-RET* transgenic mice or control non-transgenic mice analysed at this age. In some mice from established *CALC-MEN2B-RET* transgenic lines nodular CCH was observed from eight months on whereas MTC was detected in 13% of mice from one *CALC-MEN2B-RET* line, from the age of 11 months on. These result show for the first time that this MEN 2B mutation in the RET oncogene predisposes mice for MTC (Chapter 3). In view of the potentially higher oncogenic activity of mutated RET51, it would be interesting to generate transgenic mice that express a RET51 gene in which the MEN 2B mutation M918T is introduced.

2. Calc-Cre transgenic mice for C-cell specific inactivation of P53 and APC

The immunohistochemical identification of the involvement of P53 and Wnt deregulation in MTC development in patients and in the *CALC-MEN2B-RET* mice indicates that the mouse model is representative for the human situation. Involvement of these pathways in RET-induced MTC development was supported by *in vivo* studies. *CALC-MEN2B-RET* mice were crossed with P53 knockout mice and APCmin/+ mice, respectively. However, both models used have the drawback of undesired phenotypic expression, which leads to relative early death of the mice as a result of cancers not related to the studied MTC. Due to the detrimental effects of loss of P53 on the lymphoid compartment, P53ko mice suffer from aggressive lymphomas, which cause early death of these mice. APCmin/+ mice succumb to intestinal tumors from the age of 3 months onwards and do not live to more than one year of age. Better mouse models are therefore necessary if we want to further study the synergistic actions of RET with these and other tumor susceptibility pathways. One of the ways to obtain these models is the use of cre-recombinase technology.

The Cre/loxP site-specific recombination system combined with embryonic stem cell-mediated technology has provided a large number of mouse strains in which tumor suppressor genes can be inactivated in a tissue-specific manner. In order to be able to use such mice to study the effects of candidate tumor suppressor genes specifically on MTC development, we can produce a cre-expressing transgenic line that can provide thyroid C-cell specific recombination between loxP sites. We have used the 1.5 kb 5'region from the human CALC-1 promoter, to drive the expression of the cre-recombinase gene. This promoter fragment, however, may also drive

expression to other organs such as brain, lung and kidney, as seen in the *CALC-MEN2B-RET* and *CALC-WT-RET* mice, which may cause undesired side effects (Chapter 2). To overcome this drawback a C-cell (tissue)-specific enhancer sequence (TSE) identified in the CALC-I promoter might be used (Stolarsky-Fredman *et al*., 1990). By making a construct containing two or more repeats of this TSE in combination with only the first 129bp from the CALC-1 promoter region, C-cell specificity can be significantly enhanced (Messina *et al*., 2000). We are generating transgenic constructs with both types of CALC-1 promoter constructs to drive expression of the Cre gene. Transgenic mice obtained from either construct will be crossed with LacZ reporter mice to determine C-cell specificity of Cre-mediated recombination. CALC-Cre-lines with the desired specificity can then be used to introduce specific somatic mutations in the C-cells of these mice by cross-breeding with mice containing loxP sites flanking a gene (”floxed gene’’) of interest. Both floxed APC and P53 mice are available and can be crossed with the *CALC-MEN2B-RET* mice. The resultant hybrid mice can then be analysed for onset and progression of MTC development.

With respect to APC, compelling evidence is emerging that the classical interpretation of the two hit tumor suppressor gene concept of Knudsen does not entirely hold true. According to the Knudsen model the two hits necessary for complete inactivation of a tumor suppressor gene are independent mutation events. For the APC gene it was observed in tumors of familial colon cancer that both the type of the somatic mutation as well as the position of the somatic mutation depended on the type of germline mutation (Lamlum *et al*., 1999). Also in sporadic colon tumors there was interdependence of both APC hits (Rowan *et al*., 2000, Cheadle *et al*., 2002). Mutation analysis studies which correlated the second hit observed in tumors with the germline mutation indicated that there was a selection for APC genotypes that are likely to retain some activity in downregulating beta catenin activity. Therefore, it was proposed that the selection process is aimed at a specific degree of beta catenin signalling optimal for tumor formation (Albuquerque *et al*., 2002). This hypothesis was supported by studies using an APC mutated mouse strain in which beta catenin downregulation was not absolute. The APC 1638N mouse was generated by homologous recombination of a neomycin cassette in codon 1638 of the APC gene. For unknown reasons the mutant protein is poorly expressed due to some leakiness of the allele (Fodde *et al*., 1994). As a consequence, the heterozygous, APC1638N/+ mice develop far less colon tumors compared to APCmin/+ mice. In APCmin/+ mice more than 100 intestinal tumors per mouse are observed whereas APC 1638N/+ mice present on average with 5-6 intestinal tumors. Furthermore the APC1638N/+ mice present with a broad spectrum of extra-intestinal tumors including, mammary tumors, gastric tumors, desmoids and cutaneous cysts. It would be of interest to study this mutant gene dosage effect of APC on RET-induced MTC development by performing crossbreeding experiments. The results of such studies might indicate whether this ”just right signalling theory” for APC mediated tumor induction and progression may also hold for its synergistic effect on RET-induced MTC development.

3. Cholecystokinin/gastrin-derived peptides to treat metastatic MTC

The development of regulatory peptides as tools to visualise and to treat malignant tumors has been an important focus of interest in oncology over the past years (Behr *et al*., 1999, deJong *et al*., 1999). In contrast to mAbs, peptides have the advantage of much faster clearance. This results in much lower bone marrow dose, thereby avoiding the necessity of bone marrow transplantation. The pentagastrin-induced CT secretion test, which is used to detect the presence, persistence and recurrence of malignant C-cells, has an outstanding diagnostic accuracy. Therefore, the cholecystokinin (CCK) B/gastrin receptor was considered an interesting candidate for targeting of MTC's. The presence of CCKB/gastrin receptors was demonstrated in more than 90% of human MTCs (Reubi and Waser, 1996).

Pre-clinical and initial clinical evaluation of In-111-labeled (CCK)-8 analog for receptor targeted scintigraphy and radionuclide therapy of MTC has been reported (de Jong *et al*., 1999, Behr *et al*., 1999). Behr *et al*. (1999) demonstrated that In-111-labeled derivates of "minigastrin" showed an excellent targeting of CCK-B receptor-expressing tissues. Their data showed that gastrin analogs might be a useful new class of receptor-binding peptides for diagnosis and therapy of CCK-B receptor expressing tumors, such as MTC. This holds true even more, because no dependence of their quantitative degree of receptor expression upon the degree of tumor differentiation seems to exist for CCK-B receptors. This stands in clear contrast to somatostatin receptors, for which in accordance to *in vitro* data, a loss of somatostatin receptors in dedifferentiated and clinically more aggressive forms of MTC was demonstrated *in vivo* (Behr *et al*., 1997). Previous preclinical studies were performed in nude mice bearing a human MTC cell line TT (Behr *et al*., 1998, 1999) or rats bearing the CA20948 pancreatic tumor cell line (de Jong *et al*., 1999). These experiments in nude mice bearing human MTC xenografts demonstrated the feasibility of radiolabeled gastrin to target the CCKB/gastrin receptor of these tumors. Subsequently, CCK/gastrin analogues were developed as suitable radioligands for targeting of CCKB/gastrin receptor-bearing MTC xenografts (Behr *et al*., 1998, 1999). An initial evaluation in a human MTC patient showed uptake in lymphnode metastases in the neck region (De Jong *et al*., 1999). However, a variety of problems remain to be resolved. Further studies are warranted to show whether radiolabeled CCKB/gastrin receptor-binding peptides can actually improve the staging of MTC cancer patients. Furthermore, a drawback of CCKB/gastrin receptor-binding radiolabeled analogues is the high stomach and gallbladder uptake due to CCKB/gastrin receptor expression of these organs. Therefore, new candidate receptors that are more specific for MTC are needed.

4. MTC specific GFR(alpha)4-binding peptides for diagnosis and therapy

The RET tyrosine kinase can form multicomponent receptor complexes with each of four high affinity glycosyl-phosphatidyl-inositol (GPI)-anchored components (GFR(alpha)1-GFR(alpha)4). These complexes serve as receptor for the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs). There are currently four known GFLs: GDNF, neurturin, artemin and persephin. For each GFL there is a preferred GFR(alpha) receptor, to which the GFL binds with highest affinity and most potently activates the RET tyrosine kinase.

A comprehensive homologue-scanning mutagenesis study of GDNF identified sites along the second “finger” of GDNF that were critical for the activation of the GFR(alpha)1 and (alpha)2-RET receptor complexes. Replacement of these segments with corresponding segments derived from the other GFL members demonstrated the specificity of these regions for a specific GFR(alpha) (Baloh *et al*., 2000). The GFR(alpha)4 receptor was found to be specifically expressed in normal and malignant thyroid C-cells (Lindahl *et al*., 2001). Of 27 different human adult tissues studied, GFR(alpha)4 transcripts were detected only in the thyroid gland, while 8/8 MTC samples analysed showed high expression levels. Co-expression of RET and GFR(alpha)4 in humans seems to be restricted to C-cells and may be necessary for MTC development. It has been demonstrated that RET receptor triggering significantly increased the oncogenic capacity of MEN 2B mutation-bearing RET molecules *in vitro* (Bongarzone *et al*., 1998). GFR(alpha)4 was identified as the GPI-linked co-receptor of RET to form a functional receptor complex for persephin (Enokido *et al*., 1998). Therefore, persephin-derived peptides may provide unique tools for a novel therapeutic approach for MTC, which can work two-fold. On the one hand such peptides may provide a tool to deliver radio-iodine or radio-metal or other cytotoxic molecules to MTC cells with high selectivity and specificity for diagnostic and therapeutic use. On the other hand such peptides may interfere with binding of the actual ligand persephin and by this means cause reduction of the oncogenic potential of MEN 2 mutation-bearing RET molecules.
Thus persephin most probably represents the member of the GDNF family of neurothrophic growth factors (GFLs) involved in the differentiation and maintenance of thyroid C-cells (Lindahl *et al*., 2001). Peptides can be selected from the region of mouse persephin, that is required for the interaction with the GFR(alpha)4 co-receptor of RET. These peptides are selected based on homology to the GFR(alpha)-binding regions previously identified for mouse GDNF (Baloh *et al*., 2000). Such RET/GFR(alpha)4 receptor-binding peptides provide a novel class of tools to target MTC cells. The comprehensive homologue-scanning study of GDNF has indicated that critical residues for the GFR(alpha) receptor interaction and specificity are located in the second finger (F2) region of the GDNF molecule. Replacement studies of these regions have provided evidence that regions F2a and F2c are likely to be directly involved in the binding to GFR(alpha)s. Peptides, derived from the corresponding regions from mouse persephin: F2a (QPTSYAD) and F2c (WQQLPQLS) can be generated. These regions have been shown not to be involved in binding to GFR(alpha)1 and GFR(alpha)2 and are thus most likely to be specific for binding to GFR(alpha)4. In addition, a “mini-persephin” molecule corresponding to the entire F2 region (residues 65-96) can be used. The peptides can be labelled with iodine-125 and tested for their ability to bind specifically to murine MTC cells. These studies will initially be performed *in vitro*, using primary cell cultures derived from murine MTC as described previously (Chapter 3). Peptides that show binding can be used for evaluation of binding *in vivo,* using MTC-bearing *CALC-MEN2B-RET* transgenic mice, with a protocol analogous to the procedure described above for the gastrin-derived radio-metal-labelled peptides.

5. RET as a target for MTC therapy

The number of signal transduction pathways initiated by the RET proto-oncogenes is still increasing. The contribution of deregulation of each of these pathways to cancer formation however is still a matter of speculation. Oncogenic RET probably accounts for multiple mechanisms leading to a transformed phenotype since the expression of oncogenic RET variants alone suffices to transform NIH3T3 fibroblasts. This establishes a role of RET as a target for MTC therapy.

The expression of dominant negative RET mutants has been reported for the treatment of MTC (Drosten *et al*., 2002, 2003). Mutant RET variants were used which are hampered in their ability to be transported to the cell surface but which are still capable to dimerize in the cytoplasm with oncogenic RET. Transduction of TT cells with an adenoviral dominant negative RET construct resulted in the induction of apoptosis. Furthermore, the neoplastic properties of these MEN 2A RET expressing tumor cells were reverted by the dominant negative RET protein, as indicated by a strong inhibition of tumor growth *in vivo* and in increased MTC bearing animal survival (Drosten *et al*., 2003).

Other means of blocking the oncogenic activity of RET have been exploited. Targeting of RET in patients with MTC or PTC using monoclonal antibodies has been reported (Yano *et al*., 2000). Therapies aimed at the abrogation of gene expression using ribozymes or RNA interference have been envisaged. A RET MEN 2A selective ribozyme has been studied for its ability to interfere with RET activity. The ribozyme was capable of specifically cleaving MEN 2A-derived RET RNA and blocked RET-mediated transformation (Parthasarathy *et al*., 1999). Inhibition of RET has also been achieved by the expression of SHP1, a protein tyrosine phosphatase (Hennige *et al*., 2001). The test of these approaches in clinically relevant models of MTC is anxiously awaited.

Since RET function as protein tyrosine kinase has become more appreciated as a prime target for treatment of MTC, much emphasis has been put on inhibiting its kinase function. Small molecule tyrosine kinase inhibitors are an important new class of cancer treatment drugs. These molecules compete with ATP, for binding the catalytic domain of the tyrosine kinases. Upon binding, auto-phosphorylation is obstructed and downstream signal transduction is blocked. STI571 for the treatment of myeloid leukaemia and gastrointestinal stromal tumors (reviewed by Gschwind *et al*., 2004) and ZD1839 for the treatment of small cell lung carcinoma (Minna *et al*., 2004) are two examples of successfully established anti-cancer tyrosine kinase inhibitors. Two pyrazolopyrimidines (PP1 and PP2) have been shown to exert RET inhibition and were shown to inhibit RET oncogenic effects in culture. In addition, they induce RET protein destruction through proteosomal degradation at nM concentrations (Carniti *et al*., 2003). CEP-701 and CEP-751, indolocarbazole derivatives, were effective in inhibition of tumor growth in MTC cell xenografts (Strock *et al*., 2003). The most promising anti-RET drug seems to be ZD6474 which inhibits RET with an IC50 of 100nM (Carlomagno *et al*., 2002, 2004) and which is currently in advanced phase of clinical development, showing no significant toxicity in phase I trials.

The success of mutant-RET specific inhibitors may be determined for a great deal by whether MTC growth is still dependent on the deregulated or constitutively active RET. In this respect, the multistep nature of tumor development is a great concern. However, growing evidence supports a significant role for several oncogenes not

only in the initiation but also in the maintenance of tumor development. Prominent examples include myc (Felsher *et al*., 1999) and BCR-ABL (Huettner *et al*., 2000) for which it was reported that even brief inactivation of these transforming oncogenes can revert the neoplastic phenotype, at least partially. Reversal of the transformed phenotype has been reported, even in tumors with a background of multiple genetic defects, such as loss of tumor suppressor genes or further oncogenic activation (Karlsson *et al*., 2003; Gunther *et al*., 2003)

6. P53 manipulation as a potentiator of cancer therapy

The realisation of the importance of P53 in tumor initiation and progression has inspired the development of several tactics to manipulate mutant P53 in cancer cells. These strategies are aimed at the “correction” of mutant P53 to restore its normal wildtype (wt) functions. In tumors, P53 activity is frequently abolished by point mutations and this has been interpreted as a gain of function. Specifically with respect to tumor initiation, mutations in one P53 allele are sufficient to inhibit the product of the wildtype allele through a dominant negative action. Cells expressing mutant P53 can be more tumorigenic than cells lacking endogenous P53 (Dittmer *et al*., 1993). Most mutated P53 proteins are able to drive co-translated wildtype P53 into a mutant conformation, resulting in tetramers that are inactive in DNA binding (Milner and Medcalf, 1991). Tumor progression is accompanied with loss of heterozygosity, which abolishes wild type P53 activity. The vast majority of mutations in P53, which are found in cancer, fall within the DNA-binding domain, disrupting the P53-DNA target interaction and transcriptional activation of downstream targets.

Therapeutic strategies to interfere with cancer progression are aimed at the restoration of some of the wildtype properties of P53 by targeting drugs to the mutant-P53 form. Restoration of inactive or suppressed wt-P53 or reactivation of mutant-P53 has been demonstrated using antibodies, peptide constructs and small molecular weight synthetic molecules (Bykov *et al*., 2003).The use of synthetic peptides derived from the C-terminus of P53 for this purpose was prompted by the observation that a P53 c-terminus-directed antibody was capable of activating latent P53 (Hupp and Lane, 1994).

PRIMA-1 (P53 reactivation and induction of massive apoptosis-1) is a small molecular weight molecule that was selected for its ability to suppress the growth of cells expressing a P53 mutant (Bykov *et al*., 2002). In mutant-P53 bearing cells, PRIMA-1 was able to impose sequence specific DNA binding and transcriptionally activate P53 targets. PRIMA-1 displayed anti-tumor effects in mouse experiments without detectable toxicity (Bykov *et al*., 2002). Cp-31398 is a small synthetic molecule reported to confer wt-P53 conformation to a mutant-P53 and to induce transcriptional activity. Cp 31398 was able to inhibit growth of human melanoma and colon carcinoma cells in nude mice with no noted toxicity (Foster *et al*., 1999). Another innovative approach to selective reactivation of mutant-P53 has been achieved by the construction of a chimeric adaptor, which acts as a bridge to link mutant-P53 and P53 specific target sequences. The adaptor was able to activate some but not all mutants, thereby promoting the activation of target genes. The effects were mutant-P53 specific as this adaptor molecule failed to act in cells lacking P53 or expressing wtP53 (Roth *et al*., 2003).

Ellipticine, an alkaloid extracted from the plant Ochrosia elliptica, appears to preferentially regenerate wt-P53 function in mutant-P53, but the mechanism of its action has not yet been elucidated (Shi *et al*., 1998).

7. Deregulated Wnt signalling as target for cancer therapy

Only few reports describe efforts with respect to targeting of the deregulated Wnt signalling pathway for cancer therapy. These studies have focused on the question if influencing the inappropriate beta-Catenin transcriptional activity can affect the tumor phenotype of transformed cells. The level of beta-Catenin in the cytosol is normally regulated by the beta-Catenin destruction complex, which consists of the scaffold protein Axin, the product of the colon cancer gene APC (adenomatous polyposis coli), two Ser/Thr kinases CK1alpha and GSK-3beta, and beta-Catenin itself (reviews by Peifer and Polakis, 2000; Seidensticker and Behrens, 2000). In colon cancer cells, components of this complex are frequently mutated, which results in a constitutive increase in beta-Catenin levels and enhanced formation of the beta-Catenin/TCF complex (reviews by Polakis, 2000; van Noort and Clevers, 2002). The importance of developing an inhibitor that is able to counteract this deregulation of beta-Catenin has made the beta-Catenin/TCF complex a drug target for cancer treatment (reviewed by Hecht and Kemler, 2000).

Chemoprevention is emerging as a promising strategy that can reduce colon cancer risk. Non-steroidal anti-inflammatory drugs (NSAIDs) are the prototypical chemopreventive agents against colon cancer as evidenced by a plethora of data, including more than 30 epidemiological studies, and by recent human interventional trials using aspirin (ASA) (Levy *et al*., 2002). However, following NSAID treatment, there was no consistent relationship between reduced cell proliferation, induction of apoptosis and changes in beta-Catenin protein levels or beta catenin related transcription. NSAIDs decreased nuclear beta-Catenin content and cyclin D1 protein levels in parallel with their antiproliferative activity. However, cyclin D1 downregulation occurred prior to a decrease in total beta-Catenin protein levels and there was no correlation with changes in beta-Catenin related transcription, suggesting the existence of beta-Catenin-independent effects of NSAIDs on cyclin D1 expression (Gardner *et al*., 2004). NO-donating NSAIDs (NO-NSAIDs) have been introduced as a class of safer compounds with minimal damage to the gastric mucosa as compared to traditional NSAIDs (Rigas and Williams, 2002). NO-ASA inhibits beta-Catenin-mediated TCF activity by preventing the formation of the beta-Catenin/TCF-4 complex. This effect, occurring at NO-ASA concentrations far below those required to inhibit cell growth, may be a critical early event in the chemopreventive activity of NO-ASA against colon cancer (Nath *et al*., 2003)

ICAT (inhibitor of beta-Catenin and TCF), a negatively charged 81 residue protein, is a physiological inhibitor of the Wnt signaling pathway that prevents the binding of TCF to beta-Catenin (Tago *et al*., 2000; Tutter *et al*., 2001). ICAT is required for normal embryonic development in *Xenopus* (Tago *et al*., 2000), and ICAT and an ICAT homologous gene, LZIC, are localized in a human chromosome region that is frequently rearranged or deleted in various cancers (Katoh, 2001). Reduced ICAT transcript levels were found in more than 65% of malignant melanomas tested (Reifenberger *et al*., 2002). ICAT was shown to selectively inhibit beta-

Catenin/TCF binding *in vivo*, without disrupting beta-Catenin/cadherin interactions (Graham *et al*., 2002). Thus, it should be possible to design cancer therapeutics that inhibit beta-Catenin-mediated transcriptional activation without interfering with cell adhesion. At present, there is little biochemical or structural information available concerning the interaction of beta-Catenin with transcription factors other than TCF/LEFs. The crystal structure of ICAT, bound to the armadillo repeat region of beta-Catenin, was reported. This structure showed that ICAT consists of two distinct modules that occupy the TCF/LEF and CBP/p300 binding sites on beta-Catenin. ICAT thus acts as a bipartite inhibitor of transcription capable of simultaneously blocking binding of both TCF/LEF and p300 to beta-Catenin (Daniels and Weis, 2002). These data suggest new avenues for the design of inhibitors of beta-Catenin-mediated transcription

To identify small molecule antagonists of this pathway, transformed colorectal cells were challenged with a secondary structure-templated chemical library, looking for compounds that inhibit a beta-Catenin-responsive reporter. ICG-001, a small molecule that down-regulates beta-Catenin/T cell factor signaling by specifically binding to cyclic AMP response element-binding protein was identified. ICG-001 selectively induced apoptosis in transformed cells but not in normal colon cells, reduced *in vitro* growth of colon carcinoma cells, and was effective in the Min mouse and nude mouse xenograft models of colon cancer (Emami *et al* 2004).

8. P18 and cell cycle targeted therapies

Cell division in eukaryotes occurs in four phases of the cell cycle. In the G1 phase the cell is prepared for DNA replication. The chromosomes are replicated during S phase. The G2 gap period allows preparation for mitosis, before chromosome segregation and cytokinesis in the M phase. Before returning to the G1 phase, cells can enter an inactive period referred to as G0 phase, which can occur during development or differentiation. The cell cycle is regulated by a number of important protein families, which are common targets for mutational inactivation or overexpression in human tumors. The regulators of the cell cycle control the cell cycle checkpoints, which ensure the faithful chromosome replication and separation, thereby maintaining genetic stability. Failure of these checkpoints to arrest the cell after the appropriate stimuli is a hallmark of cancer. One crucial checkpoint occurs in mid-G1 phase, after passing through this point the cell commits to cell division.

Disruption of the G1-checkpoint control is a common feature in cancer. Two protein classes control cell cycle progression: The cyclins and the cyclin dependant kinases (cdks). The cyclin-D and E family of proteins, which can bind and activate cdks, coordinates passage through the G1 checkpoint. The D-type cyclin activity is required for regulated progression from G1 into S phase. This is mediated, at least partly, through coordinated phosphorylation of the retinoblastoma protein (Rb). Progressive phosphorylation of Rb leads to transcription of genes necessary for entry into the S phase, whereas unphosphorylated Rb represses the transcription of these genes. Members of the cdk inhibitors, ink4, family of proteins regulate activity of the complex of cyclin-D with cdk4 or cdk6. P18 is a member of the ink4 family and is also referred to as ink4c or cdkn2c. P18

interacts with and inhibits cdk4 and preferentially cdk6, and inhibits the action of the cyclin-D/cdk4 or cyclin-D/cdk6 complexes. Upon interaction with P18 these complexes are not able to phosphorylate Rb.
Better understanding of the cell cycle and of the role of deregulation of cell cycle control in tumorigenesis have led to the development of compounds with the potential to restore control of cell division in human cancers. Molecules that mimic physiological cdk inhibitors, such as p18, have been designed to target the ATP binding site of the cdk molecules. Flavoperidol is one such agent of which clinical trials have begun. Phase I trials found dose limiting adverse effects, such as arterial and venous trombosis (Thomas *et al*., 1997, Senderowicz *et al*., 1998). Phase II trials indicated stabilisation of disease in a subset of the patients treated (reviewed by Shah and Schwartz , 2003). Application of flavoperidol in combination with standard therapeutic agents was synergistic in the induction of apoptosis and induced stable disease and minor response in part of patients with oesophagus, lung prostate and colorectal cancer (Shah and Schwartz, 2003). Pharmacological strategies to interfere with cell cycle regulation processes have also been aimed at agents that target the proteosome. These will interfere with the degradation and expression of molecules that control G1 to S phase transition. Bortezomib has potent proteosome inhibitory activity. Bortezomib may lead to stabilisation of both cdki and cyclins Promising results in phase II trials with bortezomib in relapsed multiple myeloma have been published (Richardson *et al*., 2003).
The molecule UCN-01 has a complex mode of action and can promote or inhibit cdk activity. UCN-01 was discovered to have many direct and indirect effects on cell cycle targets. With this molecule promising phase I and II data have been generated (Sausville *et al*., 2001)

Mouse deletion studies show that p18 is a candidate tumor suppressor gene involved in MTC development. Mice lacking P18 are predisposed to a variety of other tumors including pituitary adenomas, testicular tumors and adrenal phaeocromocytomas. Other tumors observed are B-cell lymphomas, angiosarcomas, renal carcinomas, thymic lymphomas and medullary thyroid carcinomas (Franklin *et al*., 1998, 2000, Latres *et al*., 2000). These mice also display an increased propensity to carcinogen-induced tumorigenesis (Bai *et al*., 2003).
The 1p32 genomic locus where the P18 gene is located is commonly deleted in human tumors. Homozygous deletion of P18 or mutations in P18 have only rarely been found in a large number of tumor types analysed.
Table 1 lists the types and numbers of tumors in which mutations of *P18* have not been detected. Considering its important role in cell cycle control it is intriguing why *P18* mutations have only rarely been found in large numbers of different types of cancers analyzed. Tumor types in which p18 mutations or homologous deletions have been described include meningiomas, breast carcinomas, myeloid leukaemias and medullary thyroid carcinomas.
The apparent involvement of p18 in only specific types of tumors may indicate a tissue specific role for P18 function in cell cycle regulation in thyroid C-cells. Possibly there may be redundancy of P18 function in most other cell types due to expression of other CDKIs (P15, P16 or P19), which control function of Rb. This suggests that in thyroid C-cells P18 is the CDKI that plays the major role in suppressing tumorigenesis by controlling Rb function. Whether this also holds for other cells of neuroendocrine origin and

the tumors derived thereof (e.g. adrenal chromaffin cells and phaeochromocytomas) is an interesting question that can be investigated with phaeochromocytomas from MEN 2 or sporadic patients.
Another challenging explanation may be that the presence of constitutively active RET makes the C-cells more susceptible to effects of P18 mutation. The mutations in *P18* reported were detected in a familial MTC (with a germline *RET* mutation) and in two sporadic MTCs. Interestingly, both sporadic tumors had also acquired a somatic activating mutation in the *RET* gene. Also in the TT cell line, loss of P18 in combination with RET activation is observed. These data suggest that loss of P18 function and activation of RET collaborate by deregulating pathways which act synergistically in MTC induction and progression.

Table 1. List of tumor types and and number of cases studied, in which P18 mutations were detected. For references see chapter 5.

nrs	Type of tumor
103	childhood lymphoblastic leukemias
46	myeloid leukemias
71	primary or metastatic lung cancers
52	Osteosarcomas
23	various other sarcomas
71	non-small-cell lung cancers
81	primary lymphoid tumors
25	Neuroblastomas
20	primary multiple myelomas
44	adult T-cell leukemias
101	non-Hodgekin's lymphomas
25	parathyroid adenomas
14	Hepatoblastomas
44	Primary melanomas
15	Ovarian granulosa cell tumors
26	invasive primary breast cancers
20	acute myelogenous leukemias

Further study of pathways, which may be deregulated in multistep MTC development, may unravel a coherent set of rules and principles for MTC tumorigenesis. The useof high throughput techniques such as gene expression profiling and proteomics will be needed to identify other relevant pathways.

9. Gene expression profiling of RET-induced MTC

An ever-increasing number of genes have been shown to contribute to the distinct steps involved in neoplastic transformation of the more than 100 distinct types of human cancer as well as in different cancers from the same tissues. The development of cancer in humans involves a complex succession of events that usually occur over many decades. This complexity makes it questionable whether these mutations can be rationalised to a coherent set of rules and principles rather than a phenomenology of unlimited complexity. This is partly due to the fact that the initiating cause for cancer development is very heterogeneous. One of the challenges in cancer research is to dissect this complexity, in order to reduce this to a more generally applicable set of tumor pathways common to most, if not all, types of cancers (Hahn and Weinberg, 2002).
By studying familial cancers (in which the same initiating genetic event is present in all patients), the complexity of the findings can be reduced. The use of genetically identical inbred mice to generate *in vivo* models for familial cancer is likely to reduce this complexity even more. The in principle unlimited source of material, in combination with the use of the micro-array technique (with its capacity to analyse thousands of genes, which can be clustered based on many different criteria), promises not only to identify

cellular and genetic principles for tumorigenesis, but also to provide prognostic and diagnostic markers as well as novel molecular targets for therapy.
The *CALC-MEN2B-RET* mouse model for multistep MTC development in which the initiating 'first hit' is known offers the opportunity to identify additional genetic events associated with the transition to subsequent stages of tumor development. The ability to query the expression of thousands of genes simultaneously, using DNA micro-arrays, affords us a new way of analysing and understanding disease progression. Gene expression DNA chips can be applied for the discovery of genes that are relevant to disease pathways. Such RNA expression profile analyses can be performed making use of whole genome mouse micro-arrays, which contain 20-30.000 oligonucleotides representative for a similar number of mRNA species. Tissue samples from subsequent stages of MTC development (e.g. diffuse CCH, nodular CCH, microscopic MTC, and macroscopic MTC) can be used. For this purpose the thyroid glands from mice identified to be at a specific stage of MTC development, by basal or stimulated plasma calcitonin levels, can easily be collected. To isolate C-cell specimens free from surrounding normal tissue, the laser assisted micro-dissection technique on freshly frozen samples can be applied. This technique is optimised for dissection of very small samples (up to single cells) from frozen tissues and allows the simultaneous preparation of control slides for histo-pathological evaluation of the tissue. Total RNA isolation and cDNA synthesis, especially from the early stages may not yield sufficient material. In that case the technique of cDNA amplification with T7 promoter-tailed primers (Eberwine, 1996) or the exponential cDNA amplification method described and evaluated for micro-array analysis (Iscove *et al*., 2002) can be applied. Alternatively, one can consider to pool tissue samples obtained from several mice at an identical early stage of tumorigenesis to obtain sufficient amounts of RNA without the need for amplification. For an initial comparison of the different stages, expression profiles of at least four RNA samples from each stage can be compared with a reference RNA that consists of a pool of equal amounts of RNA from all four stages of MTC development. Normalisation of the data by the addition of an equal amount of 9 different *in vitro* synthesized RNA species (external controls) to each sample to be analysed (van de Peppel *et al*., 2003) will always provide a good positive control for cDNA synthesis in each sample and correction for experimental variation.
These data can provide the basis for further experimental analysis of the relevant genes using the *CALC-MEN2B-RET* transgenic mouse model and available MTC cell lines.

10. Concluding answers.
The study presented in this thesis has provided answers to the questions asked in chapter 1
I. In this thesis I described the generation and characterisation of a transgenic *CALC-MEN2B-RET* mouse model, expressing a mutated RET oncogene, as a model to study MTC development. Predisposition for MTC development in these mice, but not in control mice expressing non-mutated RET, provided experimental evidence for the oncogenic potential of the M918T mutation in RET.
II. Tumorigenesis of MTC in these mice was driven by multiple events, comparable to the human situation, as observed in MEN 2 patients. By using tumor material from familial and sporadic MTC patients and MTCs from our transgenic mouse model I found evidence

for the involvement of the P53 pathway, the P18 cell cycle regulation pathway and deregulation of the Wnt signal transduction pathway as additional oncogenic events in RET-induced MTC development.
III. Using transgenic mouse models I obtained further experimental evidence for involvement of P53 inactivation and deregulation of Wnt signalling in RET-induced MTC development *in vivo*.
IV. The observation that P18 mutations seem to be restricted to a very small number of tumor types including familial and sporadic MTC and that in sporadic MTC there is coincidence with somatic RET mutations may indicate synergistic cooperation of these two specific pathways.
V. Further study of P18 involvement in other RET-induced (neuro-endocrine) tumors such as phaeochromocytoma. or high throughput techniques, such as RNA expression profiling, applied to familial and sporadic MTC and MTC from our *CALC-MEN2B-RET* mouse model may provide us with more indications for cooperation of RET and P18 or may identify other specific sets of synergistic pathways in MTC development.
VI. Implementation of the finding that RET, P53, Wnt deregulation and P18 are involved in the tumorigenesis of MTC results in new therapeutic options for metastatic MTC.

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Summary

Medullary thyroid carcinoma (MTC) originates from the calcitonin (CT)-producing thyroid gland C-cells. MTC is found as a sporadic tumor and MTC is the most important clinical manifestation of the Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome. MEN 2 is associated with germline mutations in the *RET* gene. The *RET* proto-oncogene encodes the RET receptor tyrosine kinase which serves as the receptor for the GDNF family of neurothrophic factors. RET is an essential component of signaling pathways required for kidney organogenesis and enteric nerve development. Mutations in the *RET* gene are associated with Hirschsprung's disease and with sporadic medullary and papillary thyroid cancer and with MEN 2. There are two subtypes of MEN 2. In MEN 2A the mutations affect cysteine residues in the extracellular domain of the protein, which results in constitutive kinase activity. In MEN 2B the most common mutation results in a Met→Thr substitution at codon 918 (M918T) in the intracellular domain of the protein. This mutation is also found as a somatic mutation in a high percentage (30-40 %) of sporadic MTC cases. This MEN 2B mutation alters the substrate specificity of the protein.

To study the tumorigenesis of MTC we generated transgenic mice. We used the human CT gene promoter to generate transgenic mice expressing either the human non-mutated *RET* proto-oncogene (*CALC-WT-RET*) or the human *RET* oncogene with the M918T mutation (*CALC-MEN2B-RET*) in the C-cells. Thyroid abnormalities were never observed in *CALC-WT-RET* transgenic mice or control non-transgenic mice analyzed up to an age of more than two years. A phenotype was observed in the *CALC-WT-RET* mice. Endogenous RET expression in kidneys is restricted to the embryonic stage, transgenic RET expression was also observed in postnatal kidneys. *CALC-WT-RET* transgenic mice developed renal cystic disease depending on the level of RET protein expression in the kidneys. Two founders succumbed to renal cystic disease after 10 months. In several transgenic lines mild renal cystic disease developed after one year. Increasing RET expression, by breeding homozygous or compound heterozygous offspring, resulted in a phenotype of progressive renal cystic disease and growth retardation. These findings stress the importance of regulated RET expression in normal kidney development and function, and may indicate a role for RET in human kidney pathology. In *CALC-MEN2B-RET* mice a thyroid phenotype was observed. At the age of 20-22 months three out of eight *CALC-MEN2B-RET* transgenic founders presented with macroscopic bilateral MTC. In two founders nodular C-cell hyperplasia (CCH) was observed. In some mice from transgenic lines nodular CCH was observed from eight months on whereas MTC was detected in 13% of mice from one *CALC-MEN2B-RET* line, from the age of 11 months on. These result showed for the first time that the MEN 2B mutation in the *RET* oncogene predisposes mice for MTC. The *CALC-MEN2B-RET* mice developed MTC, preceded by CCH, with low incidence and a variable latency period, suggesting the involvement of additional oncogenic events. The nature of the additional oncogenic events involved in MTC progression was unknown.

We studied a potential role for the P53 tumor suppressor in human and mouse

RET-induced MTC development. In a panel of human MEN 2 and sporadic MTC samples immunohistochemistry revealed P53 staining, which is indicative for mutated P53, in three of 15 tumors analysed. In MTC samples from four *CALC-MEN2B-RET* transgenic mice P53 staining was observed. A possible causal relation between *P53* gene inactivation and MTC development was studied by monitoring *CALC-MEN2B-RET* transgenic mice lacking one or both alleles of the *P53* gene. Four of six composite *CALC-MEN2B-RET* ;*P53-/-* mice analysed presented with MTC at the age of 3-5 months, while the other two had CCH. At this age MTC was not detected in *P53-/-* or *CALC-MEN2B-RET* transgenic control mice. A significantly increased incidence of RET-induced MTC development was also observed at later age in composite *CALC-MEN2B-RET;P53+/-* mice compared to controls. These data indicate that inactivation of P53 contributes to Ret-induced MTC development.

Deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway has been associated with several forms of cancer. Potential involvement of this pathway in RET-induced MTC development was investigated by analysis for beta-Catenin expression of thyroid gland C-cells and MTC from *CALC-MEN2B-RET* transgenic mice. While in normal C-cells beta-Catenin staining is observed solely associated with the plasma membranes, in MTC tumor cells beta-Catenin staining was also observed in the nucleus in 6/7 cases analysed, indicating tumor-specific deregulation of the Wnt/beta-Catenin/Tcf signal transduction pathway. The significance of these findings for human MTC was evaluated using tumor samples derived from MEN 2 patients and sporadic MTC cases. Nuclear beta-Catenin staining was detected in tumor cells from 6 out of 15 human MTC samples investigated. CCH and microscopic MTC from MEN 2 patients or *CALC-MEN2B-RET* mice were not associated with nuclear beta-Catenin staining, suggesting that this is a late event in tumorigenesis. To study the effect of deregulated Wnt/beta-Catenin/Tcf signalling on RET-induced MTC development *in vivo,* we generated *CALC-MEN2B-RET* transgenic mice also carrying the *APC(min/+)* mutation. These mice could be monitored for one year. A significant increase in CCH prevalence was detected in these mice. The increased CCH incidence was age dependent and associated with nuclear beta-Catenin in hyperplasic C-cell areas in part (1/6) of the mice analysed. These data indicate that deregulation of Wnt/beta-Catenin/Tcf signalling contributes to RET-induced MTC development.

A frequent finding in MTC is loss of heterozygosity at chromosome 1p, indicating a potential tumor suppressor gene of MTC development on this chromosome arm. Therefore, we performed mutation analysis of the *P18* gene, located on 1p32 and involved in cell cycle regulation, from eight human MTC samples. In one MEN 2 and two sporadic MTC cases mutations in the *P18* gene were detected. Both sporadic MTCs had also acquired a somatic *RET* mutation. Homozygous deletion of the *P18* gene was observed in the *RET* mutation-bearing MTC cell line TT. These data strongly indicate, that P18 can act as a tumor suppressor of human MTC and suggest collaboration of loss of P18 function with activation of RET during MTC development.

Identification of these three pathways as additional oncogenic events in RET-induced MTC development provides us with important novel targets for therapy, as there are currently no effective treatment modalities available for metastatic MTC.

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Acton DS, Velthuyzen D, Goldschmeding R, van Buul-Offers S, Lips CJM and Höppener JWM. Aberrant renal expression of RET proto-oncogene in transgenic mice induces progressive renal cystic disease associated with growth retardation. *(submitted)*

Acton DS, Höppener JWM and Lips CJM Mutation and homozygous deletion of cell cycle regulator P18/ink4c/cdkn2c in human medullary thyroid carcinoma and in the MTC cell line TT. *(submitted)*

Acton DS, de Heus R, Roijers JFM Lips CJM and Höppener JWM. Involvement of P53 in RET-induced medullary thyroid carcinoma development in human patients and transgenic mice. *(submitted)*

Acton DS, van Veelen W, de Heus R, den Ouden K, Lips CJM and Höppener JWM. Deregulation of Wnt/beta-Catenin/Tcf signalling contributes to RET-induced medullary thyroid carcinoma development in transgenic mice and human patients. *(submitted)*

Selected and invited oral presentations and awarded abstracts

EuroMen Business Meeting, Hamburg, Germany (1998)
Invited oral presentation: **Acton DS**. Transgenic mouse models for MEN 2

7th international workshop on Multiple Endocrine Neoplasia, Gubbio, Italy (MEN1999)
Award winning abstract: **Acton DS,** Velthuyzen D, Lips CJM and Höppener JWM. Characterization of transgenic mice expressing wildtype or mutated c-ret as a model for Multiple Endocrine Neoplasia type 2.

8th international workshop on Multiple Endocrine Neoplasia, Grand Rapids, USA (MEN 2002)
Selected for oral presentation: **Acton DS**, de Heus R, Roijers J, Lips CJM and Höppener JWM. P53 inactivation contributes to RET-induced MTC development in transgenic mice and human patients
Selected for oral presentation: **Acton DS**. de Heus R, van Veelen W, den Ouden K, Lips CJM and Höppener JWM. Characterisation of RET-induced medullary thyroid carcinoma in transgenic mice and human patients indicate deregulation of wnt signal transduction.

38th annual meeting of the European Society for Clinical Investigation, Utrecht, The Netherlands (ESCI 2004)
Invited oral presentation: **Acton DS**. Multistep carcinogenesis in medullary thyroid carcinoma.
J. Clin. Invest. 34 (Suppl 1), 23 (2004)

9th international workshop on Multiple Endocrine Neoplasia, Bethesda, USA (MEN2004)
Selected for oral presentation: **Acton DS**, Höppener JWM and Lips CJM. Involvement of the cell cycle regulator P18/ink4c/cdkn2c in human medullary thyroid carcinoma development
J. Intern. Med. 255. 714 (2004)

10th symposium of the Dutch Thyroid Club, Amsterdam, The Netherlands (2004)
Invited oral presentation: **Acton DS**. Pathogenesis of medullary thyroid carcinoma

Curriculum Vitae

Dennis Stanley Acton werd geboren op 8 februari 1963 in Paramaribo Suriname waar hij korte tijd op de St. Aloysius kleuterschool heeft gezeten. In 1967 emigreerde hij met zijn familie naar Nederland en zij gingen wonen in Utrecht. Hij bezocht de Engel Gabriel kleuterschool en hij heeft een deel van zijn lagere school periode doorgebracht op de Charles de Foucaultschool. In 1974 verhuisde hij naar Woerden, waar hij nog twee jaar op de Mariaschool heeft gezeten. Vervolgens ging hij naar de Rijksscholengemeenschap F.A. Minkema in Woerden. In deze periode heeft hij zijn vriendin Annet van den Bosch ontmoet met wie hij in 1980 verkering kreeg. In 1981 heeft hij zijn VWO diploma gehaald. Hetzelfde jaar is hij met de studie Biologie begonnen aan de Rijksuniversiteit Utrecht waarvoor hij in 1982 zijn Propedeuse haalde. In 1983 is hij met Annet gaan samenwonen in Utrecht, waar hij nu nog steeds woont. Voor het Kandidaatsexamen in 1985 heeft hij de specialisatierichting B5*, Medische Biologie gevolgd. Voor het Doctoraalexamen Biologie heeft hij onderzoek gedaan bij de Vakgroep Functionele Morfologie, Faculteit Diergeneeskunde: *Ontwikkeling in vitro kweek methode voor eencellige varkensembryo's*. Tevens bij Vakgroep Hematologie, Faculteit Geneeskunde: *Interactie van antithrombine III met bloedplaatjes en endotheelcellen*. En bij de Vakgroep Moleculaire Celbiologie, Faculteit Biologie: *Biogenese van fimbriae van uro-pathogene micro-organismen*. In 1987 is zijn zoon Igor Nelson geboren en In hetzelfde jaar is hij getrouwd met Annet. Zijn doctoraaldiploma (oude stijl) ontving hij in 1988. Zijn eerste echte baan als onderzoeker was bij de Vrije Universiteit (VU) van Amsterdam bij de Vakgroep Verloskunde en Gynaecologie. Het betrof een Preventiefonds Project: *Zuivering en biochemische en biologische karakterisering van vruchtwater-prolactine*, in het VU ziekenhuis. Van 1989 tot 1994 werkte hij als onderzoeker bij afdeling Moleculaire Genetica van het Nederlands Kanker Instituut Amsterdam, op een Koningin Wilhelmina Fonds (KWF) project: *Functional analysis of bcl-2 in vivo*, in het Antoni van Leeuwenhoek Ziekenhuis. In 1991 is zijn dochter Noëlle Teresa geboren. Van 1995 tot 1999 is hij onderzoek gaan doen bij de afdeling Klinische Endocrinologie, Divisie Interne Geneeskunde en Dermatologie te gast op het laboratorium van de afdeling Pathologie aan de Universiteit Utrecht. Het KWF project: *Generation of transgenic mouse models for MEN 2A* werd van 1999 tot 2000 voortgezet als "genvlag project" van het Academisch Ziekenhuis Utrecht: *Characterisation of transgenic mouse models for MEN 2*, bij dezelfde afdelingen. Van 2001 tot 2004 heeft hij onderzoek gedaan op een TS-project van Crucell Holland BV en het Universitair Medisch Centrum Utrecht (UMCU): *Antibodies and vaccines for type 2 Diabetes*, ook bij de afdeling Klinische Endocrinologie maar nu te gast op het laboratorium van de afdeling (Pediatrische) Endocrinologie in het Wilhelmina Kinderziekenhuis (WKZ) van het UMCU. Eind 2001 is het door hem geschreven onderzoeksproject: *MEN2B-RET transgenic mice as a model for medullary thyroid carcinoma: study of cooperating oncogenic events and pre-clinical evaluation of novel diagnostic and therapeutic strategies*, voor financiering door het KWF gehonoreerd. In 2004 heeft hij nog korte tijd in het WKZ gewerkt bij de afdeling Metabole en Endocriene Ziekten op een door Pepscan Systems BV geleid Europese Unie onderzoeksproject: *Binding Gastrin*. Eind 2004 heeft hij de overstap gemaakt naar de biotech business. Hij werkt nu als Senior Scientist bij MucoVax BV in Leiden.

Samenvatting in het Nederlands

Medullair schildkliercarcinoom (MTC) is een kwaadaardige tumor die ontstaat uit de calcitonine producerende C-cellen van de schildklier. MTC komt voor als sporadische tumor in ongeveer 75% van de gevallen. Daarnaast is MTC de belangrijkste klinische manifestatie van het Multipele Endocriene Neoplasie type 2 (MEN 2) syndroom. MEN 2 is geassocieerd met kiembaanmutaties in het RET gen. Het RET proto-oncogen codeert voor de RET receptor tyrosine kinase, die de receptor vormt voor de GDNF familie van groeifactoren. RET is een essentiële component van signaleringspaden die nodig zijn voor embryonale niervorming en ontwikkeling van de darmzenuwen. Mutaties in het RET gen zijn geassocieerd met de ziekte van Hirschsprung, met sporadische medullaire en papillaire schildklierkanker en met MEN 2. Er zijn twee subtypen van MEN 2. Bij MEN 2A hebben de mutaties effect op cysteine residuen in het extracellulaire deel van het eiwit, wat resulteert in constante kinase activiteit. Bij MEN 2B veroorzaakt de meest voorkomende mutatie een aminozuur Met→Thr verandering op positie 918 (M918T) in het intracellulaire deel van het eiwit. Deze mutatie wordt ook gevonden als somatische mutatie in 30-40% van de sporadische MTC gevallen. De MEN 2B-geassocieerde M918T mutatie verandert de substraatspecificiteit van het eiwit.

Om de tumorgenese van MTC te bestuderen hebben we transgene muizen gemaakt. We hebben de promoter van het humane CT gen gebruikt om muizen te maken die enerzijds het niet-gemuteerde humane RET gen (*CALC-WT-RET*) of het humane RET gen met de MEN 2B M918T mutatie (*CALC-MEN2B-RET*) in de C-cellen van de schildklier tot expressie brengen.

Schildklierafwijkingen zijn nooit gevonden in *CALC-WT-RET* muizen of in niet-transgene muizen die tot meer dan twee jaar zijn vervolgd. Andere fenotypische afwijkingen werden wel gezien in *CALC-WT-RET* muizen als gevolg van de transgene RET expressie in de nieren. Normaal komt endogeen RET tot expressie in de nieren tijdens de embryonale ontwikkeling. Transgene RET expressie werd daarentegen ook gezien in de nieren na de geboorte. *CALC-WT-RET* muizen ontwikkelden een cysteuze nierziekte afhankelijk van de mate van RET eiwitexpressie in de nieren. Twee founders bezweken aan cysteuze nierziekte na 10 maanden. In verschillende transgene lijnen ontwikkelde zich milde cysteuze nierziekte na een jaar. Verhoging van RET expressie, door homozygote of samengesteld heterozygote muizen te fokken, resulteerde in progressieve cysteuze nierafwijkingen die gepaard gingen met groeivertraging. De mate nierziekte varieerde en was sterk gecorreleerd met de mate van groeivertraging. Deze resultaten benadrukken het belang van goed gereguleerde expressie van RET voor normale nierontwikkeling en functie en duiden op een mogelijke rol van RET bij nierziekten bij de mens.

In *CALC-MEN2B-RET* muizen werd wel een schildklier fenotype gezien. Op de leeftijd van 20-22 maanden bleken 3 van de 8 founders een macroscopisch bilateraal medullair schildkliercarcinoom te hebben ontwikkeld. In 2 founders werd nodulaire C-cel hyperplasie (CCH) geconstateerd. In muizen van verkregen lijnen werd nodulaire CCH gezien in sommige muizen vanaf 8 maanden en in een lijn werd MTC gezien in 13 % van de muizen vanaf een leeftijd van 11

maanden. Deze resultaten toonden voor het aan dat de MEN 2B mutatie in het RET gen muizen predisponeert voor MTC ontwikkeling. De *CALC-MEN2B-RET* muizen ontwikkelen MTC, voorafgegaan door CCH, met een lage incidentie en een variabele latentietijd, wat de betrokkenheid van additionele oncogene gebeurtenissen suggereert. De aard van de additionele oncogene gebeurtenissen die betrokken zijn bij MTC ontwikkeling en verspreiding was onbekend.

Wij hebben een mogelijke rol van de tumor suppressor P53 bij RET-geïnduceerde MTC ontwikkeling bij de mens en bij de transgene muizen bestudeerd. In een panel van humane MEN 2 en sporadische MTC specimen werd met imuunhistochemie P53 kleuring gezien, wat een indicatie is voor een gemuteerd eiwit, in drie van de 15 tumoren. In MTC van *CALC-MEN2B-RET* muizen werd in twee van de vier samples P53 kleuring gezien. Een mogelijk causale relatie tussen P53 gen inactivatie en MTC ontwikkeling werd bestudeerd door *CALC-MEN2B-RET* muizen te vervolgen die een of beide allelen van het P53 gen misten. Vier van de zes samengesteld *CALC-MEN2B-RET;P53-/-* muizen hadden een MTC ontwikkeld op de leeftijd van 2-5 maanden en de andere twee hadden CCH. Op deze leeftijd werd MTC niet geconstateerd in vijf P53-/- of 14 *CALC-MEN2B-RET* muizen. Een significante toename in de incidentie van RET-geïnduceerd MTC werd ook gezien op latere leeftijd in samengesteld *CALC-MEN2B-RET;P53 +/-* muizen ten opzichte van controle muizen. Deze data tonen aan dat inactivatie van P53 bijdraagt aan RET-geïnduceerd MTC ontwikkeling.

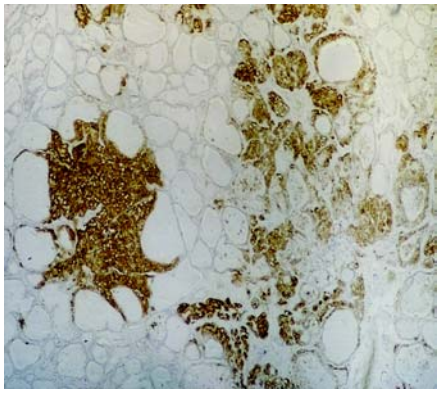
Deregulatie van het Wnt/beta-Catenin/Tcf signaaltransductiepad is geassocieerd met diverse vormen van kanker. Een mogelijke betrokkenheid van dit pad in RET-geïnduceerd MTC ontwikkeling werd bestudeerd met imuun-histochemische analyse voor beta-Catenin expressie in C-cellen en MTC van *CALC-MEN2B-RET* transgene muizen. In normale C-cellen wordt beta-Catenin kleuring uitsluitend geassocieerd met de celmembraan gezien. In MTC tumorcellen werd beta-Catenin kleuring ook gezien in de kernen in 6/7 onderzochte tumorsamples, wat aangaf dat er tumorspecifieke deregulatie van het Wnt/beta-Catenin/Tcf pad was opgetreden. De significantie van deze bevinding voor humaan MTC werd onderzocht met het humane MTC panel. In 6 van de 15 tumoren werd beta-Catenin in de kern gedetecteerd. In CCH en micro-MTC van patiënten en transgene muizen werd geen beta-Catenin kernkleuring gezien, wat suggereert dat deregulatie van dit signaaltransductiepad een late gebeurtenis in MTC tumorgenese is. Om het effect van een gedereguleerd Wnt/beta-Catenin/Tcf pad op MTC ontwikkeling *in vivo* te bestuderen hebben we *CALC-MEN2B-RET* muizen die ook de APC(min/+) mutatie gefokt. Deze muizen konden tot maximaal een jaar vervolgd worden. Een significante toename in het percentage muizen met CCH werd geconstateerd. De toename in CCH incidentie in *CALC-MEN2B-RET;APC(min/+)* muizen was leeftijdsafhankelijk en geassocieerd met kernkleuring voor beta-Catenin in de hyperplastische C-cel gebieden in een deel (1/6) van de geanalyseerde muizen. De resultaten geven aan dat deregulatie van het Wnt/beta-Catenin/Tcf signaleringspad bijdraagt aan RET-geïnduceerd MTC ontwikkeling.

Verlies van heterozygotie van chromosoom 1p wordt vaak gezien in MTC, wat aangeeft dat er een potentieel tumorsuppressor-gen op deze arm van het chromosoom ligt. We hebben mutatie analyse uitgevoerd van het P18 gen, dat ligt op 1p32 en betrokken is bij de regulatie van de celdelingcyclus. We hebben 8 humane MTC specimen geanalyseerd en in een MEN 2A en twee sporadische tumoren werden mutaties in het P18 gen gevonden. Beide sporadische MTC's hadden ook een somatische RET mutatie. Homozygote deletie van het P18 gen werd gedetecteerd in de humane MTC cellijn

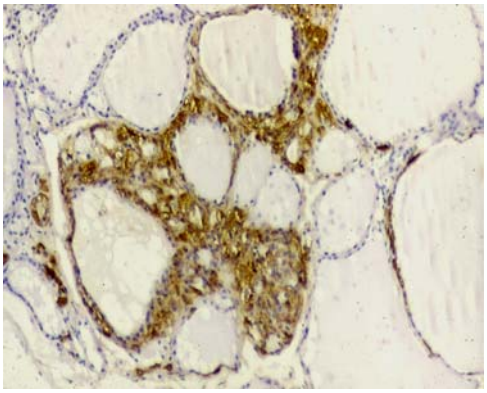
TT die ook een RET mutatie herbergt. Dit wijst erop dat P18 als tumorsuppressor van humaan MTC ontwikkeling kan werken en suggereert dat verlies van P18 functie met activatie van RET kan samenwerken in de tumorgenese van MTC.

De identificatie van deze drie additionele gebeurtenissen in RET-geïnduceerd MTC ontwikkeling biedt nieuwe mogelijkheden voor therapie. Er zijn op dit moment geen effectieve behandelingsmogelijkheden voor gemetastaseerd MTC.

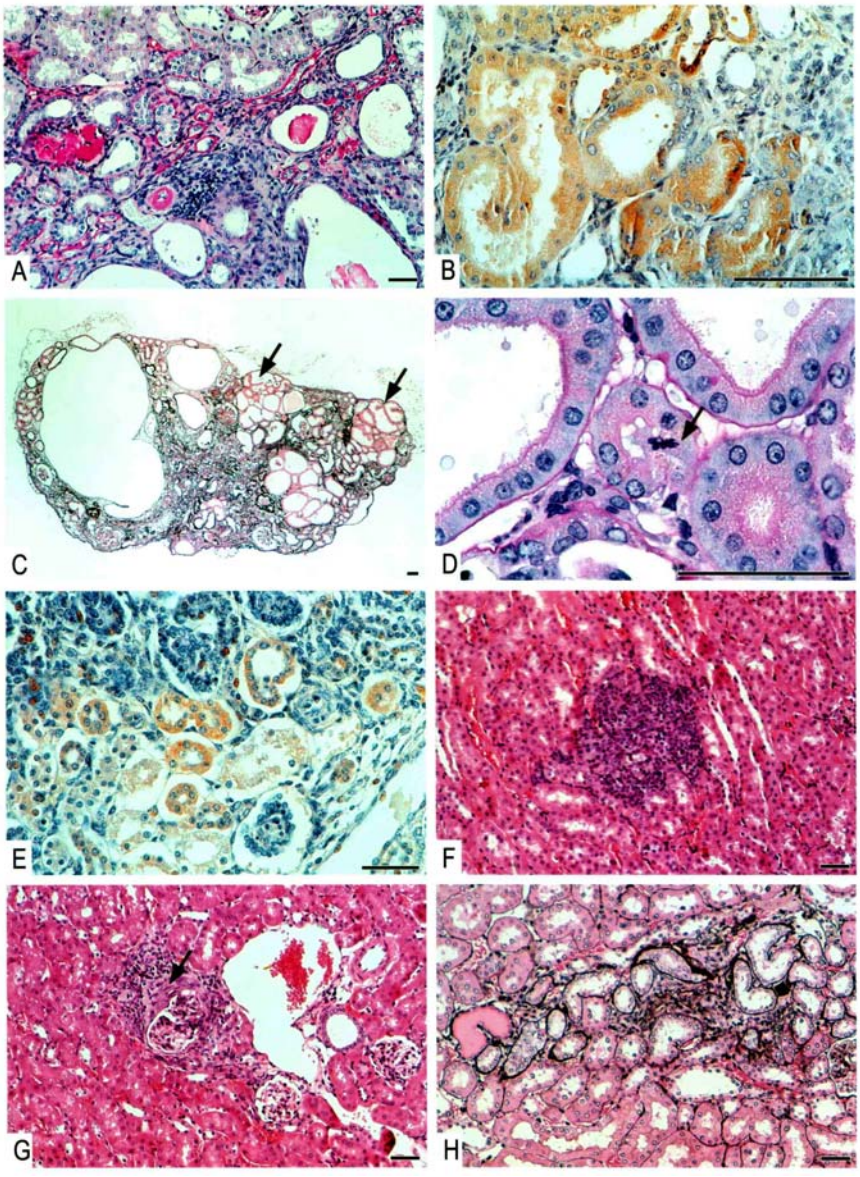
Color photos and figures



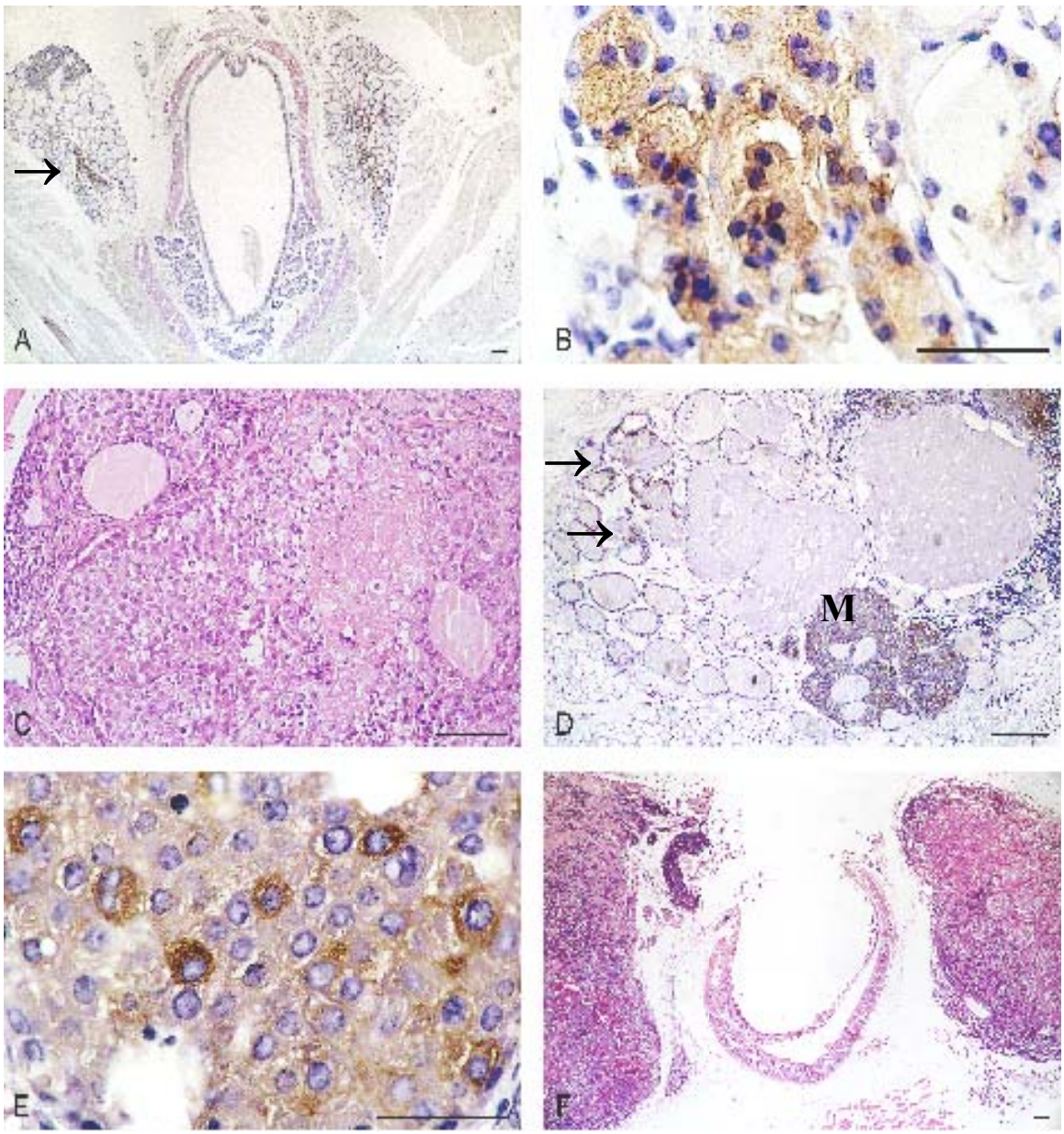
Chapter1, Photo1A.



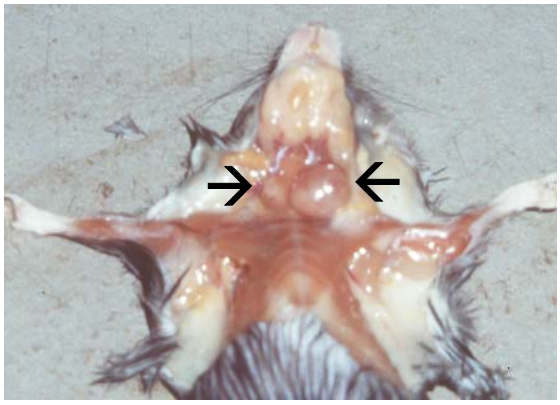
Chapter1 photo 1B



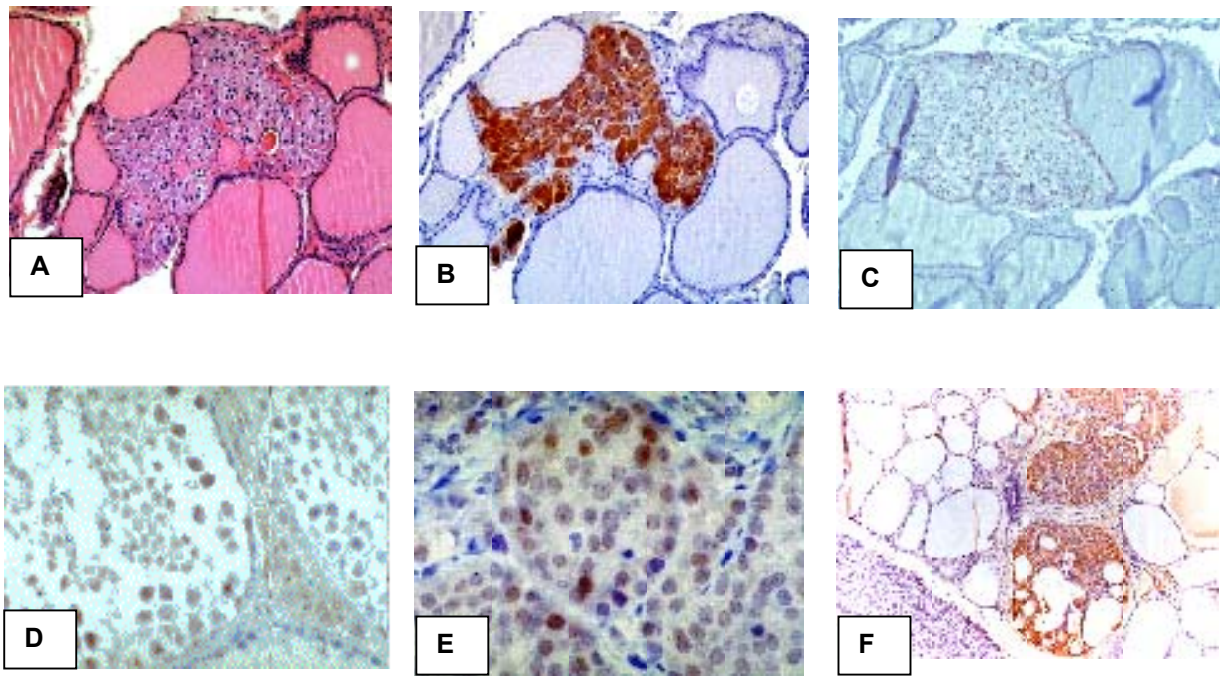
Chapter 2, Figure 2.



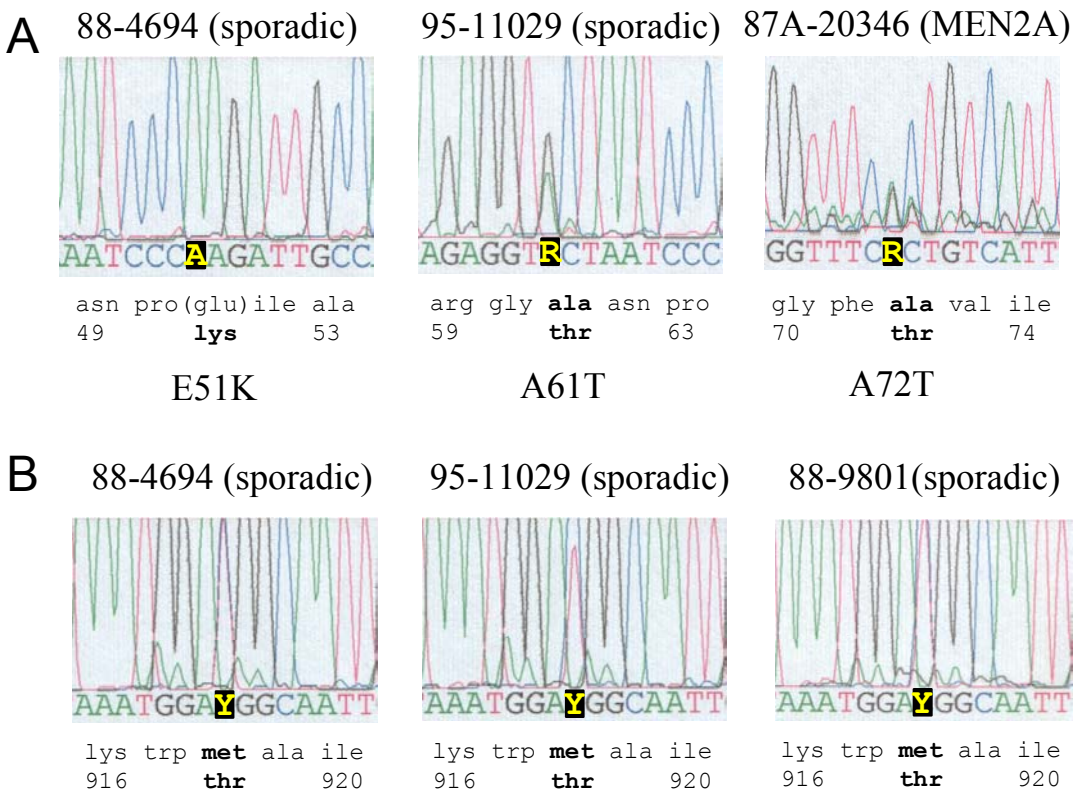
Chaper 3, photo 1.



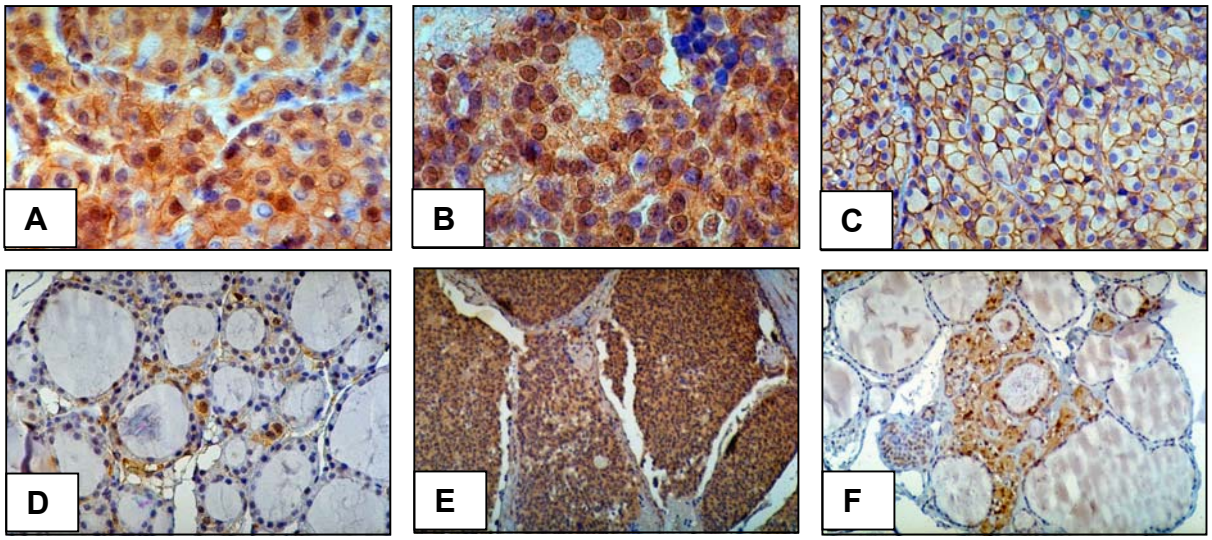
Chapter 3, figure 3.



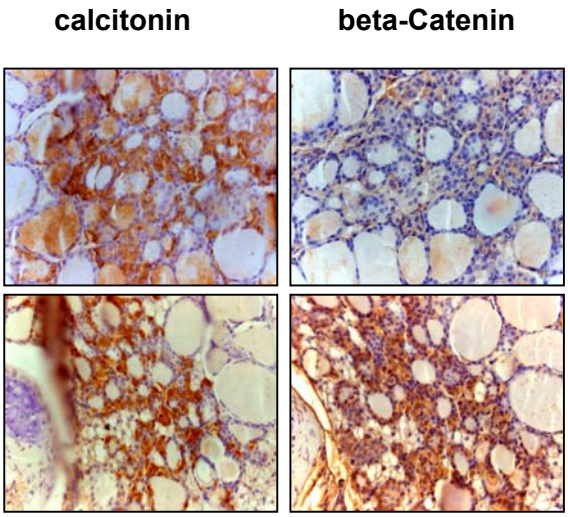
Chapter 4, photo 1.



Chapter 5, figure 2.



Chapter 6, figure 2.



Chapter 6, figure 6.

Content