SUMMARY

The hereditary cancer syndromes Multiple Endocrine Neoplasia type 2A and 2B (MEN2A en 2B) and the related syndrome Familial Medullary Thyroid Cancer (FMTC) are caused by mutations in the *RET* oncogene. The *RET* gene encodes a transmembrane tyrosine kinase receptor which can be activated by extracellular ligands, like glial-derived neurotrophic factor (GDNF), which leads to the activation of intracellular signalling pathways (signal transduction). The research described in this thesis focused on the functional characterization of several specific MEN2-associated RET mutations in order to connect the signaling properties of the mutated receptors with their associated disease phenotypes. Furthermore, we tested the tyrosine kinase inhibitor Imatinib (Glivec) as a potential inhibitor of (oncogenic) RET, as a first step towards it use as a potential therapeutic drug in patients with MEN2.

In **chapter 1** the genetic aspects concerning *RET*, signalling by wild type RET and the *RET*-associated diseases are reviewed.

In **chapter 2** we present a study on the signalling properties of two FMTC-associated RET mutants (Y791F, S891A), both mutations affecting the intracellular RET tyrosine kinase domain. We demonstrate that these mutant receptors signal independently of GDNF and as monomeric oncoproteins. Moreover, using reporters assays and western blotting, we could show that these mutants strongly activate the STAT3 signalling pathway when compared with the wild type RET receptor. This was also confirmed in tumours from patients carrying a RETY791F germline mutation. Furthermore, by interfering with various signalling pathways, we could show that aberrant activation of STAT3 by RETY791F and RETS891A was mediated by a SRC/JAK1/JAK2-dependent mechanism. This SRC/JAK1/JAK2 dependency of FMTC-RET mutants differs from previous findings obtained with a MEN2A-RET mutant, RETC634R, which showed SRC/JAK1/JAK2 independency. In order to get insight into the structural-molecular mechanisms by which these intracellular point mutants affect the tyrosine kinase domain of RET, we modeled the tyrosine kinase domain of these FMTC RET mutant proteins. We were able to show that the amino acid substitutions probably change the structure of the activation loop and thereby change the activation loop to a more open substrate and ATP binding conformation. Nevertheless, the exact molecular

mechanisms by which SRC and JAK1 and 2 interact with oncogenic RET still needs to be investigated further.

In **chapter 3**, we demonstrate that oncogenic RET (RETC634R, RETY791F and RETS891A) can induce constitutive phosphorylation of STAT3 Ser727. RET wild type is also able to induce ligand-dependent phosphorylation on Ser727, but is unable to phosphorylate STAT3 on Tyr705. Using various strategies, we demonstrated that RET induced STAT3 Ser727 phosphorylation through a pathway involving RAS-RAF-MEK1/2-ERK1/2 was required to achieved maximal transcriptional activition by STAT3. Moreover, inhibition of ERK1/2 resulted in both, decreased phosphorylation of STAT3Ser727 and reduced proliferation of MTC-TT cells, a metastatic tumor cell line expressing RETC634W. In biopsies from patients carrying a germline RETS891A mutation, strong nuclear staining of phosphorylated ERK1/2 and Ser727 phosphorylated STAT3 was observed in the tumor tissue. These data suggest that oncogenic RET mutants strongly activate STAT3 through STAT3-Y705 (Chapter 2) and Ser727 (Chapter 3) phosphorylation, which could play an important role in the development of MTCs.

Chapter 4 Despite clear genotype-phenotype correlations in the RET-associated MEN2 cancer syndrome, the molecular mechanisms connecting the mutated receptors with their distinct clinical subtypes are far from completely understood. In chapter 4**,** we tried to find specific signalling properties for the different disease associated RET mutants by reporter assays, western analysis in combination with structural modeling studies. In this study we show that the ERK1/2, STAT3 and SRC signaling pathways are differentially activated by specific MEN2-RET oncoproteins and that the level of activation of these pathways is correlated with the degree of RET Tyr1062 phosphorylation, a tyrosine important for activation of the ERK1/2 pathway, and RET Tyr981 phosphorylation, a tyrosine involved in the activation of STAT3 and SRC. Moreover, phosphorylation levels of RET Tyr1062 and Tyr918 were upregulated by activated SRC, suggesting a direct implication of SRC in the regulation of RET activation and signaling.

In **chapter 5 and 6** we describe the use of Imatinib (Glivec), a tyrosine kinase inhibitor known to target the receptor tyrosine kinases BCR-ABL, PDGFR and c-KIT, as an

potential inhibitor against RET. Currently, Imatinib is clinically used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST) as well as dermatofibrosarcoma protuberans.

We studied the effect of Imatinib on RET in two MTC derived cell lines expressing MEN 2 associated mutant RET receptors, MTC-TT and MZ-CRC-1, respectively.

We could show that Imatinib inhibits RET Tyr1062 phosphorylation in a dose-dependent manner after 1.5 hours of exposure. Interestingly, after 16 hours of exposure to Imatinib, both RET Tyr1062-phosphorylation and RET protein levels were decreased. A dose-dependent decrease in cell proliferation of both cell lines after exposure to Imatinib with IC_{50} concentrations of 23 ± 2 μM and 25 ± 4 μM was seen. These IC_{50} values for MTCs are high compared to those for CML and GIST. We further could show that Imatinib induced cell cycle arrest, apoptotic and non-apoptotic cell death. In conclusion, we showed that Imatinib inhibits RET-mediated MTC cell growth-proliferation affecting both phosphorylation and protein levels of RET in a dose-dependent manner. However, the concentration of Imatinib necessary to inhibit RET phosphorylation (5 to 10 μM) did not match the concentrations required to inhibit the proliferation of the tumor cell lines (IC_{50}=23 ± 2 μM for MTC-TT and 25 ± 4 μM for MZ-CRC-1). These suggest that other alterations are implicated in the tumorigenesis of MTCs and this makes it yet impossible to conclude that Imatinib is a good candidate for systemic therapy of MTC.

In **chapter 7** The observations presented in this thesis are discussed in view of the signalling properties of mutant RET-MEN2 proteins and the possible use of RET in designing systematic treatment for MEN2 is discussed.