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Wnt4 expression in thyroid cells is modulated by the transcription factor Pax8

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A tutti coloro
che cercano di conciliare
con passione
il desiderio della felicita'
e il senso del dovere
e sanno che
le sole cose che contano
sono quelle
che non possono essere
contate

François Garagnon

A Filoma…

To all those
who seek with passion
to reconcile a desire for
happiness
with a sense of duty
and know that
the only things that count
are those
that cannot be counted

François Garagnon

To Filoma…

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Abstract

The transcription factor Pax8 is expressed during thyroid development and is involved in the morphogenesis of the thyroid gland and maintenance of the differentiated phenotype. To date, Pax8 has been shown to regulate all the genes that are considered markers of thyroid differentiation. To identify novel Pax8 target genes we performed a genome-wide expression analysis following Pax8 siRNA. The silencing of Pax8 expression in rat thyroid differentiated FRTL-5 cells and the subsequent analysis of the gene expression profile by microarray identified Wnt4 among the down-regulated genes. As the other members of the Wnt family, Wnt4 has been implicated in several developmental processes including regulation of cell fate and patterning during embryogenesis. Up to now, the only data on Wnt4 in thyroid concern its down-regulation as necessary for the progression of thyroid epithelial tumors. Currently, we believe that it could be involved in thyroid morphogenesis, development and in the maintenance of the epithelial phenotype.

We focused our attention on the elucidation of the molecular mechanisms by which Pax8 could regulate Wnt4 expression in thyroid cells. Analysis of the 5'-flanking region of the Wnt4 gene identified a putative Pax8 binding site confirmed by EMSA and ChIP analysis. Moreover, transfection of FRTL-5 cells with different regions corresponding to progressive deletions of the 5'-UTR of Wnt4 showed that all the generated constructs possess a thyroid-specific activity due to the presence of the transcription factor Pax8 and other transcription factors recently demonstrated to be its interactors. The results obtained during this study show that Pax8 is indeed involved in the transcriptional modulation of the Wnt4 promoter. Interestingly, we also revealed that the expression of Wnt4 is TSH dependent in FRTL-5 cells.

Taken together, our data indicate that in thyroid cells the transcription factor Pax8 participates to Wnt4 gene expression directly binding to its 5'-flanking region suggesting that Wnt4 is a new target of this master regulatory gene. We propose that in thyroid cells the expression of Wnt4 correlates with the integrity of the epithelial phenotype and is reduced when this integrity is perturbed. Moreover, we would like to suggest that the over-expression of Wnt4 in thyroid cancer cellular models is able to revert the mesenchymal phenotype.

1. BACKGROUND

1.1 Introduction

Wnts are powerful regulators of cell proliferation and differentiation, and their signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion (Nelson and Nusse 2004). Over the past two decades, 19 members of the Wnt protein family have been found in mammals and have been subdivided into canonical signaling, with transforming activities in mammary epithelial cells, and non canonical signaling involved in the planar cell polarity (PCP) and in Calcium signaling (Eisenmann 2005).

Member of the Wnt family, Wnt4 is classified as a non-canonical Wnt protein even though there is evidence that it is also able to activate the canonical signaling pathway (Li et al. 2013). Wnt4 knockdown highlighted its crucial role in the development of several organs such as kidney, ovary and mammary gland (Brisken et al. 2000; Vainio et al. 1999). Moreover, Wnt4 null mice die within 24h of birth, probably because of severe lack of kidney functions (Stark et al. 1994). In kidney development, Wnt4 plays a key role in the mesenchymal to epithelial transition and morphogenesis required for tubule formation (Tanigawa et al. 2011) and is activated by the transcription factor Pax2 that is expressed during normal kidney development and is able to influence outgrowth and branching of the ureteric bud (Torban et al. 2006). Interestingly, the clinical and molecular features of the SERKAL syndrome have been identified as associated with a homozygous null mutation in WNT4 in humans, demonstrating that this gene plays an essential role in human sex-determination and organogenesis (Mandel et al. 2008).

Moreover, Wnt4 was shown to be strongly down-regulated in human anaplastic carcinomas and to behave as a key factor in Ras-mediated transformation of rat epithelial cells (De Menna et al. 2012).

We recently observed that the silencing of the transcription factor Pax8 in FRTL-5 differentiated thyroid cells and the subsequent analysis of the gene expression profile identifies Wnt4 among the down-regulated genes defining Wnt4 as a novel target of this transcription factor (Di Palma et al. 2011). Pax8, a member of the Pax genes family, is expressed almost exclusively in thyroid and kidney (Plachov et al. 1990; Poleev et al. 1992) and is required for both the morphogenesis of the thyroid gland and the maintenance of the thyroid-differentiated phenotype (Pasca di Magliano et al. 2000). In Pax8 knockout mice, the thyroid gland is barely visible and lacks the follicular cells (Mansouri et al. 1998); accordingly the expression of the thyroid specific markers, like Thyroglobulin (Tg) and Thyroperoxidase (TPO), cannot be detected. All together, the above studies demonstrated that Pax8 plays a crucial role in thyrocytes differentiation. Interestingly, mutations in the Pax8 gene have been associated with congenital hypothyroidism in humans (Di Palma et al. 2000).

Here, we investigated the molecular mechanisms by which Pax8 could regulate Wnt4 expression in thyroid cells. The bioinformatic (in silico) analysis of the 5'-flanking region of the Wnt4 gene identified several putative Pax8 binding sites and our results show that indeed Pax8 is involved in the transcriptional regulation of the Wnt4 gene. Since it is known that the expression of Pax8 is inhibited by TGF-β1 treatment of FRTL-5 cells in a concentration dependent manner (Kang et al. 2001), we have therefore studied the mechanisms by which the over-expression of Wnt4 could prevent the EMT induced by TGF-β1. Indeed, we show that TGF-β1 treatment is no longer able to induce EMT in a cellular model over expressing Wnt4.
Taken together, our data indicate that in thyroid cells Pax8 participates to Wnt4 gene expression directly binding to its 5'-flanking region and that the reduced expression of Wnt4 correlates with the alteration of the epithelial phenotype. In conclusion, we suggest that the over-expression of Wnt4 in thyroid cancer cellular models is able to revert the mesenchymal phenotype.

1.2 The Thyroid gland

The thyroid is one of the largest endocrine gland in the body and is composed of two cone-like lobes or wings: lobus dexter (right lobe) and lobus sinister (left lobe); and it is also connected with the isthmus. The thyroid gland is an organ situated on the anterior side of the neck and its name is due to its shield shape (from the greek tireòs = shield). (Figure1)

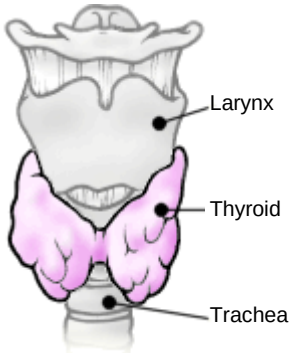

Figure 1: *Thyroid localization.*

In a fully differentiated thyroid we can find two cellular types: the follicular cells and the parafollicular cells (De Felice and Di Lauro 2004) (Figure 2).

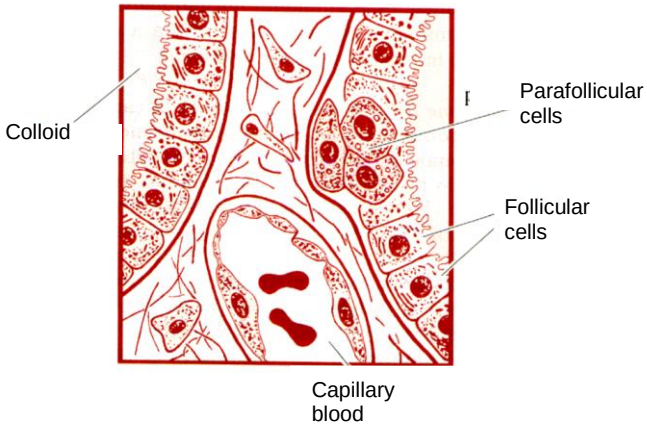

Figure 2: *Follicular and parafollicular cells of the thyroid gland*.

The follicular cells produce thyroid hormones T3 and T4 while the parafollicular cells produce calcitonin. Thyroid hormones are synthesized into colloids by iodination and condensation of thyroxin molecules bound to thyroglobulin (Tg) by a peptide bond. Tg is a glycoprotein that acts both like a substrate for the synthesis of thyroid hormones and as a storage of them. Via a reaction with the enzyme thyroperoxidase, iodine is covalently bound to tyrosine residues in Tg molecules, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroxine (T4) is produced by combining two molecules of DIT while triiodothyronine (T3) is produced by combining one molecule of MIT and one molecule of DIT. (Figure 3)

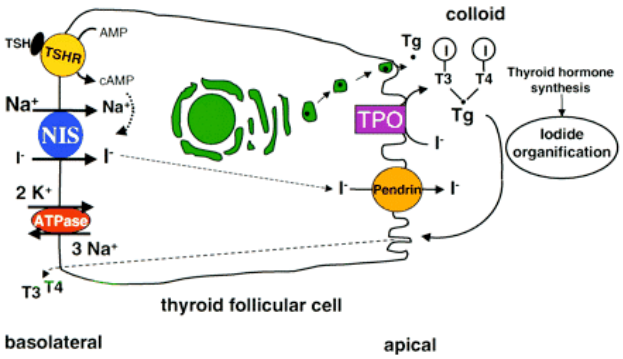

Figure 3: *Mechanism of thyroid gland functioning*.

Most of the thyroid hormones circulating in the blood is bound to transport proteins. Only a very small fraction of the circulating hormone is free (unbound) and biologically active, hence measuring concentrations of free thyroid hormones is of great diagnostic value. When thyroid hormone is bound, it is not active, so the amount of free T3/T4 is what really matters. For this reason, measuring total thyroxine in the blood can be misleading. T3 bind almost exclusively to Thyroxin Binding Globulin (TBG) while 80% of T4 bind to TBG, 15% to Thyroxin Binding Prealbumin (TBPA) and remaining 5% to seroalbumin. It is likely that all the cells in the body are targets for thyroid

hormones. While not strictly necessary for life, thyroid hormones have profound effects on many fundamental physiologic processes, such as development, growth and metabolism. Thyroid hormones stimulate diverse metabolic activities in a lot of tissues, leading to an increase in basal metabolic rate. One consequence of this activity is to increase body heat production, which seems to result, at least in part, from increased oxygen consumption and rates of ATP hydrolysis. Thyroid hormones regulate lipid metabolism; in fact, increased thyroid hormone levels stimulate fat mobilization, leading to increased concentrations of fatty acids in the plasma. They also enhance oxidation of fatty acids in many tissues. Finally, plasma concentrations of cholesterol and triglycerides are inversely correlated with thyroid hormone levels - one diagnostic indication of hypothyroidism is increased blood cholesterol concentration. Thyroid hormone play also a role in the carbohydrate metabolism. They stimulate almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis and glycogenolysis to generate free glucose.

Thyroid hormones are clearly necessary for normal growth in children and young animals, as evidenced by the growth-retardation observed in thyroid deficiency. Not surprisingly, the growth-promoting effect of thyroid hormones is intimately intertwined with that of growth hormone, a clear indication that complex physiologic processes like growth depend upon multiple endocrine controls. Also it is of critical importance in mammals the fact that normal levels of thyroid hormone are essential to the development of the fetal and neonatal brain. Thyroid hormones also acts on the cardiovascular system where they increases heart rate, cardiac contractility and cardiac output. They also promote vasodilatation, which leads to enhanced blood flow to many organs, like for example: the central nervous system, in which both decreased and increased concentrations of thyroid hormones lead to alterations in mental state (too little thyroid hormone, and the individual tends to feel mentally sluggish, while too much induces anxiety and nervousness); the reproductive system in which normal reproductive behaviour and physiology is dependent on having essentially normal levels of thyroid hormone (hypothyroidism in particular is commonly associated with infertility). The thyroid gland is under the control of the pituitary gland, a small gland the size of a peanut at the base of the brain. When the level of T3 and T4 drops too low, the pituitary gland produces Thyroid Stimulating Hormone (TSH) which stimulates the thyroid gland to produce more hormones. Under the influence of TSH, the thyroid will manufacture and secrete T3 and T4 thereby raising their blood levels. The pituitary senses this and responds by decreasing its TSH production. The pituitary gland itself is regulated by another gland, known as the hypothalamus. The hypothalamus is part of the brain and produces TSH Releasing Hormone (TRH) which tells the pituitary gland to stimulate the thyroid gland by releasing TSH. (Figure 4)

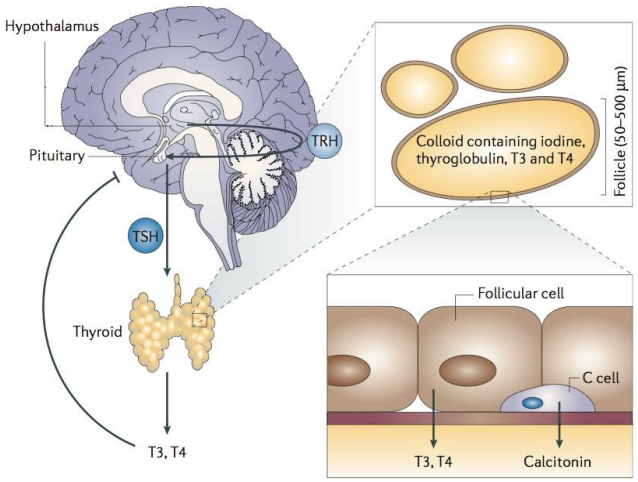

Figure 4: *Schematic representation of the axis Hypothalamus-Pituitary gland-Thyroid.*

1.3 Gene expression regulation and thyroid-enriched transcription factors

Gene expression includes all the events that go from the transcription of a gene to the production of the corresponding protein. All these events are finely regulated. Studying gene expression means to understand in which tissue a gene is expressed, how it is expressed and what causes its expression. In fact, phenotypic differences among different cell types are mainly due to differences in gene expression patterns. Gene expression can be regulated at different levels: activation of DNA structure, start of transcription, maturation of the transcript, transport into the cytoplasm, translation of mRNA and protein stability. Nevertheless, the most important step is the start of the transcription process which determines whether a gene is transcribed or not. RNA polymerase II, as well as RNA polymerase I and III, needs proteins to find the right transcription start site and these proteins necessary for the start of the transcription process are called general initiation factors. Given that RNA polymerases and general initiation factors are the last targets of regulatory events, during the assemblage of the RNA polymerases complex there are several points for regulation to take place. It is important to note that purified RNA polymerases and corresponding general initiation factors have an intrinsic ability to accurately transcribe DNA templates through core promoter elements, thus allowing the fundamental transcription mechanisms to be elucidated, but these activities are generally suppressed in the cell by the packaging of DNA within chromatin and by negative cofactors that directly interfere with the function of the basal transcription factors. As discussed below, this imposes requirements for transcriptional activators and corresponding cofactors that act in a gene-specific manner both to reverse the repression (anti-repression) and to effect a net activation above the intrinsic activity of the basal transcription machinery.
It is possible to subdivide transcriptional factors that cooperates directly with RNA polymerase II in three different categories:

a. general transcription factors;
b. upstream transcription factors;
c. inducible transcriptional factors.

a. General transcription factors are necessary for the start of RNA synthesis in all the genes of class II (genes that encodes for proteins). They form a complex with the RNA polymerase II close to the transcription start site and this complex is called basal complex of transcription.

b. Upstream transcriptional factors are proteins that recognize short consensus sequence situated upstream the transcription start site (an example is the Sp1 factor that recognizes GC boxes). These factors are ubiquitous and they act increasing the transcription efficiency of all the genes containing a GC box in their promoter.

c. Inducible transcriptional factors work like upstream factors, but they have a regulatory role. They are synthesized or activated in a tissue-specific manner and they recognize sequences called response elements.

In general, transcription factors acts directly recognizing specific and well conserved cis-sequences situated in promoters or enhancers. Some of these factors and of these sequences are common to more than a promoter and they are used in a constitutive manner; some other are specific and their activity is finely regulated. During the assembly process of these factors onto the regulatory sequences there are a lot of checkpoints and the speed of transcription can be increased or decreased in response to different regulatory signals. Nevertheless, not all the transcription factors bind these specific sequences on the promoter, they can also recognize other proteins already bound to the promoter, and sometimes they can bind directly to the RNA polymerase. Often DNA-binding factors that regulate the transcription of protein-coding genes act together with an expanding group of cofactors that act either through modifications of the chromatin structure or, more directly, regulating formation or function (transcription initiation or elongation) of the pre-initiation complex. Requirement for cofactors involved more directly in transcription is somewhat surprising in view of the specificity intrinsic to the various DNA-binding regulatory factors, the structural complexity of their ultimate target (the basal transcription machinery), and documented interactions between regulatory factors and components of the basal transcription machinery. Early studies of activator functions in systems reconstituted with DNA templates and purified RNA polymerase II and corresponding general initiation factors revealed that additional ‘‘co-activator’’ or ‘‘mediator’’ activities were required for the activator function but not for basal (activator-independent) transcription. Co-activators function in a variety of ways and often contain the enzymatic activities necessary for an alteration in chromatin structure from a quiescent state to one allowing active gene transcription. Broadly speaking, co-activators can be divided in three classes. One class of proteins modifies histones in ways that allow greater access of other proteins to the DNA. Examples of these are p300 and CBP, powerful histone acetyltransferases (HATs) that interact with a wide variety of

transcription factors and other proteins (Hermanson et al. 2002). These proteins support transcription in vitro from chromatinized templates. A second class of co-activators are members of the TRAP/DRIP/Mediator/ARC complex, proteins that bind to transcription factors, recruit RNA polymerase II and interact with the general transcription apparatus. The Mediator complex supports transcription in vitro from DNA templates but does not support efficient transcription from chromatinized templates. Lastly, protein complexes of the yeast SWI/SNF family (or their mammalian homologs BRG1 or BRM) contain ATP dependent DNA unwinding activities, necessary for efficient gene transcription in vivo. These groups of proteins will not support transcription from naked DNA, but raise transcription from chromatinized templates in vitro (Lemon et al. 2001). Co-repressors have the opposite effect on chromatin structure, making it inaccessible to the binding of transcription factors or resistant to their actions. These proteins (such as NcoR) are often associated with histone deacetylase (HDAC) activity, though other mechanisms for gene silencing clearly exist (Hermanson et al. 2002). Although co-activators are defined as proteins that increase transcriptional activity without binding to DNA, it is useful to think of those that bind directly to transcription factors and contain relevant enzymatic activities as primary co-activators. Those that dock on transcription factors and serve as scaffolds for the recruitment of other proteins containing these enzymatic activities can be considered secondary co-activators. This distinction rapidly blurs as proteins that can function as primary co-activators on some transcription factors can also be used as enzymatic tools assembled by secondary co-activators in other contexts. The expression of thyroid-specific proteins is responsible for the characteristic phenotype of the differentiated thyroid follicular cell. The promoter region of the thyroid markers Thyroglobulin (Tg), Thyroperoxidase (TPO), symport sodium-iodine (NIS) and TSHr has been characterized and it is known, at least in part, the mechanism responsible for their transcriptional activation in thyroid cells. The promoter region that allows the thyroid-specific transcription of the Tg gene is located between bases -170 and +1 with respect to the start site of transcription (Damante and Di Lauro 1994; Sinclair et al. 1990). In particular, the promoter region of the Tg gene presents three binding sites for the transcription factor TTF-1, which are located in three regions named A, B and C: the region A and region C are essential for promoter activity. In the region C, besides to the binding site for TTF-1 there is also a binding site for the transcription factor Pax8 which physically overlaps that one for TTF-1 (Zannini et al. 1992). In the region A, the binding site of a ubiquitous protein (UFA, ubiquitous factor A) overlaps with TTF-1; less relevant is the function of the region B (Sinclair et al. 1990). Between the regions B and C is placed the region K, which presents the binding site for the transcription factor Foxe-1 (Figure 5).

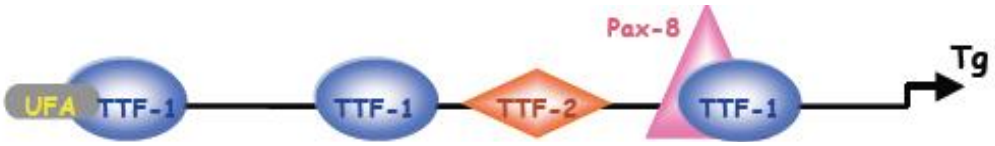

Figure 5: *Organization of the Tg gene promoter*.

The organization of the promoter of the TPO gene is very similar to the promoter of the Tg gene; in fact, it presents, three binding sites for TTF-1 in regions A, B and C. In the region C the binding site for Pax8 overlaps with the binding site for TTF-1, while in region B the binding site of a ubiquitous protein (UFB, ubiquitous factor B) overlaps with of TTF-1. The binding site for Foxe1 is located in the region Z located between the regions B and C (Francis-Lang et al. 1992) (Figure 6).

Figure 6: *Organization of the TPO gene promoter*.

The expression of characteristic markers of the differentiated thyroid phenotype is guaranteed by the presence of three transcription factors thyroid-specific, essential for the correct development of the thyroid gland: TTF-1, Foxe1 and Pax8. These factors, individually, are also present in other tissues both embryonic and adult, but their co-expression is observed only in thyroid cells mature and its precursors; it has been demonstrated that these transcription factors are essential for the expression of the thyroid differentiation markers, such as the thyroglobulin (Tg), the thyroidperoxidase (TPO), the symport sodium-iodine (NIS) and the TSH receptor (TSHr).

TTF-1/Titf1, also named T-EBP or Nkx2.1, is a member of the Nkx-2 subfamily, originally identified as a protein able to bind to a DNA sequence that is present three times on both the Tg and the TPO promoters, and it is involved in the activation of thyroid-specific gene expression. TTF-1 is also expressed in the lung, and its presence is required for the transcriptional activation of surfactant proteins A, B, and C and Clara secretory protein. The critical role of TTF-1 for both thyroid and lung morphogenesis was clarified in TTF-1 null mice that resulted not viable and lacked the thyroid, the pituitary gland and the lung parenchyma. Deep study revealed that in these mice at the begin of the embryogenesis the thyroid primordium forms in its correct position, but subsequently, the thyroid precursor cells undergo apoptosis (Kimura et al. 1999) and disappear at E10.5–11.5. During mouse development TTF-1 expression starts at day E8.5 in endodermal cells that later will become

the pharynx and its onset clashes with the proliferation of the cells of the endodermal layer. This event is the first step of the development of the thyroid.

Foxe-1 is a member of FOX (Forkhead box) proteins which are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation and differentiation. FOX proteins are able to bind DNA through the forkhead domain that is a domain common among several transcription factors involved in development and differentiation. In the mouse, Foxe-1 expression starts at 8 dpc into the endodermal cells of the pharynx and it is more evident in the posterior area of the pharynx, where TTF-1 is not expressed. Hence, this expression pattern suggests that Foxe-1 has a less important roles during the first steps of development whereas it is very important for the migration of the thyroid gland during embryogenesis. In fact, the analysis of Foxe1 knock-out mice revealed that thyroid starts to develop normally but, at day E10.5, the thyroid primordium fails to migrate towards its correct position in front of the trachea attached to pharyngeal endoderm suggesting that Foxe-1 might be important during the migration of the thyroid during its development, probably regulating the expression of genes involved in cellular migration.

1.4 The transcription factor Pax8

Pax8 is a member of the Pax gene family, a family of genes that encodes for DNA binding proteins involved in the regulation of the development of a variety of tissues in different species. The Pax gene family is composed of nine proteins highly conserved in vertebrates and all these proteins are characterized by the presence of the paired box. This specific sequence encodes for the paired domain, a very important domain for recognizing the target sequences on DNA. The paired domain is composed of 128 amino acids and is subdivided in two fundamental sub domains, one at C-terminus the other at N-terminus, called PAI and RED respectively. Each of these domains contain an helix-turn-helix motif and both domains are united by a linker region (Mansouri et al. 1998) (Figure 7).

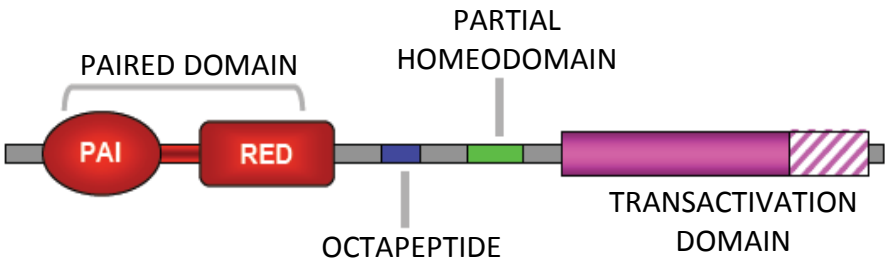

Figure7: *Schematic structure of the protein Pax8.*

We can explain the subdivision of the paired domain into two independent domains with the ability of Pax proteins family to bind DNA in different ways and overall to recognize a lot of sequences. Within the homology rate of the paired domains we can subdivide the nine Pax proteins in different groups. One of these groups is composed by Pax2, Pax5 and Pax8. These three proteins are characterized also by the presence of an octapeptide and a partial homeodomain. During embryonic development Pax8 is expressed in thyroid, kidney and the nervous tissue. Pax8 has been demonstrated to be required both for the morphogenesis of the thyroid gland and for the maintenance of the thyroid-differentiated phenotype.

In the endoderm Pax8 mRNA is present only in the thyroid anlage (Plachov et al. 1990). Like Titf1/Nkx2-1, Pax8 is detected in the developing thyroid from E8.5, i.e., at the time of specification.

Expression of Pax8 is maintained in TFCs during all stages of development and in adulthood. In the nervous system, Pax8 mRNA is transiently expressed in the myelencephalon and through the entire length of the neural tube. No signals are detected in the brain at later stages of the development, nor are they present in the adult brain. In the excretory system, Pax8 mRNA is present in the nephrogenic mesenchyme, which gives rise to the epithelial structures of nephrons as a consequence of the instructive interactions of the growing nephric duct and ureter. Indeed, Pax8 mRNA is expressed in the nephrogenic cord at E10.5, in mesenchymal condensations at E13, in the cortex of the metanephros at E16, and in the adult kidney.

Analysis of $Pax8^{-/-}$ mice revealed the role of this transcription factor during embryonic life. Whereas no phenotype has been detected in heterozygous $Pax8^{+/-}$ mice, homozygous $Pax8^{-/-}$ mice are born at the expected Mendelian frequency but show growth retardation and die within 2–3 wk. These mice do not display any apparent defects in the spinal cord, midbrain/hindbrain boundary, or kidneys. On the contrary, in $Pax8^{-/-}$ mice the thyroid gland is severely affected because neither follicles nor TFCs can be detected, and the rudimentary gland is composed almost completely of calcitonin-producing C cells. Hypothyroidism is the cause of death of the mutated animals: the administration of T4 to $Pax8^{-/-}$ mice allows the animals to survive.

In addition, important roles in morphogenesis of the TFC component of the thyroid gland, it has been shown, in cell culture systems, that Pax8 is a master gene for the regulation of the thyroid-differentiated phenotype (Pasca di Magliano M. et al. 2000). Pax8 not only is required for the survival of the thyroid precursor cells but also holds a specific upper role in the genetic regulatory cascade, which controls thyroid development and functional differentiation. These functions of Pax8 in thyroid development are consistent with the findings that in other organs Pax genes have a relevant role both in initiating and maintaining the tissue-specific gene expression program (De Felice and Di Lauro 2004).

1.5 Wnt proteins

The WNT genes encode for a large family of secreted glycoproteins highly conserved among species. During development, Wnts have different roles in several biological processes as cell fate, stem cell maintenance, proliferation, migration, polarity, and death. In adults, Wnts function in homeostasis, and inappropriate activation of the Wnt pathway is implicated in a variety of cancer. The majority of WNT proteins share a sequence identity of 35% there are though different subgroups that share even higher grade of sequence identity (58% - 83%). The 19 human WNT protein so far identified have different common features like: very similar size within a range of 39 kDa – 46 kDa, a secretory signal at the N-terminus, several highly charged amino acids residues and many glycosilation sites (Miller 2001 and Clevers 2006). Moreover, WNT glicoproteins share a 23 or 24 cysteine residues sequence evolutionarily conserved, suggesting that Wnt proteins folding depends on the formation of multiple intramolecular disulfide bonds. The primary amino acid sequences of these proteins suggest that they should be soluble instead secreted Wnts are highly hydrophobic and strictly associated at the extracellular matrix and cell membrane. The high hydrophobicity of these proteins is due to palmytolation of the first cysteine residue and of a serine residue in the middle of the protein. In fact, although Wnt proteins are secreted from cells, secretion is usually inefficient and previous attempts to characterize Wnt proteins have been hampered by their high degree of insolubility (Willert et al. 2003). Once palmytolated in the endoplasmatic reticulum, Wnt proteins are transported and secreted in vesicles. Once secreted the extracellular trafficking of Wnt proteins is mediated by large lipoproteins present on the external face of plasma membrane. (Hausmann et al. 2007) (Figure 8).

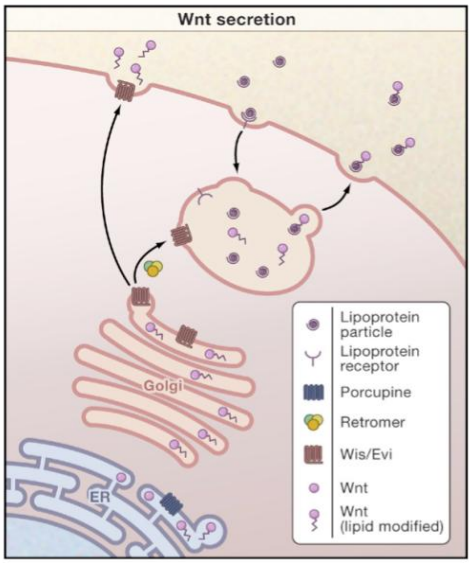

Figure 8: *Schematic representation of Wnt secretion.*

1.6 Wnt signaling

Wnt signaling is transduced through binding with different receptors. The most studied are the Frizzled proteins, which are seven transmembrane receptors with an extracellular N-terminal rich domain (CRD). Another important regulator of Wnt signaling, is the LDL receptor related proteins 5/6 (LRP5/6) that acts promoting the WNT/Fz binding. (Kikuchi et al. 2007).
Through the binding with a complex comprised by Frizzled and LDL receptor-related proteins 5/6 (LRP5/6) Wnt proteins are able to activate canonical signaling instead the binding with Fz, Ryk, or Ror2 preferentially activates non-canonical pathways. The canonical pathway is mainly involved in cell fate determination, differentiation and stem cell renewal while the non-canonical one is responsible of morphological changes and tissue organization (Figure 9).

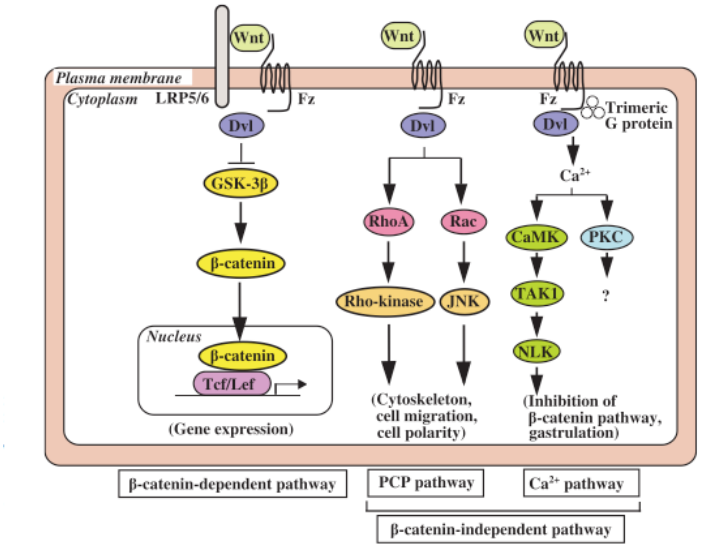

Figure 9: *Outline of the Wnt signaling pathway.*

The best known Wnt pathway is the Wnt/β-catenin pathway also defined "canonical" Wnt signaling (Figure 10). The signaling function of β-catenin is regulated principally through alteration of its stability. Wnt signalling induces the stabilization of the 'free' cytoplasmic pool of β-catenin. In the absence of Wnt signaling, β-catenin is rapidly degraded by a destruction complex consisting of APC, axin (conductin homolog), glycogen synthase kinase 3-beta (GSK3β) and casein kinase (CKI). CKI and GSK3b induce the phosphorylation of β-catenin. Thus, The phosphorylated β-catenin is linked to ubiquitination and proteosomal degradation. Wnt proteins bind to Fz and LRP5/6 activate the cytoplasmic protein DVL (Dishvelled) that subsequently recruits the complex APC/axin/CKI/GSK3β to the cell membrane. β -catenin is so allowed to accumulate in the cytosol. Once in the cytosol β-catenin can either associate with E-cadherin mediating cell adhesion (Brembeck et al. 2006) or translocate to nucleus were in collaboration with T-cell factor (TCF) and lymphoid enhancer-binding protein transcription factor (LEF) activates target genes transcription.

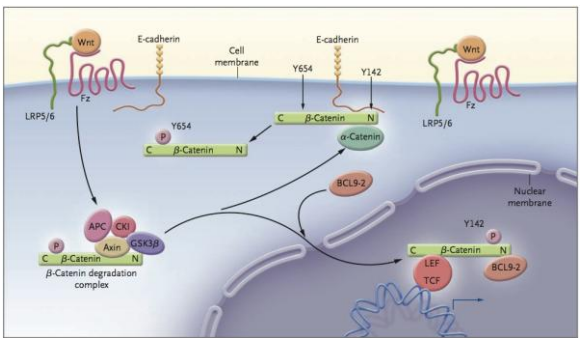

Figure 10: *Wnt canonical signaling and dual function of β –catenin.*

The non-canonical pathway involves the signalings mediated by Wnt without β-catenin. The Planar Cell Polarity (PCP) pathway is considered as non-canonical pathway. In vertebrate, Wnt/polarity signalling is thought to control polarized cell movement during gastrulation and neurulation. Another non-canonical way through which Wnt proteins can act is the Ca^{2+} dependent pathway (Kohn et al. 2005). This signalling involves an increase in intracellular Ca^{2+} and activation of PKC. It could be activated by a distinct group of ligands and Fzd receptors; at first, the Wnt/Ca^{2+} pathway involves activation of a heterotrimeric G protein, an increase in intracellular Ca^{2+} and activation of calcium/calmoduline-regulated kinase II (CamKII) and PKC. (Schlessinger et al. 2008) (Figure 11).

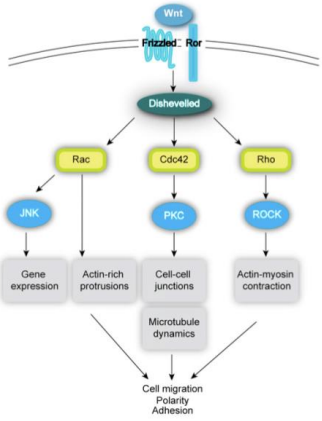

Figure 11: *A proposed Wnt/GTPase pathway.*

The non-canonical pathways can act through different cellular mechanisms such as the inhibition of the canonical pathway, the regulation of cell migration and cell polarity. The complexity of the Wnt pathway is constantly increasing thanks to discoveries of novel receptors and co-receptors able to transduce Wnt signaling.

1.7 Wnt4

Wnt4 is a member of the Wnt family and is common classified as a non-canonical Wnt protein even though it has been demonstrated that it is also able to activate the canonical signaling, depending on the cellular context (Schlessinger et al. 2009). Wnt4 null mice die after birth probably for severe kidney dysfunctions (Stark et al. 1994). More in details, female mice lacking Wnt4 show a regression of the Mullerian duct, partial female to male sex reversal and oocytes degeneration (Vainio et al. 1999). At early stage of development Wnt4 is expressed in both female and male gonad and after sex determination Wnt4 is down-regulated in the male: this event is needful to induce Mullerian duct regression and to allow the blood coleomic vessel formation, a vasculature structure characteristic of male gonads. (Jeays-Ward et al. 2003). Interestingly, Wnt4 plays a key role in the mesenchymal to

epithelial transition and morphogenesis required for tubule formation in kidney development (Vainio et al. 2003).
To date, it has been described only an autosomal recessive disorder caused by a loss of function mutation in Wnt4 gene named SERKAL Syndrome (Mandel et al. 2008). In particular, this syndrome is characterized by different overlapping features including female sex reversal and dysgenesis of kidneys, adrenals and lungs. The Wnt4 missense mutation associated with this syndrome was shown to adversely affect mRNA stability, most probably as a result of distruption of a highly conserved mRNA secondary structure. Defective Wnt4 activity was found to affect the development of three major organs (gonads, kidneys, and adrenal glands), all of which originate from the primordial urogenital ridge, thus suggesting a pivotal role for Wnt4 at an early embryological stage of development. More specifically, the authors suggested that WNT4 suppresses 5 α-reductase activity in humans. The most important role of 5 α-reductase in humans is the conversion of testosterone to 5 α-DHT, which is much more potent than testosterone and is responsible for the morphogenesis of the human male external genitalia.
Unfortunately, Mechanisms underlying the regulation of Wnt4 expression in thyroid during development are complex and have not been defined in their molecular details.
The only available information concern the identification of Wnt4 as a key player in Ras-mediated
transformation of rat thyroid epithelial cells, being involved in the inhibition of aberrant motility of neoplastic cells (De Menna et al. 2012). In particular these data indicate Wnt4 is strongly down-regulated in human anaplastic carcinomas thus suggesting that Wnt4 could be involved in advanced human thyroid cancer.

2. Aims of the study

Transcriptional regulation is a fundamental process for cellular differentiation and development.
In an adult organism differentiated cells are characterized by the expression of a specific subset of genes and this expression is regulated mainly at the transcriptional level. A good model for studying transcriptional regulation is represented by thyroid follicular cells that express a very specific set of genes, called differentiation markers, that are thyroglobulin (Tg), sodium-iodide symporter (NIS), thyroperoxidase (TPO) and TSH receptor (TSHr).
The most important transcription factor for the development and the differentiation of thyroid follicular cells is Pax8 (Pasca di Magliano et al. 2000). In this context, Pax8 interacts with another crucial transcription factor, TTF-1, and these two factors are able to synergistically induce the expression of the thyroglobulin gene (Di Palma et al. 2003). Moreover, Pax8 and TTF-1 are part of an heterocomplex that has an apparent molecular mass greater than the sum of the two factors suggesting the presence of other co-factors.
The silencing of Pax8 expression in thyroid differentiated FRTL-5 cells recently generated in our laboratory and the following analysis of the gene expression profile by microarray indicated Wnt4 among the down regulated genes (Di Palma et al. 2011).
Wnt4 was initially classified as a non-canonical Wnt protein, but more recently it has been described to activate also the canonical signaling pathway. Wnt4 knockdown underlined its crucial role in the development of several organs such as kidney, ovary and mammary gland.
Interestingly, recent data have been demonstrated that Pax2, another member of the Pax gene family, is able to activate Wnt4 promoter activity in a proximal tubule cell line promoting nephrogenesis (Torban et al. 2006).
We focalized this research project on the study of the role of the Wnt4 gene in correlation with the transcription factor Pax8 in thyroid differentiated cells and the possible involvement of Wnt4 in human thyroid tumors.

3. Materials and methods

3.1 RNA interference

FRTL-5 cells were plated ($8x10^4$ well) in a 24-well plate and were transfected in triplicate with 50 nM rat Wnt4 siRNA (Ambion, Life Technologies Ltd, UK), 100 nM Pax8 siGENOME siRNA or siGENOME Non-Targeting #3 (Dharmacon, Lafayette, CO) as scramble using DharmaFECT 1 transfection reagent, following the manufacturer's protocol. 72 h after transfection, the cells were harvested and the total RNA was extracted.

3.2 RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen, San Diego, CA) according to the manufacturer's directions. Total RNA (1 µg) was retrotranscribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories). Real-time PCR analysis was performed using an iCycler-iQ real-time detection system and SYBR green chemistry (Bio-Rad Laboratories). To design the qRT-PCR assays, we used the Primer Express software, and the primers sequences are:

Rat Oligonucleotides	Sequence
Act Fw	5'-GGCAATGAGCGGTTCCGATG-3'
Act Rw	5'-ATGGTGGTGCCACCAGACAG-3'
Pax8 Fw	5'-CAGCTATGCCTCTTCCGCTATT-3'
Pax8 Rw	5'-TGTGGCTGTAGGCATTGCC-3'
Wnt4 Fw	5'-TCCTCGTCTTCGCCGTGTTC-3'
Wnt4 Rw	5'-GAGCTTCTCGCACGTTTCCTC-3'
E-cadh Fw	5'-CCTGGGACTCCAGTTACAGG-3'
E-cadh Rw	5'-CTCAGACCCTGTGAAAGCTGG-3'
α-SMA Fw	5'-ACAATGGCTCCGGACTGGTG-3'
α-SMA Rw	5'-AGAGTCAGGATGCCTCGCTTG-3'
Vim Fw	5'-GAATACCGGAGACAGGTGCAG-3'
Vim Rw	5'-CGGCCAATAGTGTCCTGGTAG-3'
Snail I Fw	5'-GATGCACATCCGAAGCCACAC-3'
Snail I Rw	5'-GGAGCAGGAGAAAGGCTTCTC-3'
Fbn Fw	5'-CTACCAAGGCTGGATGATGGTGG-3'
Fbn Rw	5'-GGAGCAGGTTCCCTCTGTTG-3'

HUMAN Oligonucleotides	Sequence
GAPDH Fw	5'-GTCTTCACCACCATGGAGAA-3'
GAPDH Fw	5'-ATCCACAGTCTTCTGGGTGG-3'
Pax8 Fw	5'-CCCTTCCAACACGCCACT-3'
Pax8 Rw	5'-CTGCTTTATGGCGAAGGGTG-3'
Wnt4 Fw	5'-CATGAGTCCCCGCTCGT-3'
Wnt4 Rw	5'-CGAGTCCATGACTTCCAGGT-3'
E-cadh Fw	5'-CGAGAGCTACACGTTCACGG-3'
E-cadh Rw	5'-CTTTGAATCGGGTGTCGAGGG-3'
α-SMA Fw	5'-ACAATGGCTCCGGACTGGTG-3'
α-SMA Rw	5'-AGAGTCAGGATGCCTCGCTTG-3'
Vim Fw	5'-CGGGAGAAATTGCAGGAGGAG-3'
Vim Rw	5'-CAAGGTCAAGACGTGCCAGAG-3'
Snail I Fw	5'-GATGCACATCCGAAGCCACAC-3'
Snail I Rw	5'-GGAGCAGGAGAAAGGCTTCTC-3'
Fbn Fw	5'-AGCGGACGCATCACTTGCAC-3'
Fbn Rw	5'-TGCACTGGAGCAGGTTTCCTC-3'

Reactions were carried out in duplicate in three independent experiments. For each gene, values are means ± SD of three independent experiments, normalized by the expression of housekeeping gene, and expressed as a percentage of the value measured in parental FRTL-5 or BcPAPcells. To calculate the relative expression levels, we used the 2^{-}DDCT method (Livak and Schmittgen 2001)

3.3 ChIP assay

Genomic sequences including 3 kb upstream of the start codon were retrieved from Genome website (http://www.genome.ucsc.edu) and were explored to predict potentially transcription factor binding sites using GEMS, Genomatix software (http://www.genomatix.de). Chromatin immunoprecipitation (ChIP) was performed as follows. The cross-linking solution, containing 1% formaldehyde, was added directly to cell culture media. The fixation proceeded for 10 min and was stopped by the addition of glycine to a final concentration of 125 mM. FRTL-5 cells were rinsed twice with cold PBS plus 1 mM PMSF, and then scraped. Cells were collected by centrifugation at 800xg for min at 4 C. Cells were swelled in cold cell lysis buffer containing 5 mM piperazine-N,Nbis (2-ethanesulfonic acid) (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, and inhibitors cocktail (Sigma) and incubated on ice for 10 min. Nuclei were spun down by microcentrifugation at 2000xg for 5 min at 4 C, resuspended in nuclear lysis buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.8% sodium dodecyl sulfate (SDS), 1 mM PMSF and inhibitors cocktail (Sigma), and then incubated on ice for 10 min. Samples were broken by sonication into chromatin fragments of an average length of 500/1000 bp and then microcentrifuged at 16,000xg. The sonicated cell supernatant was diluted 8-fold in ChIP Dilution Buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl, and precleared by adding Salmon Sperm DNA/Protein A Agarose (Upstate Biotechnology, Inc., Lake Placid, NY) for 30 min at 4 C. Precleared chromatin from $1x10^6$ cells was incubated with 1 mg of affinity-purified rabbit polyclonal antibody anti-Pax8 (kindly provided by Prof. R. Di Lauro), or no antibody and rotated at 4 C for 16 h. Immunoprecipitates were washed five times with RIPA buffer containing 10 mM Tris-HCl (pH 8), 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM PMSF; twice with LiCl buffer containing 0.25 M LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), and then three times with TE (10 mM Tris-HCl, pH 8; 1 mM EDTA). Before the first wash, the supernatant from the reaction lacking primary antibody was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Immunoprecipitates were eluted by adding 1% SDS, 0.1 M NaHCO3 and reverse cross-linked by addition of NaCl to a final concentration of 200 mM and by heating at 65 C for 16 h. Recovered material was treated with proteinase K, extracted with phenol-chloroform-isoamyl-alcohol (25:24:1) and precipitated. The pellets were resuspended in 30 µl of TE and analyzed by PCR using specific primers for the analyzed regions. The input sample was resuspended in 30 µl of TE and diluted 1:10 before PCR.
PCR was performed using the following specific primers: Forward, 5'-GATCCAGAAGCGAGGTTTCGGAT–3' and Reverse, 5'–AACCTAGTCACTAGCGCTCGGG-3'.

3.4 Promoter constructs and plasmids

Specific proximal promoter fragments of rat Wnt4 gene were amplified by PCR using the following primers:

Construct name	Oligo Forward	Oligo Reverse
3100Wnt4LUC	5'-CGACGCGTAGCCTCCCTGCCTTTGGCCC-3'	5'-GGAAGATCTGGTGCCGTGGGTGTCCGCCC-3'
920Wnt4LUC	5'-CCGCTCGAGACAGAAAGGCGAGGGGTTCA-3'	5'- CCCAAGCTTGGTGCCGTGGGTGTCCGCCC-3'
640Wnt4LUC	5'-CCGCTCGAGGCTCTCGCTCCGGTGGCT-3'	5'- CCCAAGCTTGGTGCCGTGGGTGTCCGCCC-3'
300Wnt4LUC	5'-CCGCTCGAGGGGGGGGGTGTCCCGCGCC -3'	5'- CCCAAGCTTGGTGCCGTGGGTGTCCGCCC-3'
190Wnt4LUC	5'- CCGCTCGAGGCTGACAGTCGGGTCCGTCG-3'	5'- CCCAAGCTTGGTGCCGTGGGTGTCCGCCC-3'

The 3100Wnt4LUC was cloned into MluI/BglII sites of the pGL3basic vector (Promega, Madison, WI) , while all the other construct were subcloned into XhoI/HindIII of the same vector.
Wnt4 cDNA (NCBI Reference Sequence NM_005923) was obtained by PCR from mouse thyroid cDNA using the forward primer 5'-CGGAATTCCGGGAGCCTTGCGGCCGCTG-3' and the reverse primer 5'-GCTCTAGATCACCGGCACGTGTGCATCT-3'. Wnt4 cDNA was digested with EcoRI and XbaI restriction enzymes and cloned in both pCEFL Puro vector carrying puromycin resistance.
CMV Pax8 construct was already described (Zannini et al. 1992).

3.5 Cell culture and transfection assays

Rat thyroid follicular FRTL-5 cells were maintained in Coon's modified F12 medium (EuroClone, MI, Italy) supplemented with 5% newborn bovine serum (HyClone, Logan, UT), penicillin and streptomycin 100X (EuroClone, MI, Italy), 20ng/ml Glycyl-histydyl-lysine (Sigma-Aldrich, St. Louis, MO), 3.6ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 10µg/ml bovine pancreas insulin (Sigma-Aldrich, St. Louis, MO, USA), 5µg/ml Apo-Transferrin Human, 10ng/ml Somatostatin (Sigma-Aldrich, St. Louis, MO, USA) and 0.5mU/ml thyroid stimulating hormone (TSH).
The starvation medium consisted of Coon's modified Ham's F12 medium supplemented only with 0,2% BSA. TSH treatment was performed by addition of 1 mU/ml TSH (Sigma-Aldrich, St. Louis, MO) to the culture medium at 12, 24, and 48 h after starvation.
HeLa cells and BcPAP cells were grown in DMEM (EuroClone) supplemented with 10% vol/vol fetal calf serum (HyClone). NIH3T3 cells were grown in DMEM (EuroClone) supplemented with 10% newborn bovine serum (HyClone, Logan, UT).

Transfections were carried out with the FuGENE 6 reagent (Roche Diagnostics, Munich, Germany) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:3 in all the experiments.
The plasmid pRL-TK (Promega) was used as internal control in the transfection assays. Cells extracts were prepared 48 h after transfection to determine the levels of the firefly and renilla luciferase with the Luciferase and Renilla Reporter Assay System (Promega).
48h after transfection cells were lysed in Passive lysis buffer (Promega Corporation, Madison, WI, USA) supplemented with 100X protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Firefly and renilla luciferase activity were assayed, respectively, with the Luciferase Assay System (Promega Corporation, Madison, WI, USA) and the Renilla Assay System (Promega Corporation, Madison, WI, USA) following manufacturer's instructions.
Luminescence was measured with LUMAT LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity was normalized on the activity of TK-Renilla vector to correct each sample for transfection efficiency. Transfection experiments were done in duplicate and repeated at least three times.

3.6 Electrophoretic mobility shift assay (EMSA)

Double-strand oligonucleotides were labeled with γ-^{32}P ATP and T4 polynucleotide kinase and used as probes. The binding reactions were carried out in a buffer containing 20 mM Tris-HCl (pH 7.6), 75 mM KCl, 1 mM dithiothreitol, 10% glycerol, 1 mg/ml BSA, and 3 mg/ml polydeoxyinosinic deoxycytidylic acid. After 30 min of incubation at room temperature, free DNA and DNA-protein complexes were resolved on a 6% non denaturating polyacrylamide gel and visualized with a by autoradiography.
The oligonucleotides used in the competition assay and the antibodies used in the supershift experiments were incubated with the protein extract for 20 min before adding the probe.
Oligonucleotides derived from the MatInspector analysis were: probe A, 5'-GCGGGTCCAGCGGCGCGGGACACCCCCC-3' and probe B, 5'-TCCGCCGCCACCGCCGCATCCCGGCTCTG-3'. The oligonucleotide mutated in the core sequence was: 5'-GCGGGTCCAGCGGATATGGACACCCCCCC-3'.

3.7 Protein extract and immunoblotting

Cells were washed twice with ice-cold PBS and lysed in a buffer containing 10 mM HEPES (pH 7.9), 400 mM NaCl, 0,1 mM EGTA (pH 7.8), 5% glycerol, 1 mM dithiothreitol, and 1 mM phenylmetilsulfonil fluoride. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

For Western blot analysis, proteins were separated on SDSPAGE, and gels were blotted onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The primary antibodies used were: mouse monoclonal anti-Wnt4 (Zymed, Invitrogen). Secondary antibody Rabbit IgG Horseradish peroxidase linked whole antibody (GE Healthcare) were used as suggested by manufacturers.
The filters were developed using an enhanced chemiluminescence detection method (Pierce, Rockford, IL) according to the manufacturer's directions.

3.8 TGF-β1 treatment

FRTL-5, FRTL-5 Wnt4 cl 31 and FRTL-5 Wnt4 cl 35 cells were cultured in complete medium. At 70% of confluence, 5 and 10 ng/ml of TGF-β1 (SIGMA) was added to culture media for 48 hours. RNA was subsequently isolated for q-RTPCR experiments.

3.9 Wound-healing assay

Confluent BcPAP, BcPAPpoolpCEFLpuro and BcPAPpoolWnt4 cells plated on tissue culture dishes were wounded by manual scratching with 10-µl pipette tip, washed with PBS and incubated at 37 C in complete media. At the indicated time points, phase contrast images at specific wound size were captured.

4. Results and Discussion

4.1 Wnt4 is a novel Pax8 target gene identified in FRTL-5 cells

The differentiation program of thyroid follicular cells (TFCs), by far the most abundant cell population of the thyroid gland, relies on the interplay between sequence-specific transcription factors and the transcriptional co-regulators with the basal transcriptional machinery of the cell. However the molecular mechanisms leading to the fully differentiated thyrocytes are still the object of intense study. The transcription factor Pax8, a member of the *Paired-box* gene family, has been demonstrated to be a critical regulator required for proper development and differentiation of thyroid follicular cells. Despite being Pax8 well-characterized with respect to its role in regulating genes involved in thyroid differentiation, genomic approaches aiming at the identification of additional Pax8 targets are lacking and the biological pathways controlled by this transcription factor are largely unknown. Therefore, to identify new downstream targets of Pax8, we investigated the genome-wide effect of Pax8 silencing comparing the transcriptome of silenced versus normal differentiated FRTL-5 thyroid cells. In total, 2815 genes were found modulated 72 hours after Pax8 RNAi, induced or repressed. As shown in the volcano plot representation below, red dots represent differentially expressed genes and gray dots represent all the other genes (Figure 12).

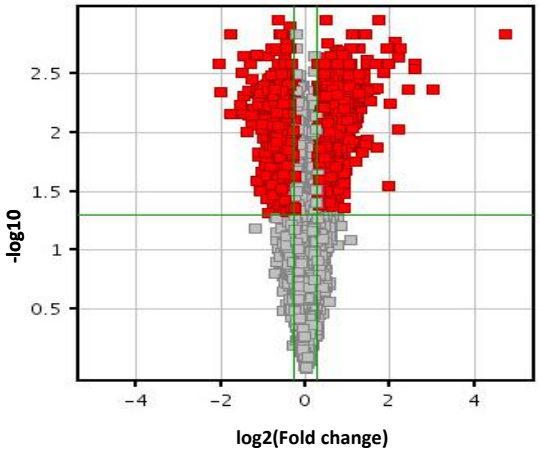

Figure12: ***Volcano plot representation of dysregulated genes.*** *The negative log10-transformed p-values are plotted against the log ratios (log2 fold change) between conditions. Red dots represent differentially expressed genes that show both large fold changes and high statistical significance. Gray dots represent all the other genes.*

Among the down-regulated genes we identified Wnt4 that belongs to the signal transduction category. This gene has been demonstrated to play an important role in the development of several organs such as kidney, ovary and mammary gland. It has been demonstrated that Pax8 is able to activate Wnt4 promoter

activity in a proximal tubule cell line promoting nephrogenesis (Torban et al. 2006). At the same time, a cooperative role for Pax2 and Pax8 in metanephric branching morphogenesis and nephron differentiation has been uncovered (Narlis et al. 2007).
To validate the expression data obtained by the microarray analysis, we performed qRT-PCR for Wnt4 on RNA samples prepared 72 h after Pax8 siRNA transfection. The FRTL-5 cells transfected with a non targeting siRNA were used as control. As shown in Figure 13 the down-regulation of Pax8 in thyroid cells closely correlates with reduced expression levels of Wnt4. Hence, the results of the microarray analysis were validated by the qRT-PCR analysis.

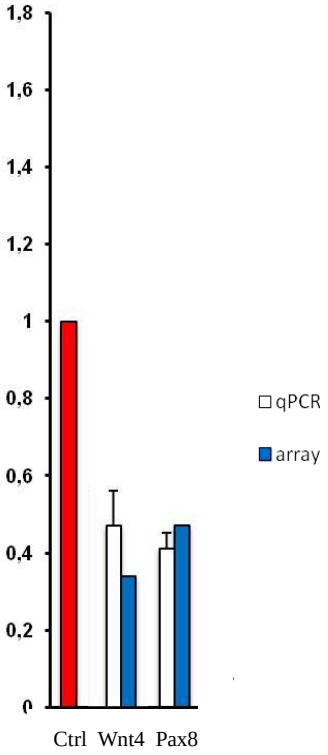

Figure 13: ***Validation of the microarray results by qRT-PCR.*** *Relative gene expression in siPax8 transfectants was calculated using non-targeting siRNA transfectant control (Ctrl) as reference. Differential gene expression in siPax8 vs Ctrl cells, 72 h after Pax8 silencing: comparison between qRT-PCR and array data.*

The effect of Pax8 silencing on Wnt4 expression was also evaluated 24h after siRNA transfection. This time point was chosen because the half-life of the Pax8 protein was found to be approximately 16 hours.
These data strongly suggested Wnt4 as a downstream targets of Pax8, in fact reduced levels of the transcription factor Pax8 directly correlate with a reduction of the levels of the secreted glycoprotein Wnt4.

4.2 Analysis of Wnt4 gene 5'-flanking region

To determine whether Pax8 could regulated Wnt4 gene expression by directly binding to its regulative genomic sequence, we performed a computational analysis using the MatInspector Software 8.0. We searched for Pax8 binding sites in a region of about 3000 bp in Wnt4 5'-flanking region, and the analysis showed the presence of several Pax8 consensus sequences. Through this in silico analysis of the 3000 bp upstream the ATG of the rat Wnt4 gene, several putative binding sites for transcription factors of the Pax family have been identified, three of which have already been described in literature as binding sites of the transcription factor PAX2 expressed in kidney (Torban et al. 2006).
To characterize more in detail the 5'-UTR of Wnt4 and to assess the transcriptional activity of this region in thyroid cells, five different regions corresponding to progressive deletions of the 5'UTR were subcloned into XhoI/HindIII sites of the pGL3-basic vector upstream the luciferase reporter gene (Figure 14).

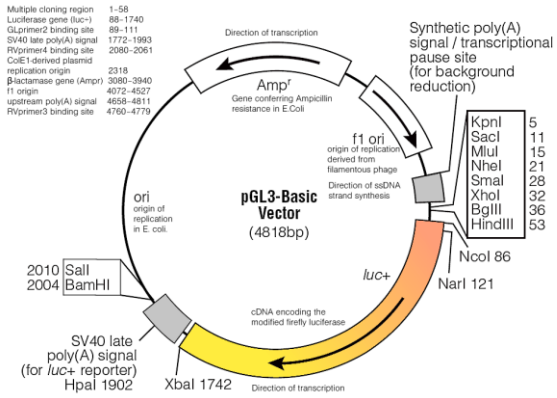

Figure 14: ***pGL3 basic vector used to subclone the progressive deletions of the 5'UTR of Wnt4.*** *The five different regions corresponding to progressive deletions of the 5'UTR were subcloned into XhoI/HindIII sites of the pGL3-basic vector in front of a luciferase (luc) cDNA..*

Particularly, the deletions were engineered taking into account the position of the Pax8 binding sites predicted by the MatInspector software. To verify the expression level and tissue specificity of each fragment, the deletion constructs were transiently transfected in FRTL-5 thyroid cells, in HeLa and in NIH3T3 cells. As positive control we used the RSV reporter construct, whose value was set at 100% (Figure 15).

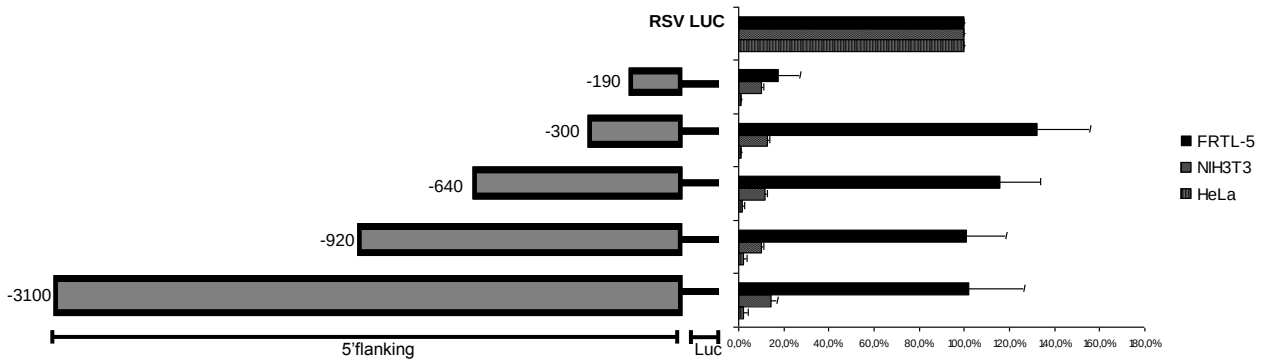

Figure 15: ***Capacity of the proximal promoter fragments of the Wnt4 gene to induce luciferase reporter gene expression.*** *The constructs were transfected into FRTL-5, NIH3T3 and HeLa cells. Luciferace enzyme activity was determined as Wnt4 promoter region activity. All the constructs generated have the highest activity in thyroid cells. No induction of the transcriptional activity was observed in the -920, -640, -300 deletions and, only the smallest construct carrying 190 bp of the Wnt4 5'-flanking region showed a strongly reduced activity in rat FRTL-5 cells.*

Interestingly, the results obtained showed that all the constructs generated have the highest activity in thyroid cells. No reduction of the transcriptional activity was observed in the -920, -640, -300 deletions and only the smallest construct carrying 190 bp of the Wnt4 5'-flanking region showed a strongly reduced activity in rat FRTL-5 cells.

These results suggest that the 5'-flanking region of Wnt4 is transcriptionally active preferentially in thyroid cells and prompted us to study the role of Pax8 in the modulation of Wnt4 expression.

To this end, we tested the effect of transient Pax8 expression on the Wnt4 promoter region (300Wnt4LUC and 190Wnt4LUC respectively) by luciferase assays. Specifically, we performed transactivation assays in HeLa cells transfecting the reporter constructs 300Wnt4LUC and 190Wnt4LUC together with increasing concentration of an expression vector encoding Pax8. As shown in Figure 16, Pax8 is able to activates transcription from the 300Wnt4LUC in a dose dependent manner, but not from smallest construct 190Wnt4LUC.

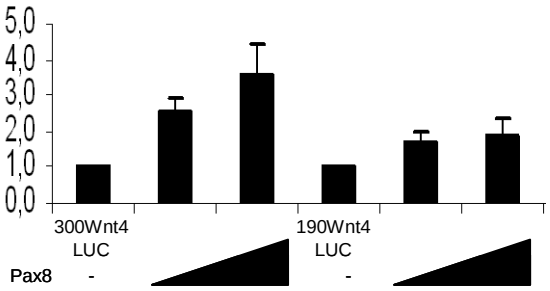

Figure 16: ***Pax8 activates transcription from the Wnt4 promoter region.*** *HeLa cells were transiently transfectated with 300Wnt4LUC and 190Wnt4LUC alone and in combination with increasing concentration (100 and 200 ng) of the expression vector encoding Pax8 (CMV5-Pax8) 48h after transfection the transcriptional activity was determined as the firefly over renilla luciferase activity. Data are sxpressed as fold induction over the transcription obtained with with 300Wnt4LUC and 190Wnt4LUC, whose values were set at 1,0. Data are means from three independent experiments, each performed in duplicate.*

These results suggest that the region between 300 bp and 190 bp upstream the ATG of the Wnt4 gene represents a crucial region for Pax8 regulation of this gene.

4.3 EMSA analysis of the predicted binding sites

The in silico analysis by the MatInspector software predicted two binding sites for Pax8 within 300 bp and 190 bp upstream the translational start site of the Wnt4 gene that we named A and B respectively (Figure 17).

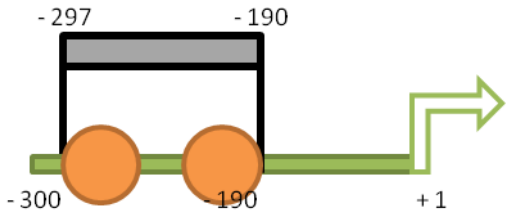

Figure 17: ***Putative Pax8 binding sites in the rat Wnt4 promoter region.*** *The 300 bp 5'-flanking sequence of the rat Wnt4 gene was analyzed with MatInspector software for possible Pax8-binding motifs. In orange the two predicted binding sites.*

Specifically, site A was identified on the negative strand, while site B was identified on the positive strand of the DNA sequence, respectively, positioned at -297 and -211 bp from the translational start site (ATG) mapped on the rat promoter. The alignment of the core sequence of the two putative Pax8 binding sites shows a high grade of conservation among Wnt4 mammalian promoter

regions, highlighting the potential relevance of these sites for the promoter activity (TABLE 1).

Species	Pax8 predicted binding site A	Pax8 predicted binding site B
Rat	TGTCC**CGCG**CCGCT	CCGCC**GCCA**CCGCC
Mouse	TGTCC**CACG**CCGCT	CCGCC**GCCA**CCGCC
Human	TGTCC**CGGG**CCGCT	CCGCC**GCCg**CCGCC
Chichen	TGTCC**CCAG**CGGCG	**CCT**CCGCT

Table 1: ***Graphic output of the sequence analysis showing the conservation in different species of the core consensus sequences (in bold) .***
The sequence alignment was obtained using whole genome comparative analysis from VISTA browser 2.0.

To further characterize and verify the prediction obtained by the MatInspector analysis, we designed two oligo probes, containing the putative binding sites A and B and we performed gel mobility shift experiments. At first, we incubated the A and B ^{32}P-labeled oligo probes with total protein extracts prepared from FRTL-5 thyroid cells. We used total protein extract of wild-type and Pax8-transfected HeLa cells, as negative and positive control, respectively. Interestingly, the retarded band observed when FRTL-5 protein extract was incubated with oligo A (Figure 18, probe A, lane 2) is identical to the retarded band of the retarded band of the positive control (Figure 18 probe A, lane 4). In contrast, the predicted binding site B could not be confirmed as a Pax8 binding site (Figure 18, probe B).

1: Free probe
2: FRTL-5
3: HeLa
4: HeLa-Pax8
5: FRTL-5 + Ab anti-Pax8
6: FRTL-5 + Ab anti-tubulin

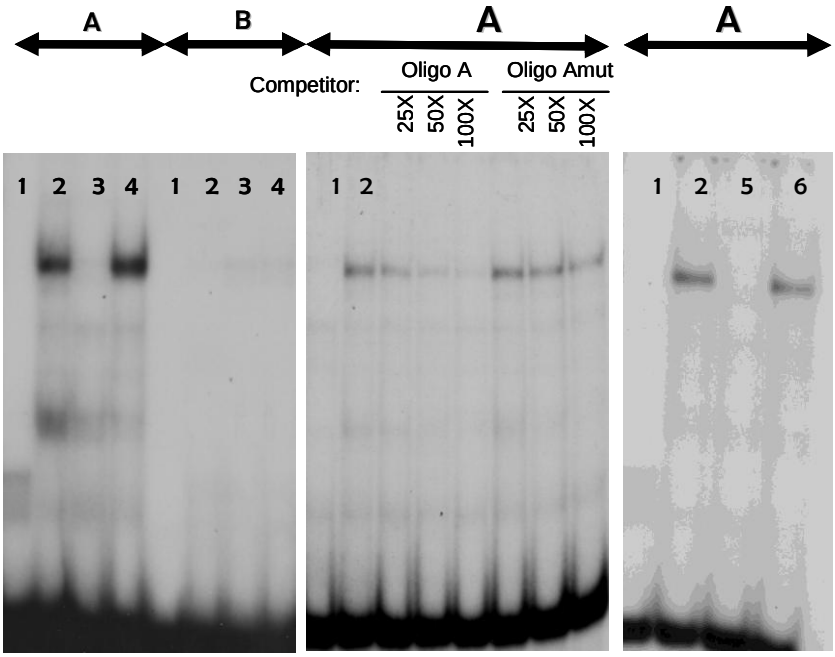

Figure 18: ***Electromobility shift assays for Pax8 binding to putative recognition motifs in the Wnt4 promoter.***
^{32}P-labeled oligo probes A and B were challenged in EMSA with total protein extracts prepared from FRTL-5 (lane 2). We used total protein extracts of HeLa (lane 3) and Pax8-transfected HeLa cells (lane 4), as negative and positive control of the EMSA. The specificity of the complexes observed with the FRTL-5 protein extract and probe A was tested by competition analysis, using an increasing amount (from 25 to 100-fold molar excess) of unlabeled oligonucleotides A or unlabeled oligonucleotides A mutated in the core sequence. Protein extract of FRTL-5 was incubated, alone or together with the antibody against Pax8 or tubulin (as control), in a supershift EMSA with labeled probe A.

Afterwards, we demonstrated by competition assays that the retarded band observed when the FRTL-5 extract is incubated with oligo probe A is the result of a specific interaction. We tested the binding specificity of the oligo probe A by competition with unlabeled oligo A and itself mutated in the core sequence. The Pax8 protein complex was specifically inhibited by competition with 25, 50 and 100 fold molar excess of unlabeled "self" but the competition with the cold mutated probe A is completely abolished (Figure 18).
To further consolidate this data of Pax8 binding on the oligo probe A, we performed a supershift assay with an anti-Pax8 specific antibody. This time, as shown in Figure 18, lane 5, we observed that a supershifted band is produced after the incubation of FRTL-5 protein extract and the probe A with the anti-Pax8

antibody. The specificity of this supershifted band is confirmed by the presence of the expected retarded band corresponding to the Pax8-oligo complex and by the absence of a supershift when the FRTL-5 protein extract is incubated with the anti-tubulin antibody (Figure 18, lane 6).
All together, these results demonstrate that Pax8 is able to specifically binds *in vitro* to the sequence identified.
Furthermore, to demonstrate the ability of Pax8 to interact with the Wnt4 promoter region *in vivo*, chromatin immunoprecipitation (ChIP) assays on FRTL-5 cells were carried out. The cross-linked chromatin was immunoprecipitated using a polyclonal Pax8 antibody. As control, to rule out unspecific background of the ChIP assay, one reaction lacking the primary antibody was also performed. The successful PCR with specific oligonucleotides to amplify the region with more predicted Pax8 binding sites upstream the coding sequence, revealed the binding of Pax8 to the 5'-flanking region of Wnt4 in vivo (Figure 19).

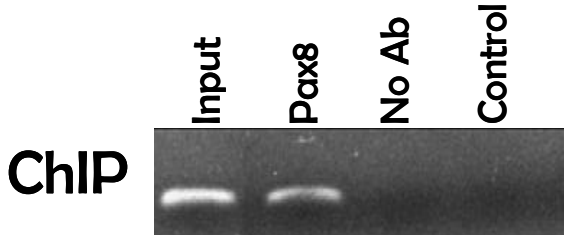

Figure 19:*ChIP experiment for Pax8 binding to Wnt4.* *Chromatin extracted from cross-linked FRTL-5 cells was immunoprecipitated using an antibody against Pax8. The immunoprecipitated was analyzed by PCR with oligonucleotides corresponding to the rat Wnt4 promoter region. Parallel PCR were performed with total input DNA obtained from unprecipitated aliquot of similarly treated chromatin sample, an aliquot treated with no antibody and from no template control.*

Taken together, the obtained results clearly highlighted that Wnt4 could be considered a novel and direct target of Pax8.

4.4 Wnt4 is responsible for the maintenance of the epithelial phenotype

The epithelial-mesenchymal transition (EMT) is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are altered to release epithelial cells from the surrounding tissue. The cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype. Essential for embryonic development, EMT is nevertheless potentially destructive if deregulated, and it is becoming increasingly clear that inappropriate utilization of EMT mechanisms is an integral component of the progression of many tumors of epithelial tissues (Radisky 2005). Induction of EMT can compromise the mechanical and physiological integrity of the tissue, and inappropriate induction of this process can have disastrous consequences. The defining property of cells that undergo EMT – the ability to separate from neighboring cells and penetrate into and through surrounding tissues – is particularly dangerous when acquired by tumor cells, and EMT processes identified in developmental studies are now being found to be involved in key steps of tumor metastasis (Kang and Massague 2004; Yang et al. 2004). EMT also acts in tumor progression by providing increased resistance to apoptotic agents (Maestro et al. 1999; Vega et al. 2004), and by producing supporting tissues that enhance the malignancy of the central tumor.
Recently, Wnt4 has been demonstrated to be essential for normal conversion of metanephric mesenchyme to the epithelia of the nephron (Carrol et al. 2005; Stark et al. 1994).
All this evidence prompted us to investigate the involvement of Wnt4 in the modulation of the EMT markers. To this end, we silenced Wnt4 with a specific siRNA and analyzed by qRT-PCR the expression level of some typical EMT markers. The transfection with a siRNA Non-Targeting (siCtrl) was used as negative control. More in details, FRTL-5 cells were transfected with siCtrl and siWnt4 respectively and 72 hours later, we analyzed the expression level of Wnt4, E-cadherin (E-cadh) as epithelial marker and α-Smooth muscle actin (α-SMA) and Vimentin (VIM) as mesenchymal markers. By qRT-PCR we showed that upon Wnt4 knockdown, the mRNA levels of E-cadh are reduced, whilst the expression of the mesenchymal markers α-SMA and Vim is up-regulated in the Wnt4 silenced cells with respect to the FRTL-5 transfected with siCtrl (Figure 20).

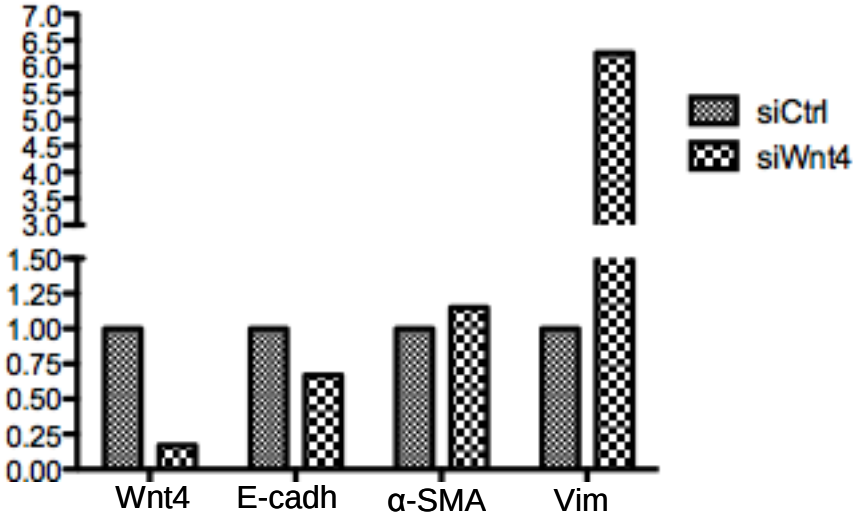

Figure 19: ***Effect of Wnt4 silencing on EMT of thyroid cells.***
qRT-PCR analysis was performed on total RNA prepared from FRTL-5 transfected either a siRNA ctrl or a siRNA Wnt4. The expression of E-cad, α-SMA and Vim was measured.

This data demonstrate that reduced levels of Wnt4 correlate with EMT suggesting a relevant role of this gene in the maintenance of the epithelial phenotype. To better analyze the involvement of Wnt4 in this process, we produced stable clones over-expressing Wnt4 in rat thyroid differentiated FRTL-5 cells. In particular, we stably transfected the cells with an expression vector encoding Wnt4 or with the empty vector as control. The screening analysis of all the clones identified two clones, named Wnt4 cl 31 and Wnt4 cl 35, that express a high level of the exogenous Wnt4 protein (Figure 21).

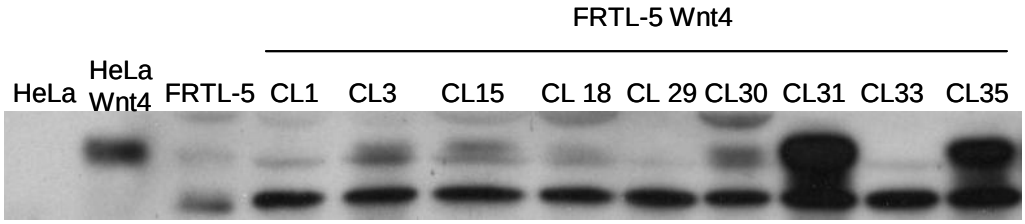

Figure 21: *Wnt4 over-expression in the Wnt4 clones analyzed by Western blot with a specific α-Wnt4 antibody.*

To further investigate the role of Wnt4 in the maintenance of the epithelial state, we analyzed the same EMT markers described above. As shown in the Figure 22 the stable over-expression of Wnt4 is able to revert the mesenchymal phenotype. In particular, the up-regulation of this gene determines a strong increase of E-cadh and a reduction of the mesenchymal markers (α-SMA and VIM).

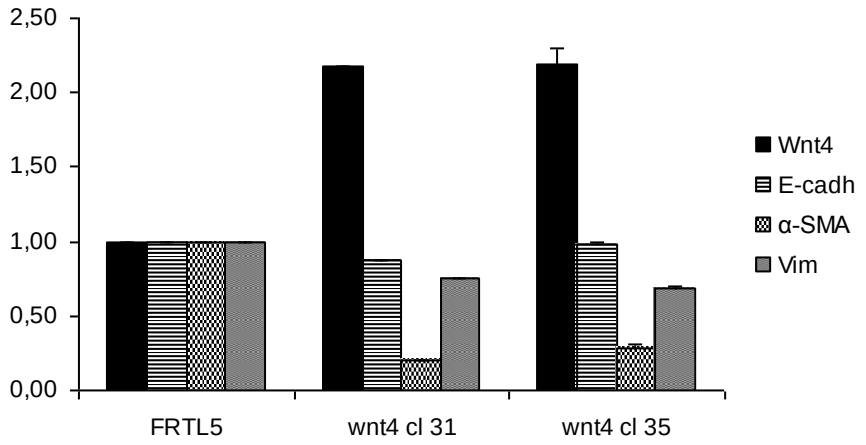

Figure 22: ***Effect of Wnt4 over-expression on EMT of thyroid cells.***
qRT-PCR analysis was performed on total RNA prepared from FRTL-5Wnt4 clones 31 and 35 and from FRTL-5 stable transfected with the empty vector. The expression of E-cad, α-SMA and Vim was measured.

4.5 The TGF-β1 inducted EMT could be reverted by the over-expression of Wnt4

Since many years it has been extensively demonstrated that Transforming Growth Factor-β1 (TGF-β1) in thyroid is a potent inhibitor of growth and DNA synthesis in rat (Morris et al. 1988), porcine (Tsushima et al. 1988), and human (Taton et al. 1993) thyroid follicular cells (TFCs) as well as in other epithelial cell systems (Sporn et al 1986; Lyons et al 1990). Differentiation markers of TFCs, such as Na^+/I^- symporter (NIS), thyroglobulin (Tg), and the TSH receptor (TSHR) are also suppressed by TGF-β1 (Franzen et al 1999). TGF-β1 synthesis at the mRNA level has been documented in follicular cell cultures derived from the thyroid glands of numerous species (Pekary et al 1995). It is also known that the treatment with TGF-β1 of thyroid differentiated cells is responsible for a severe reduction of Pax8 expression and for the EMT induction. The intringuily ability of TGF-β1 to down-regulate the transcription factor Pax8 and to induce EMT, suggested us to investigate a possible correlation between low levels of Pax8 induced by TGF-β1 and Wnt4 expression.

To investigate this correlation, we treated the FRTL-5 cells with 5 and 10 ng/ml of TGF-β1 for 48 hours and we analyzed by qRT-PCR the expression levels of Pax8, Wnt4 and of specific EMT markers (Figure 23). In agreement with the data in the literature, TGF-β1 determines a strong reduction of Pax8 mRNA level (see panel a) and induces EMT; in addition, this treatment is also able to down-regulate the expression level of the epithelial marker E-cadherin (panel c), and to significantly induce the levels of its inhibitor Snail I as well as of the mesenchymal marker FBN (panels d and e). As expected as a consequence of the reduced levels of Pax8, also Wnt4 levels are decreased (panel b).

Subsequently, the two clones over-expressing Wnt4 (Wnt4 cl 31 and 35) were treated with the same concentration of TGF-β1 described above. The Wnt4 cl 31 and 35 that stably over-expressing Wnt4 showed reduced levels of Pax8 and endogenous Wnt4 after treatment (panels a and b). Very interestingly, the qRT-PCR analysis revealed that the over-expression of Wnt4 is able to revert the mesenchymal phenotype.

Notably, the stable exogenous expression of Wnt4 is able to reduce the expression of the mesenchymal markers FBN and Snail I (panels d and e), but the 48 hours treatment with TGF-β1 is not sufficient to revert the down-regulation of the E-cadh (panel c). However, the strong reduction the E-cadh inhibitor Snail I, an early EMT marker, could suggest that the reversion of the mesenchymal state is taking place in the cells that over-express Wnt4.

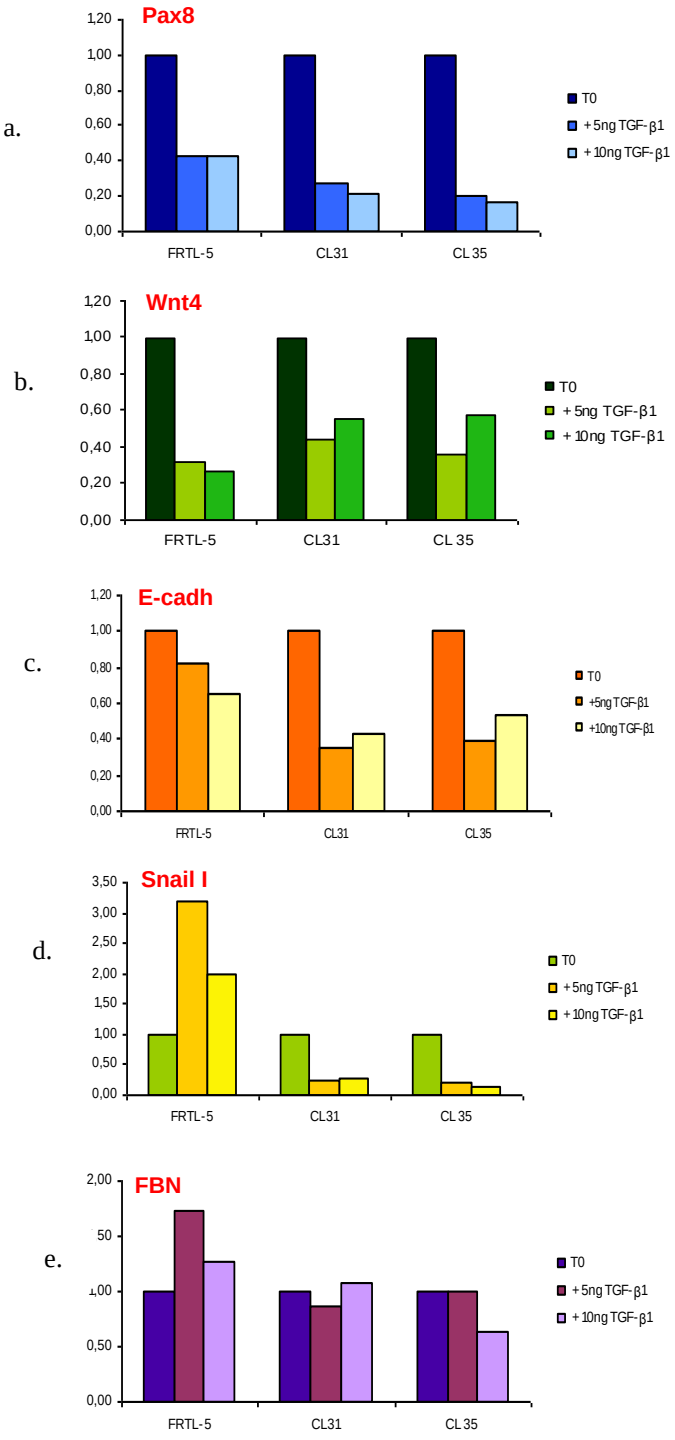

Figure 23: ***Effect of TGF-β1 on expression of EMT markers.*** *FRTL-5cells and clones 31 and 35 over-expressing Wnt4 were treated with the indicated concentration of TGF-β1for 48 hours. Total RNAs were prepared and analyzed by qRT-PCR for the evaluation of the mRNA expression levels of the EMT markers.*

4.6 TSH regulates Wnt4 expression in thyroid cells

The main physiological stimulus regulating thyroid function is given by thyrotropin (TSH) produced by the pituitary gland. Like many other hormones, TSH exerts its function through a G protein-coupled receptor, the TSH receptor (TSHR), which relies on the associated G protein to transmit and amplify the signal inside the cell. In rat thyroid cells, TSH-mediated activation results primarily in cAMP production that is likely to play a crucial role in the onset of differentiation both in vitro and in vivo. Indeed cAMP agonist, for instance forskolin (FSK), can mimic TSH-induced differentiation. The presence of TSH is also required for the maintenance of the differentiated phenotype, since withdrawal of TSH for a prolonged time causes loss of thyroid-specific gene expression in cultured cells. Several studies have led to the conclusion that TSH regulates the expression of the Tg gene by determining its rate of transcription (Avvedimento et al. 1984, Van Heuverswyn et al. 1984, Santisteban et al. 1987). A number of in vitro studies, in which cultured thyrocytes were used, have shown that the lack of TSH leads to a drastic reduction of Tg mRNA and that re-addition of TSH is able to reactivate Tg transcription (Chebath et al. 1979, Tosta et al. 1983).

In vivo studies in rat models also suggested that TSH controls Tg gene transcription via the interaction with its receptor on thyrocytes, and that cAMP is a physiological mediator of this effect (Van Heuverswyn et al. 1984, 1985). However, no cAMP-responsive elements have been found in the rat Tg promoter region (Gerard et al. 1989, Lee et al. 1991).

The thyroid transcription factor Pax8 binds to a single site on the Tg promoter, and it has been showed to activate Tg gene expression through a thyroid-specific mechanism (Pasca Di Magliano et al. 2000). In addition, Pax8 appears to be also involved in the thyroid-specific expression of the rat sodium/iodide symporter (NIS) gene (Ohno et al. 1999).

The group where I developed my PhD project investigated the role of Pax8 as a mediator of TSH action in thyrocytes, demonstrating that in a model system of rat thyrocytes in culture in the absence of TSH, Pax8 mRNA and protein rapidly disappear, whereas upon TSH stimulation, both mRNA and protein are newly synthesized (Mascia et al. 2002)

To investigate the role of TSH in the expression of Wnt4, differentiated rat thyroid FRTL-5 cells were starved for 7 days in serum-free medium containing 0.2% BSA and then treated with TSH for different times (3, 6, 12, 24 and 48 hours). The expression of both Pax8 and Wnt4 was analyzed at the mRNA level by qRT-PCR. As expected, Pax8 expression was strongly reduced upon starvation of the cells and reinduced upon TSH stimulation. Similarly, also the expression of Wnt4 turned out to be modulated by TSH, and the kinetic of induction upon TSH stimulation well correlates with that of Pax8, suggesting that TSH regulation of Wnt4 expression could be mediated by Pax8 (Figure 24).

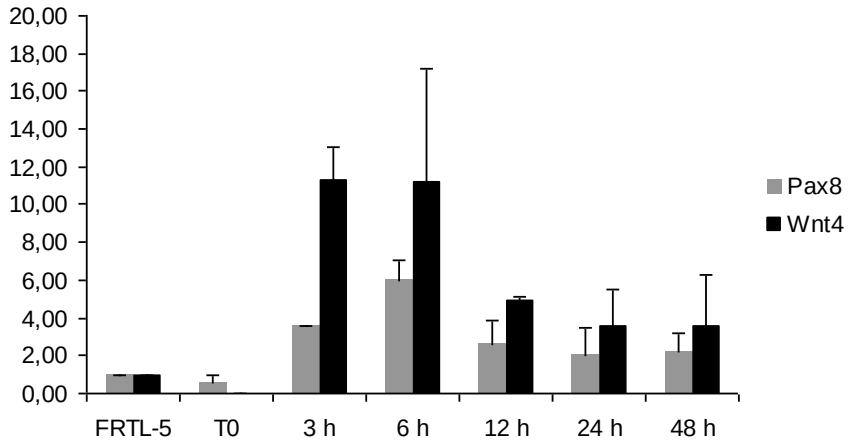

Figure 24: ***Wnt4 expression is modulated by TSH in FRTL-5 thyroid cells.*** *FRTL-5 cells were cultured in regular medium or maintained in starvation medium for 7days (T0) and then treated with 1mU/ml of TSH for different times (3, 6, 12, 24 and 48h) and analyzed by qRT-PCR analysis to measure the expression of Pax8 (gray bars) and Wnt4 (black bars) mRNA.*

4.7 Wnt4 expression is down-regulated in human thyroid cancer and its over-expression is required for cellular migration

Thyroid cancer is the most common malignancy of the endocrine system and represents approximately 1% of all newly diagnosed cancer. The most frequent type of thyroid malignancy is papillary carcinoma (PTC), which constitutes more than 80% of all cases. The overall prognosis of PTC is very good with an average 10-years survival rate of more than 90%. However, approximately 10% of PTC patients may die as a consequence of their disease. Several clinicopathological parameters have been identified to predict a worse prognosis including old age at diagnoses, extrathyroidal invasion, distant metastases and aggressive histological variants (Miccoli et al. 2009). The most recent advances in thyroid cancer research derive from an increased understanding of the mechanisms that regulate thyroid cell differentiation and proliferation and the signal transduction pathways involved. Among these pathways, the Wnt/β-catenin pathway has received most attention during the past years for its critical role in cancer (Sastre-Perona and Santisteban 2012). Many publications emphasize the role of the Wnt/β-catenin pathway in thyroid cancer. Recently it has been showed that Wnt4 down-regulation is necessary for the progression of thyroid epithelial tumors toward a fully malignant phenotype (De Menna M. et al. 2012). Moreover, it has been extensively demonstrated that a progressive decrease of Pax8 levels occurs in thyroid tumors from follicular adenoma to differentiated carcinoma and then to anaplastic carcinoma, which parallels the progressive dedifferentiation and increasing malignancy of thyroid tumors (Fabbro et al. 1994; 2008).
To evaluate the involvement of the secreted glycoprotein Wnt4 in thyroid carcinogenesis with respect to the transcription factor Pax8, we analyzed (by quantitative RT-PCR) both Wnt4 and Pax8 mRNA levels in four different human cancer cell lines, derived from follicular (WRO cells), anaplastic (Cal62 cells) and papillary (FB2 and BcPAP cells) thyroid carcinoma. As control, we used a pool of six normal thyroid tissues. As showed in Figure 25 a, all the tested thyroid cancer cell lines express much lower Wnt4 mRNA levels in comparison to normal thyroids, indicating that a strong down-regulation of Wnt4 expression occurs in thyroid carcinomas.

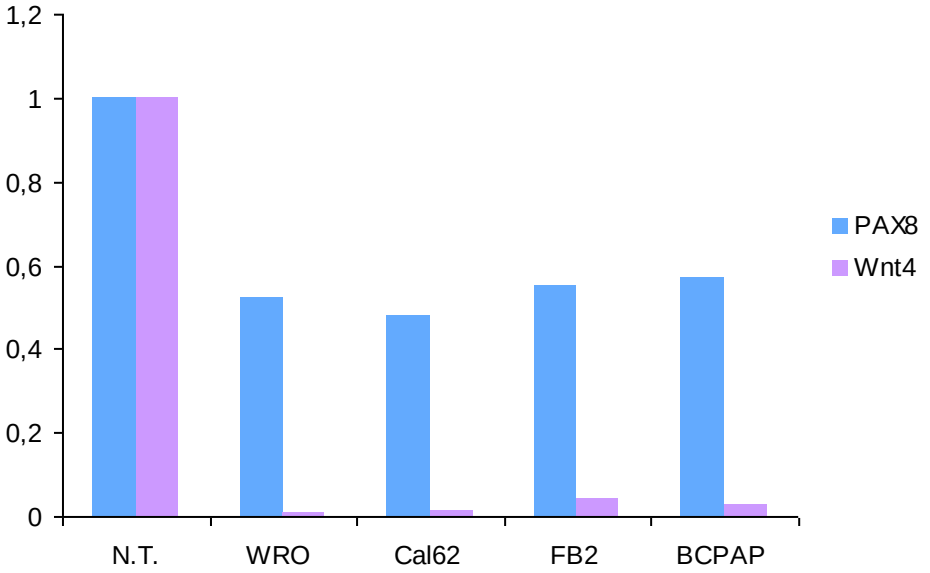

Figure 25: *Expression of Pax8 and Wnt4 in human thyroid cancer cells*. qRT-PCR analysis was performed on total RNA prepared from human thyroid cells. As control, a pool of six normal thyroid tissues (N.T.) was used.

In agreement with the data present in literature, also the mRNA levels of Pax8 were decreased in the analyzed tumor cancer cell lines.
Subsequently, to determine whether Wnt4 is able to alter the expression levels of the EMT markers, we stable transfected the BCPAP (papillary thyroid carcinoma) cell line with an expression vector encoding Wnt4 or with the parental vector. We obtained two pools of cells stable transfected with the expression vectors pCEFL and pCEFLWnt4, respectively; we analyzed by qRT-PCR the expression level of the EMT markers. As showed in Figure 26, the over-expression of Wnt4 is able to determine a strong enhancement of E-cadherin expression in human papillary cancer cells, while no modification is observed at the mesenchymal level. The high expression of the E-cadherin, that is the main epithelial marker, let us hypothesize that the over-expression of Wnt4 could influence the stabilization of the epithelial state without reverting the mesenchymal one.

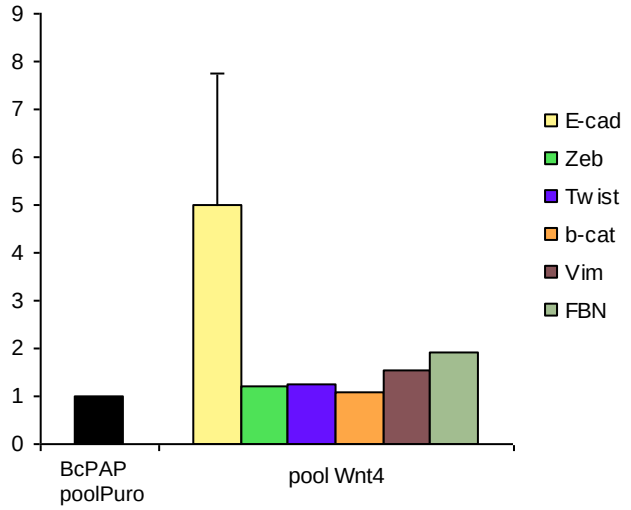

Figure 26: ***Effect of Wnt4 expression on EMT of human cancer thyroid cells.***
qRT-PCR analysis was performed on total RNA prepared from BcPAP pools of clones either expressing Wnt4 or transfected with the parental vector. The over-expression of Wnt4 is clearly responsible for the maintenance of the epithelial phenotype.

Moreover, to further study whether elevated levels of Wnt4 may have a role in cell migration, we performed wound healing assays comparing the cell mobility of BCPAP wild type cells and the pools of cells stable expressing Wnt4 or the empty vector as control. After 12 hours, the area of the wound was completely recovered by migrating cells in BcPAP cells and in the control pool. At difference, the motility of the cells stable expressing Wnt4 resulted strongly decreased, suggesting that Wnt4 over-expression significantly reduces the migration of human papillary thyroid tumors cells(Fgure 27).

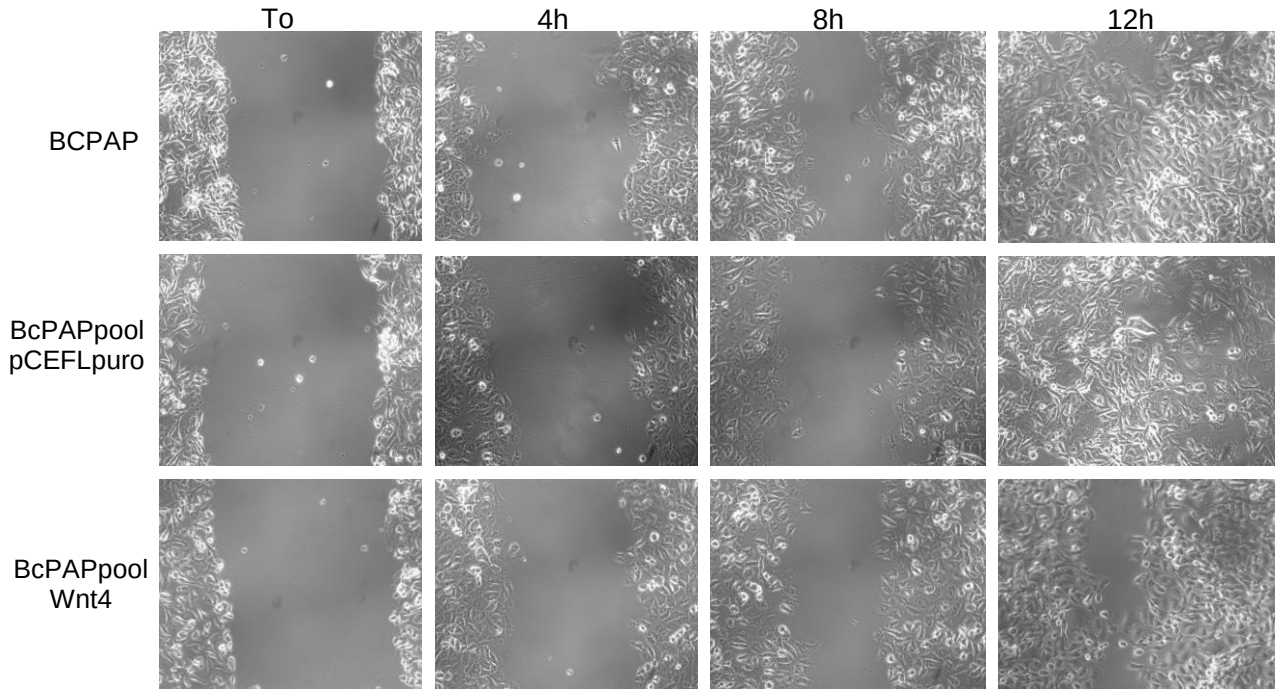

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Figure 27: ***Wnt4 over-expression in human papillary thyroid tumor cells inhibits cellular migration.***
Wound healing migration assay for BcPAP, BcPAPpoolpCEFLpuro and BcPAPpoolWnt4 was performed. The healing of wounds by migrated cells at time O, 4, 8 and 12 h was imaged. BcPAPpoolWnt4 cells migrate slower than BcPAP and BcPAPpoolpCEFLpuro cells.

5. Conclusions

During thyroid development, Pax8 is expressed upon transition from undifferentiated endoderm cells to thyroid follicular fated cells in the thyroid anlage and continues to be expressed throughout development and in the adult gland (De Felice and Di Lauro 2004). It has been clearly demonstrated that Pax8 is necessary for the expression of thyroid-specific genes, like Thyroglobulin (Tg), Thyroperoxidase (TPO), and sodium/iodide symporter (NIS) essential for the syntesis of active thyroid hormones (Ohno et al. 1999; Pasca di Magliano et al. 2000; Zannini et al. 1992). In addition, $Pax8^{-/-}$ mice have a barely visible thyroid gland that lacks the follicular cells (Mansouri et al. 1998). The critical role exerted by Pax8 in thyroid follicular cell differentiation has been demonstrated also in cell culture systems. For example, in thyroid cells expressing the polyoma virus middle T antigen (PCPy cells), loss of Pax8 expression results in loss of the thyroid-differentiated phenotype, measured as the expression of Tg, TPO and NIS genes. Re-introduction of Pax8 in PCPy cells is sufficient to reactivate the expression of the endogenous Tg, TPO and NIS genes (Pasca di Magliano et al. 2000). Given the pivotal role played by Pax8 in thyroid differentiation, many studies have been focused on the molecular mechanisms by which Pax8 modulates thyroid gene expression.

The genome wide screening carried out in the lab where I performed my PhD programme aimed at the identification of new targets of the transcription factor Pax8 in rat thyroid differentiated cells FRTL-5. In the end, 2815 genes were found modulated 72 h after Pax8 RNAi; among the down-regulated genes there was Wnt4.

Wnt4 is a secreted glycoprotein correlated and required for kidney tubulogenesis. Mice lacking Wnt4 activity fail to form pretubular cell aggregates. Moreover, Wnt4 appears to act as an autoinducer of the mesenchyme-to-epithelial transition that underlies nephron development (Stark et al. 1994). In thyroid, Wnt4 has been recently demonstrated to be a key player in Ras-mediated transformation of epithelial cells and to be involved in the inhibition of aberrant motility of neoplastic cells (De Menna et al. 2012).

The results of this study indicate Wnt4 as new direct target of the transcription factor Pax8 and we demonstrated that Wnt4 has a thyroid-specific transcriptional activity suggesting that Pax8 has an important role in the modulation of Wnt4 gene expression. More in details, we demonstrated by EMSA experiments that there is a specific binding site responsible for Pax8 binding to the Wnt4 promoter region and this site is very well conserved among the species. Furthermore, we revealed that in rat differentiated cells FRTL-5 Wnt4 is involved in the maintenance of the epithelial phenotype, in fact the EMT induced by the down-regulation of Wnt4 is completely reverted in the cellular model system that stable over-expresses Wnt4. This stable over-expression of Wnt4 is also able revert the EMT mediated by the TGF-β1 signaling.

We also revealed that, like Pax8, the expression of Wnt4 is strongly TSH-dependent in FRTL-5 thyroid cells and is significantly reduced in human thyroid cancer cells.
In conclusion, our data suggest that the over-expression of Wnt4 in human papillary cancer cells could influence the stabilization of the epithelial state and is necessary to reduce cellular migration.
Moreover, the identification of Wnt4 as a novel target of the transcription factor Pax8 prompt us to hypothesize that its expression is associated with the fully differentiated state of the thyroid cells.

6. Acknowledgements

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ORIGINAL ARTICLE

Characterization of a novel loss-of-function mutation of PAX8 associated with congenital hypothyroidism

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Summary

Background Congenital hypothyroidism (CH) is a common endocrine disease that occurs in about 1:3000 newborns. In 80–85% of the cases, CH is presumably secondary to thyroid dysgenesis (TD), a defect in the organogenesis of the gland leading to an ectopic (30–45%), absent (agenesis, 35–40%) or hypoplastic (5%) thyroid gland. The pathogenesis of TD is still largely unknown. Most cases of TD are sporadic, although familial occurrences have occasionally been described. Recently, mutations in the PAX8 transcription factor have been identified in patients with TD.

Objective Our aim was to identify and functionally characterize novel PAX8 mutations with autosomal dominant transmission responsible for TD.

Design The *PAX8* gene was sequenced in a mother and child both suffering from congenital hypothyroidism (CH) because of thyroid hypoplasia. Subsequently, expression vectors encoding the mutated PAX8 were generated, and the effects of the mutation on both the DNA-binding capability and the transcriptional activity were evaluated.

Results *PAX8* gene sequencing revealed a heterozygous mutation that consists of the substitution of a histidine residue with a glutamine at position 55 of the PAX8 protein (H55Q). When tested in cotransfection experiments with a thyroglobulin promoter reporter construct, the mutant protein turned out to be still able to bind DNA in Electrophoretic Mobility Shift Assay assays but transcriptionally inactive.

Conclusions Our findings confirm the important role of PAX8 in normal thyroid development and support the evidence that in humans haploinsufficiency of PAX8 is associated with TD.

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Introduction

Primary congenital hypothyroidism is a common endocrine disease in newborns, with an incidence of about 1:3000 live births, and is characterized by elevated TSH levels at birth in response to reduced thyroid hormone levels.[1] In 85% of the cases, CH is because of developmental anomalies of the thyroid gland (thyroid dysgenesis, TD), which result in a thyroid gland that is absent (thyroid agenesis or athyreosis), ectopically located (thyroid ectopy) or hypoplastic (thyroid hypoplasia).[2] About 5% of the TD cases are associated with mutations in the genes responsible for thyroid development and growth,[3] including genes that encode for TTF-1/NKX2.1,[4,5] FOXE1,[6,7] PAX8,[8,9] TSH receptor[10] and NKX2.5.[11]

PAX8, like other members of the Pax family, recognizes DNA via a conserved 128-amino acid domain, the paired domain. The gene maps to human chromosome 2q12-14 and contains a 4-Kb transcription unit divided into 12 exons.[12] PAX8 plays an important role in thyroid development, when the thyroid bud invaginates from the floor of the pharynx,[13] and it is required both for the morphogenesis of the thyroid gland[9] and for the maintenance of the thyroid-differentiated phenotype.[14] In the adult thyroid, PAX8 is essential for the transcriptional activation of the thyroperoxidase (TPO), thyroglobulin (Tg) and sodium/symporter (NIS) genes.[15,16] Recently, it has been demonstrated that PAX8 and TTF-1/NKX2.1 biochemically associate and synergistically activate transcription from the TPO[17] and Tg gene promoters.[18,19] Homozygous Pax8 knockout mice die shortly after weaning, presumably from severe hypothyroidism because of thyroid hypoplasia, whereas heterozygotes were described unaffected.[9]

To date, eleven heterozygous inactivating mutations in the *PAX8* gene have been identified and characterized in patients with TD.[8,20,21] Only two of these mutations, the deletion in exon 7 (c.989-992delACCC) located outside the paired domain[22] and the p.S48F located within the paired domain,[23] show normal DNA-binding affinity but are transcriptionally inactive. The remaining eight mutations already described are located within the paired domain of PAX8 and result in a severe reduction in DNA binding. The autosomal dominant transmission is

well established with variable degrees in the severity of hypothyroidism.

In this study, we report the identification and functional characterization of a novel *PAX8* mutation in a mother and child with CH because of thyroid hypoplasia. The mutation results in the substitution of histidine for glutamine at codon 55 of the PAX8 protein. Although the mutated residue is located within the paired domain, the mutated protein does not display an impairment in the DNA-binding activity but is unable to activate transcription from known PAX8 target promoters.

Our results strengthen the notion that PAX8 mutation contributes to the sporadic and familial cases of CH.

Patients and methods

Case reports

Two members of a nonconsanguineous family were diagnosed with hypothyroidism (Fig. 1a). Patient II-1 was diagnosed as hypothyroidism at birth in the frame of a neonatal screening, displaying a TSH level of 200 mU/ml (normal range: 1–10) and a low free T_4 of 13 pmol/l (normal range: 20–30). The patient had no detectable Thyroglobulin antibodies, and his serum TG was 160 μg/l (normal, 20–70). Thyroid ^{123}I scintigraphy showed a hypoplastic gland in normal position. Since birth he is receiving substitutive L-T4 treatment. His physical and psychomotor development is normal.

Patient I-1, 24-year-old woman, is the mother of patient II-1. During the CH screening, she was diagnosed as athyreotic but a recent ultrasonography showed a hypoplastic thyroid (right lobe: 0·4 × 1·4 × 1·3 cm; left: 0·9 × 0·5 × 1·5 cm.) (Fig. 1a). She was treated since birth and had normal development in terms of growth and intelligence. Ultrasonography of the kidneys did not reveal any structural abnormalities in the renal system. Her family history is unknown, and unfortunately, the other members of the family did not agree to provide DNA for the genetic studies.

DNA sequencing

All the coding region of the PAX8 gene was amplified as previously described.[8] PCR products were then purified with Antartic Phosphatase-Exonuclease I (New England BioLabs, Beverly, MA, USA) at the following conditions: 37°C for 15 min and 80°C for 15 min, respectively, and inactivation and then directly sequenced for enzymes activation bidirectionally with a 377 ABI sequencer with the same primers used for PCR amplification.

Plasmids

The plasmids were previously described and were as follows: CMV-TTF-1[24] and pCMV5-H29a (also named PAX8-WT).[12] The H55Q and H55A mutations were introduced by site-directed mutagenesis (QuikChange II-E Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). All constructs were confirmed by sequencing. The reporter plasmid TgCAT, originally named pTACAT-3, used in transient transfection experiments was previously described.[25]

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (Euroclone, Pero, MI, Italy). For transient transfection experiments, cells were plated at 3×10^5 cells/60-mm tissue culture dish 5–8 h prior to transfection. Transfections were carried out with the FuGENE6 (Roche Diagnostics, Basel, CH, Switzerland) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:2 in all experiments. The plasmid CMV-LUC was used as internal control in transfection assays. Cells extracts were prepared after 48 h to determine either the levels of CAT protein with a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics) or LUC activities by luciferase assays as previously described.[26] Transfection experiments were carried out in duplicate and repeated at least three times. CAT

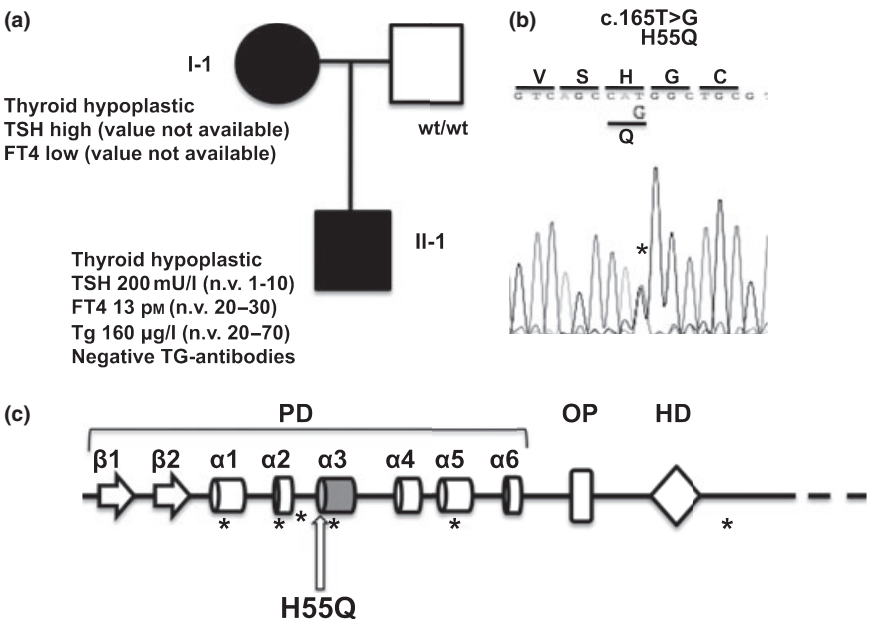

Fig. 1 Identification of a novel PAX8 mutation. (a) Pedigree of the family presenting with congenital hypothyroidism (CH) associated with the H55Q mutation of PAX8 in the heterozygous state. (b) Chromatogram of part of exon 3 of patients showing a heterozygous T → G transversion at nucleotide 165 of the paired domain PAX8 (c.165T>G). This mutation replaces a histidine residue by glutamine (H55Q). (c) Schematic representation of the paired domain (PD) and the position of the H55Q mutation in α-helix 3 (α3). The octapeptide (OP) and the residual paired-type homeodomain (HD) are shown. Also, the previously identified PAX8 mutations are indicated by asterisks.

activity values on the graph are the means of all experiments (±) SD. Statistical analysis uses two-tailed Student's *t*-test to obtain the *P* value associated with the observed fold of activation differences.

Protein production and Western blot analysis

Wild-type and mutated PAX8 cDNA constructs were transcribed and translated using the TnT Quick Coupled Transcription/Translation System from Promega Corporation (Madison, WI, USA). The level of transcription/translation products and total extract of transfected Hela, prepared as previously described,[27] were assessed by Western blot. For these analysis, proteins were separated by SDS-10% PAGE, gel were blotted onto Immobilon P (Millipore, Bredford, MA, USA) for 2 h, and the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 2 h before the addition of the anti-PAX8 antibody (kindly provided by R. Di Lauro) for 1 h. The filters were washed three times in Tris-buffered saline plus 0·5% Tween 20 before the addition of horseradish peroxidase–conjugated secondary antibody for 45 min. Horseradish peroxidase was detected with ECL (GE Healthcare, Waukesha, WI, USA).

EMSA

Double-stranded oligonucleotide C derived from the Tg promoter was labelled with [γ-^{32}P] ATP and T_4 polynucleotide kinase and used as probe as previously described.[16] The binding reactions were incubated for 30 min at room temperature in a binding buffer containing 10 mM HEPES (pH 7·9), 10% glycerol, 0·1 mM EDTA, 8 mM MgCl2, 1 mM dithiothreitol and 0·15 g/ml of poly (dI-dC). DNA–protein complexes were resolved on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography.

Results

Sequencing of PAX8

In both affected members of the family (I-1 and II-1 in Fig. 1a), sequence analysis of the *PAX8* coding region gene revealed a heterozygous T → G transversion in exon 3 (Fig. 1b), resulting in the substitution of a histidine at position 55, within the α-helix 3 of the PAX8 paired domain, with a glutamine residue (PAX8-H55Q) (Fig. 1b,c).

DNA-binding properties of the PAX8-H55Q mutant

To evaluate whether the PAX8-H55Q mutation produces a defect in the DNA-binding ability of the PAX8 protein, we carried out EMSA analysis with *in vitro*-translated PAX8 and PAX8-H55Q proteins. As shown in Fig. 2a, the PAX8-H55Q mutant is able to bind DNA similarly to wild-type PAX8 when challenged with the oligo C derived from the Tg promoter and containing a well-known PAX8-binding site. Equivalent amounts of each protein were used in the EMSA assay reactions as confirmed by the Western blot analysis (Fig. 2a). The same DNA-binding ability was observed when total protein extracts of HeLa cells transiently transfected with expression vectors encoding either the wild-type or the mutated PAX8 protein were employed in the EMSA assay (Fig. 2b). Also in this case, the amounts of each total extract used in the binding reactions were normalized by Western blot (Fig. 2b).

Therefore, our data indicate that the mutation of the histidine residue within the paired domain does not affect the ability of PAX8 to recognize its binding site on DNA, suggesting that the H55 residue is not involved in contacts with DNA. To further confirm these results, we have generated an additional mutant, named

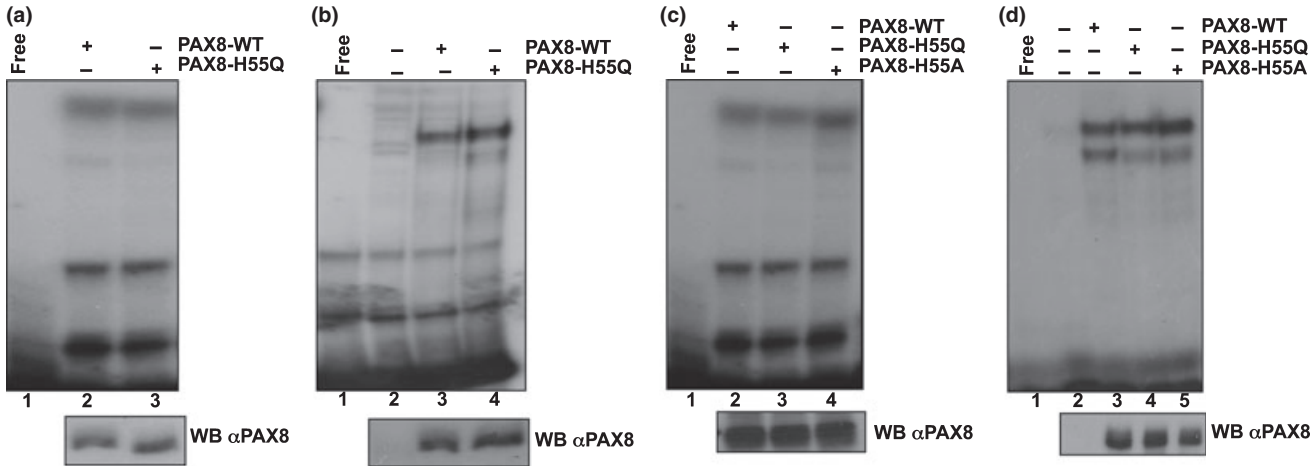

Fig. 2 DNA binding of the PAX8-WT and PAX8 mutant proteins. The DNA-binding properties of PAX8-WT, PAX8-H55Q and PAX8-H55A mutants were tested on a PAX8 response element, oligonucleotide C. EMSA were performed with *in vitro*-translated WT, H55Q and H55A mutant PAX8 proteins (a,c), and protein extract prepared from HeLa cells transiently transfected with PAX8-WT, PAX8-H55Q and PAX8-H55A (b,c). PAX8-H55Q mutant (a, lane 3; b, lane 4; c, lane 3; d, lane 4) and PAX8-H55A mutant (c, lane 4; d, lane 5) are able to form a low-mobility complex with the oligo C as the PAX8-WT (a, lane 2; b, lane 3; c, lane 2; d, lane 3). No retarded band is present in the extract prepared from nontransfected HeLa cells used as a negative control (b and d, lane 2). The analysis by Western blotting of PAX8-WT, PAX8-H55Q and PAX8-H55A proteins showed that equivalent amounts of each protein were produced.

PAX8-H55A, in which the histidine residue is substituted by an alanine. The alanine is the substitution residue of choice that does not alter the main-chain conformation,[28] and it has been proven to be unable to make contact with DNA. Consequently, we generated this mutation to clarify whether the H55 residue of PAX8 is directly involved in contacts with DNA or not. Specifically, if the H55 residue is not involved in contacts with DNA, we expected that the PAX8-H55A mutant did not show any impairment in DNA binding. Indeed, our results of the EMSA assays clearly indicated that the PAX8-H55A mutant is able to bind to the PAX8-binding site contained in the oligonucleotide C and shows no significant differences when compared to wild-type PAX8 and PAX8-H55Q (Fig. 2c,d). The same results were obtained when *in vitro*-translated proteins (Fig. 2c) or total extract of HeLa cells transiently transfected with either the wild-type or the mutated expression vectors (Fig. 2d) were employed in EMSA assays. Western blotting of wild-type and mutated proteins produced *in vitro* or transiently expressed in HeLa cells revealed no difference in the synthesis efficiency, indicating that the amino acid substitution does not cause destabilization of the mutant proteins (Fig. 2c,d).

Transcriptional activity of PAX8-H55Q mutant

To evaluate the functional relevance of the H55Q mutation, we relied on the ability of PAX8 to activate transcription from the thyroglobulin (Tg) promoter[16] and we investigated the ability of the PAX8-H55Q mutant to activate the transcription of a reporter gene under the control of the Tg promoter. HeLa cells were transiently transfected with expression vectors encoding wild-type PAX8 or PAX8 mutants, together with a reporter construct in which the minimal region of the Tg promoter is subcloned upstream of the CAT gene (pTACAT3[25]). In agreement with previous results,[16,18] wild-type PAX8 is able to stimulate transcription from the Tg promoter up to 7-fold, whereas PAX8-H55Q or PAX8-H55A shows a significantly reduced Tg reporter activation (Fig. 3a). These results led us to the conclusion that the PAX8-H55Q mutant has a significant reduced ability to activate transcription despite the fact that it has no impairment in DNA-binding activity.

Recently, it has been demonstrated that a physical interaction between PAX8 and the transcription factor TTF-1/NKX2.1 underlies the synergistic effect of both factors on Tg promoter

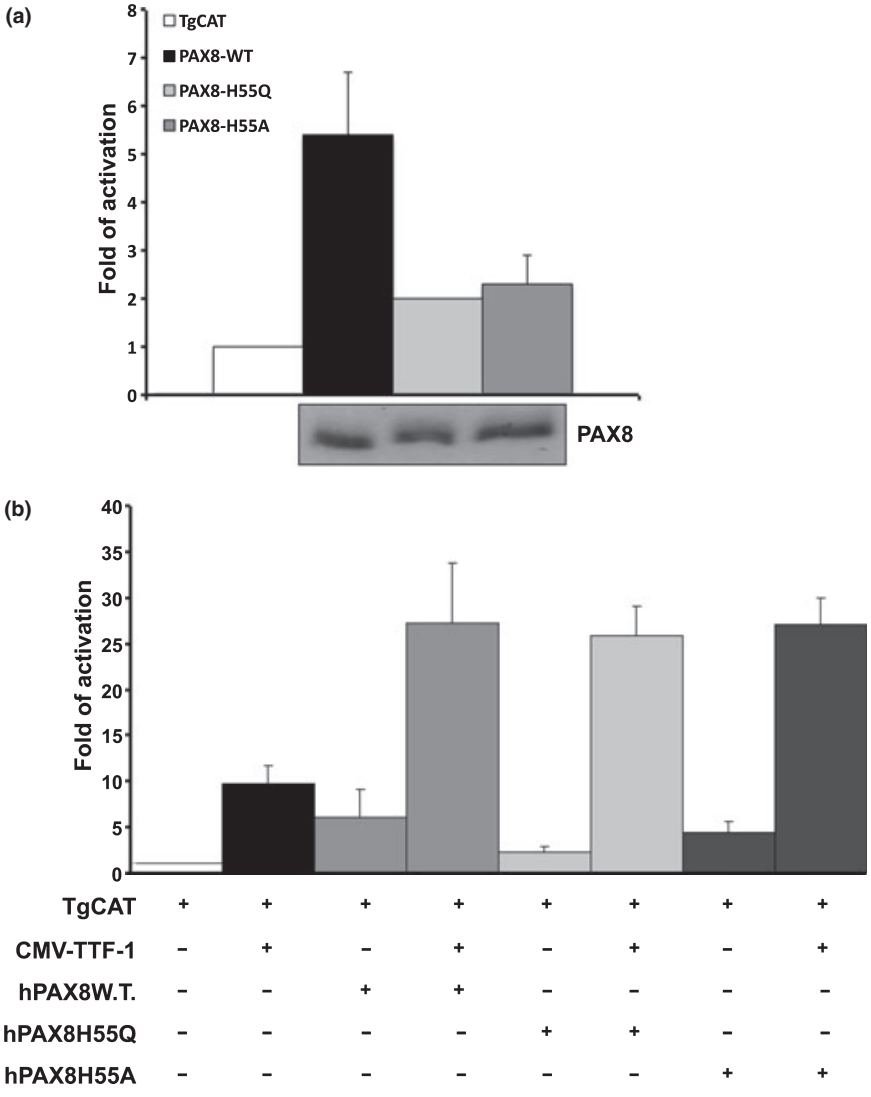

Fig. 3 Functional analysis of PAX8-H55Q. (a) HeLa cells were transiently transfected with the reporter plasmid TgCAT (2 μg), the expression vector encoding PAX8-WT (100 ng), the PAX8-H55Q mutant (100 ng) and the PAX8-H55A mutant (100 ng). The protein levels of PAX8-WT, PAX8-H55Q and PAX8-H55A after transfection were measured by immunoblot and are shown. (b) HeLa cells were transiently transfected with the reporter plasmid TgCAT (2 μg) with or without the expression vectors encoding for TTF-1 (50 ng), PAX8-WT (100 ng), PAX8-H55Q mutant (100 ng) and PAX8-H55A mutant (100 ng). Folds of activation are considered as ratio between values obtained with and without cotransfection of the expression vectors. CMV-LUC was added as internal reference and all data were normalized to the ratio CAT/LUC activity. Results are means ± SD from three independent experiments, each performed in triplicate transfection. Statistical analysis has been performed by means of an unpaired two-tailed Student's *t*-test to obtain the *P* value associated with the observed fold of activation differences ($P < 0{\cdot}1$ and $P < 0{\cdot}05$, respectively).

activation.[18] To test whether the mutant PAX8-H55Q and TTF-1/NKX2.1 could synergistically stimulate transcription from the Tg promoter, we transiently cotransfected in HeLa cells the reporter construct TgCAT together with the expression vectors encoding wild-type PAX8, PAX8-H55Q, PAX8-H55A and TTF-1/NKX2.1 separately or in combination. Our data show that although the activity of the PAX8-H55Q mutant is significantly lower than the activity of wild-type PAX8, it can still display a robust synergism with TTF-1/NKX2.1 in the transcriptional activation of the Tg promoter (Fig. 3b). A similar behaviour was observed with the PAX8-H55A mutant (Fig. 3b).

These results indicate that the H55Q mutation retains the ability to functionally interact with TTF-1/NKX2.1.

Discussion

We have identified and functionally characterized a novel loss-of-function mutation in the transcription factor PAX8, dominantly inherited in two members of a family with severe thyroid hypoplasia. The mutation, a T → G transversion in exon 3 of the *PAX8* gene, results in the substitution of a histidine at position 55 by a glutamine residue (H55Q). Although the H55Q mutation is located within the paired domain, we demonstrated that it does not alter the DNA-binding ability of the mutant; however, it modifies the functional properties of the protein. Our data are in agreement with the results previously reported by Grasberg *et al.* for the PAX8-S48F mutation,[23] showing that the mutation, even if located within the paired domain, abrogates the transactivation property of PAX8 but not its DNA-binding ability.

Based on the crystal structure models of paired domain–DNA complexes,[29,30] histidine 55 of PAX8 is located in the α-helix 3 of the paired domain and is predicted to make contacts with the DNA backbone. However, this amino acid represents a variant residue, being not conserved among the *PAX* genes.[29] It is worth noting that the structure of PAX8 bound to DNA has not been determined yet; therefore, any assessment on the PAX8–DNA complex has been made by comparison with other PAX homologues bound to DNA.[31] Our hypothesis is that the H55Q mutation within helix 3 of the paired domain is not involved in contacts with the DNA but might affect the folding and the conformation of the C-terminal region of PAX8, possibly of domains of the protein known to be crucial for the transcriptional activity because involved in physical interactions with other coactivators and/or the basal transcriptional machinery. This hypothesis is supported by the observation that also the PAX8-H55A mutation that we have generated retains the ability to bind DNA but is not able to efficiently activate transcription, as it happens with PAX8-H55Q. Because the alanine residue has a small side chain, the substitution of the histidine residue by alanine should severely interfere with the DNA recognition and consequently show an impairment in the DNA binding of PAX8-H55A. However, our results demonstrated that such a mutation affects only the transactivation property of PAX8. Hence, the H55Q mutation represents, together with the previously reported S48F mutation,[23] a new type of PAX8 mutations responsible for congenital hypothyroidism.

PAX8 is a major gene in the regulation of the thyroid-differentiated phenotype[14] and plays an important role in the morphogenesis of thyroid gland.[9] To date, all PAX8 gene mutations reported are located within the paired domain except for one mutation c.989-992delACCC,[22] located in exon 7 outside the paired domain that cause a frameshift with a premature stop codon after codon 277. The PAX8/277del protein retains the DNA-binding ability but it is not capable of activating transcription, thus indicating that the C-terminal region is essential for PAX8 transcriptional activity. Recently, it has been demonstrated that PAX8 activity can be modulated by the interaction with other proteins, forming complexes on regulatory regions of target genes.[18,27,32] Therefore, our findings suggest that the PAX8-H55Q mutant does not efficiently activate transcription because it does not allow the recruitment of other factors or coactivators to the target promoters. Furthermore, it has been demonstrated that PAX8 co-operates with the transcription factor TTF-1/NKX2.1,[17,19] and our laboratory specifically demonstrated that PAX8 and TTF-1/NKX2.1 form a functional heterocomplex responsible for the synergistic transcriptional activation of the *Tg* promoter in differentiated thyroid cells.[18] The relevance of this interaction has been further confirmed by the analysis of the *in vivo* mouse model bearing a partial deficiency of *Ttf1* and *Pax8* genes. The double heterozygous null mice show thyroid dysgenesis, a phenotype that is completely absent in either of the single heterozygous mice.[33] Our results demonstrate that the PAX8-H55Q mutant is still able to synergize with TTF-1/NKX2.1 on the *Tg* promoter, as indicated by the significantly higher stimulation of the reporter gene expression in cells expressing both PAX8-H55Q and TTF-1/NKX2.1, suggesting that the H55 residue is not essential for the functional interaction with TTF-1/NKX2.1. Therefore, it will be of interest to further investigate the effect of this novel mutation with respect to the already described interactions that PAX8 makes with other partners.

The mutation here reported in the child was inherited from his mother, in line with the dominant model of inheritance proposed for the cases of familial thyroid dysgenesis caused by PAX8 mutations.[8,34] The present case, as the others previously published, demonstrates that loss-of-function mutations of PAX8 are symptomatic in heterozygotes in humans, whereas only homozygous mice for a targeted disruption of *Pax8* display severe hypothyroidism, characterized by absence or dramatic reduction in the thyroid gland.[9] Haploinsufficiency, monoallelic expression, imprinting, dominant negative properties[8,34] and stochastic expression of the PAX alleles, as noted for Pax5,[35] are the molecular mechanism that could lead to disturbed thyroid development in humans. The patients reported here carry the H55Q mutation and both present CH with thyroid hypoplasia.

In conclusion, the novel PAX8-H55Q mutation identified in patients with congenital hypothyroidism and thyroid hypoplasia confirms the role of PAX8 in normal thyroid development and might be helpful to better understand the role of PAX8 in the pathogenesis of thyroid dysgenesis.

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Competing interests/financial disclosure

The authors have nothing to disclose.

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