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The role of WNT4 in the non-canonical WNT/PCP pathway in thymic epithelial tumors

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ABBREVIATIONS

Abbreviation	Description
AKT	AKT Serine/Threonine Kinase
APC	Adenomatous Polyposis Coli Protein
CamKII	Calmodulin-dependent Protein Kinase
CK19	Cytokeratin 19
CM	Conditioned Medium
CT	Childhood Thymus
Ct	Cycle Threshold
CTNNB1	Catenin Beta 1
DAB	3, 3'-Diaminobenzidine
DKK	Dickkopf
DVL	Dishevelled
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FOXN1	Forkhead Box N1
FZD	Frizzled receptors
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

Abbreviations

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IKK	IκB kinase
JNK	c-Jun N-terminal Kinase
LRP	The LDL Receptor–related Protein
MCS	Multicellular Spheroids
MAPK	Mitogen-activated Protein Kinase
MMP	Matrix Metalloprotease
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	Nuclear Factor of Activated T Cells
NF-κB	Nuclear Factor Kappa B
NT	Normal Thymus
PCP	Planar Cell Polarity
PKC	Protein Kinase C
PORCN	Porcupine O-acyltransferase
pTEC	Primary Thymic Epithelial Cells
RAC1	Ras-related C3 Botulinum Toxin Substrate 1
ROR2	Receptor Tyrosine Kinase Like Orphan Receptor 2
RPMI	Roswell Park Memorial Institute
RYK	Receptor Like Tyrosine Kinase
SFRP	Secreted Frizzled Related Protein
TBST	Tris-buffered Saline, 0.1% Tween® 20 Detergent

Abbreviations

TC	Thymic Carcinoma
TCA	Trichloroacetic Acid
TCF/LEF	T cell factor/ Lymphocyte enhancer factor
TCGA	The Cancer Genome Atlas
TET	Thymic Epithelial Tumor
TIMP	Tissue Inhibitor of Metalloproteinases
TLE	Transducin-like Enhancer Protein
TSQCC	Thymic Squamous Cell Carcinoma
WNT	Wingless and Int-1

1. INTRODUCTION

1.1 Thymic tumors

The thymus is a bi-lobed organ of the immune system located anatomically in the upper part of the chest under the breastbone and in front of the heart. The thymus is relatively large and active in infants' period and grows until adolescence, while in adulthood, the thymus starts to shrink and the tissue inside gradually involutes and finally would be replaced by adipose tissue [1]. Thymus is mainly made up of thymocytes that surround stromal cells, including epithelial cells, mesenchymal cells and a few myoid cells [2] [3]. The function of the thymus is mainly focused on T lymphocyte maturation through positive and negative selection requiring interaction with major histocompatibility complex (MHC) molecules on antigen presenting cells (APC) inside the thymus. Defects of the thymus could lead to deficiency of T cell immunity and infections or tumors or to autoimmune diseases like myasthenia gravis if T cell tolerance induction is insufficient. However, tumors like lymphomas and germ cell cancers could also develop from thymus, only thymomas, thymic carcinomas (TCs) and thymolipomas result from thymic epithelial cells [4].

Thymic epithelial tumors (TETs) are rare malignancy accounting for 0.2-1.5% of all tumors with an incidence of 0.15 per 100,000 population [5], but TETs are the most common tumors of anterior mediastinum with 50% incidence of all anterior mediastinal tumors [6]. The World Health Organization (WHO) classified thymic tumors into TCs [7] and thymomas histopathologically and thymomas are further subclassified into five major different types (A, AB, B1, B2 and B3) (Figure 1) according to the cytology and quantity of epithelial cancer cells with an increasing degree of atypia from type A to B3 and relative proportions of non-neoplastic lymphocytic components decreasing from type B1 to B3 [8] [9]. Compared to thymomas, TCs are also an epithelial tumors, of which the most common subtype is squamous cell carcinoma. TCs exhibit higher malignant features and a poorer prognosis with more aggressive local invasion and

more distant metastases than thymomas [4] [10]. At the time of diagnosis 30% of patients with malignant TETs have locally advanced tumors, with invasion into tissues such as the pleura, pericardium/heart, lung and vessels [9]

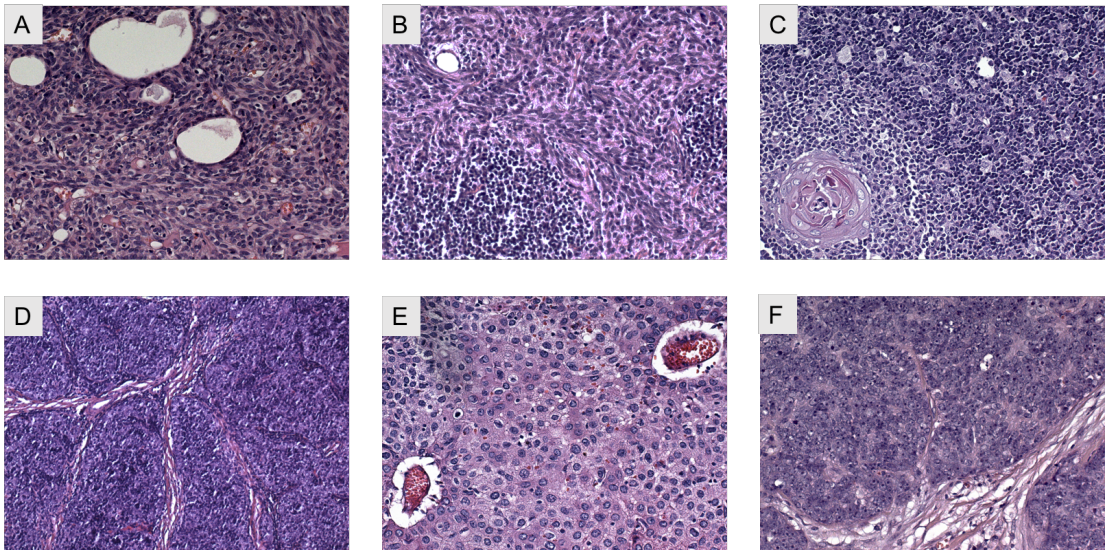


Figure 1: Pathology of thymoma and thymic carcinoma.

Hematoxylin and eosin stains different thymoma tissues at magnifications of 200x. (A) Thymoma A; (B) Thymoma AB; (C) Thymoma B1; (D) Thymoma B2; (E) Thymoma B3; (F) Thymic carcinoma.

Different types of treatments are available for patients with thymoma and TCs like chemotherapy, resection and radiotherapy, but surgery to remove the tumor is still the most important treatment, since only complete resection can result in definitive cure and has the most consistent prognostic effect on disease-free and overall survival [11] [12]. However, there are many conditions (e.g. advanced tumor stage) in which surgery is not reasonably applicable. In such settings, multimodal treatment strategies are applied, but novel therapies, including personalized, i.e. molecularly based targeted approach would be highly appreciated. However, this is challenging in these rare tumors, since genetic alterations of TETs are significantly different from those of squamous cell carcinomas of head, neck and lung [13]. The molecular pathology of TETs is still poorly understood. Previous studies have demonstrated that maintenance and functional integrity of the thymic stroma requires stimulation via Notch, bone morphogenetic protein (BMP), and WNT signaling pathways [14] [15].

1.2 Canonical and non-canonical WNT pathways

WNTs, abbreviation of wingless-type MMTV integration site family, are secreted glycoproteins that have crucial roles in the regulation of multiple biological processes, including cellular proliferation, differentiation, polarity, migration and cell stemness and has also been tightly associated with a variety of human cancers [16] [17]. Without WNT ligands, cytosolic β-catenin is involved in a multi-molecular complex consisting of Axin, the adenomatous polyposis coli protein (APC), and glycogen synthase kinase 3b (GSK-3β) in cytoplasm. Phosphorylation of Axin1/2, APC, and β-catenin (Ser33, Ser37) by GSK-3β results in β-catenin ubiquitination and proteolysis by proteasomes [18]. The WNT signaling pathway is highly conserved and is generally divided into canonical/β-catenin (CTNNB1) dependent pathway and non-canonical/β-catenin independent pathway. All these pathways are activated by WNT ligands (WNT1-19) binding to seven-transmembrane-receptors, frizzled receptors (FZD1-10), and co-receptors LRP5/LRP6 (low-density lipoprotein-receptor related protein 5 and 6), which leads to phosphorylation and increased activity of Disheveled (Dvl) and its downstream proteins [19].

Canonical WNT signaling inhibits β-catenin degradation by inactivation of GSK-3β, which is inhibited by phosphorylated Disheveled. Non-phosphorylated β-catenin then can translocate into the nucleus to activate the transcription factors of T cell factor/lymphocyte enhancer factor (TCF/LEF) family and initiate transcription of canonical WNT pathway related genes that encode proteins like MYC, CYCLIN D1, and matrix metalloproteases (e.g. MMP2, MMP7) [20] [21]. Of the 19 members of WNT ligands, WNT 1, 3, 8, 10 are known canonical members to activate β-catenin signaling through binding to Frizzle receptors and LRP5/6 [22]. However, while the canonical WNT pathway has been studied thoroughly, non-canonical pathways are more complicated and less well understood. Current common knowledge suggests

WNT5a and WNT11 as representative ligands of the non-canonical WNT pathway, which could bind to different receptors to activate non-canonical WNT signaling, including frizzled receptors and a variety of co-receptors like orphan receptor 2 (ROR2) and receptor-like tyrosine kinase (RYK) [22] [23]. Currently, the most extensively studied non-canonical WNT pathways are divided into the planar cell polarity (PCP) pathway and calcium signaling pathway. The PCP pathway is found to work through the cascade of small GTPases RAC1 and RAS and downstream c-Jun N-terminal kinase (JNK) with the binding of FZD receptors and could regulate cytoskeletal rearrangements, cell motility and metastasis formation [24], whereas the WNT/calcium signaling pathway induces the release of intracellular calcium which can activate downstream kinases including protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase (CamKII) and the calcineurin-dependent transcriptional nuclear factor of activated T cells (NFAT) [25]. Besides, frizzled receptors were also explored in many studies and many FZD receptors such as FZD2, FZD6, FZD7, FZD8 and FZD10 were reported to regulate both canonical and non-canonical pathways. Each member of FZDs can interact with several different WNT proteins to activate either the canonical WNT/β-catenin or the non-canonical WNT/PCP and WNT/Ca^{2+} signaling pathways. However, FZD1, FZD4, FZD5 were found to have more important roles in canonical WNT signaling pathways in a variety of malignancies like breast cancer [26] and glioma [27].

1.3 Mechanisms of WNT pathways in tumorigenesis

The role of WNTs in neoplasm was first reported to induce mammary cancer in mouse models in the 1980s [28]. More and more studies found alterations of WNT signaling pathways to be involved in the tumorigenic processes of various malignancies, including breast cancer [29] [30], colon cancer [31] [32], melanoma [33] [34] and so on. Mutations concerning the canonical WNT pathway play the most prominent role in many cancers. Human tumor suppressor, adenomatous polyposis coli (*APC*) gene was discovered in colon cancer and found to be associated with oncogenic transcriptional

activator β-catenin [35]. Mutational APC can result in abnormal accumulation of β-catenin in nucleus and overactive TCF/ β-catenin signaling [31]. Other components of canonical pathway were also found mutated in different cancers, like *AXIN* mutations in hepatocellular cancers [36] and mutations of the gene encoding the co-receptor LRP5 in breast cancers [37]. The non-canonical WNT/Ca^{2+} pathway, which is crucial for cell adhesion and movement, was also linked to tumor development [38] [39]. WNT5a, a representative of the non-canonical WNT signaling pathway(s), was found to play different roles in different tumors. WNT5a could work as proto-oncogene in melanoma [40] and pancreatic cancer [41] and also can be a tumor suppressor in breast cancer [42] and colon cancer [43]. There are also finding that the WNT/Ca^{2+} pathway can inhibit canonical TCF/β-catenin signaling through activating TAK1/NLK (Transforming growth factor (TGF)-β-activated kinase 1 /Nemo-like kinase) mitogen-activated protein kinase pathway [44], while WNT5a can activate the canonical WNT pathway with the combination of Fzd4 [45], which indicates the dual effect of WNT ligands between WNT canonical and non-canonical pathways in different cancers. For the correlation between tumors and WNT/PCP signaling pathway, apart from the role of WNTs and FZDs in the tumorigenesis, other components of PCP pathway are also noticeable. Van Gogh-like 1(Vangl1), the human homolog of Drosophila PCP gene, was reported to promote the invasive capability by interacting with PKC in hepatocellular carcinoma [46] and colon cancer [47]. However, considering the fact that the effects of WNT ligands on cellular functions were diverse and complex, the function of WNT pathways on tumorigenesis is still ambiguous and only partially discovered.

As far as WNT signaling in the thymus and thymic epithelial tumors (TETs) is concerned, there are some interesting findings from studies in recent years. WNT signaling plays a key role in the development of the thymus, where expression of WNTs decreases during or after thymic involution [48] [49], and regulates T cell development and maturation in the thymus [50]. In the thymus, WNT4 ligand is expressed primarily in the cytosol of thymic epithelial cells and secreted depending on the WNT secretion

proteins, Porcupine O-acyltransferase (PORCN) and WNT ligand secretion (WLS) mediator [51] and could activate a highly complex signaling network via G-protein dependent Frizzled receptors [52]. In mice animal models, premature thymic involution is induced upon deregulation of WNT signaling [53]. Given these findings, it is not surprising that maintenance of thymic epithelial cells requires a correct regulation of WNT signaling, including diminished expression of WNT proteins or increased levels of WNT inhibitors, which is associated with the senescence of thymic epithelial cells [54] [55]. Understanding the WNT signaling mechanisms in regulating thymus organogenesis and maintenance of thymic epithelial might open new insights into thymic involution [52]. The effect of aberrant autocrine loops of the canonical and non-canonical WNT signalling is not only restricted to cancer cells, but dynamically interacts with the tumor microenvironment [56].

Against this background, I studied the autocrine loop of WNT4 secretion in TECs that were cultured in conventional 2-dimensional (2D) cell culture plates and as three-dimentional (3D) spheroids using extracellular matrix (ECM). In addition, I quantified - on the RNA level - the so far unknown expression of WNT ligands, their corresponding Frizzled receptors and their inhibitors across the histological spectrum of thymomas and TCs. 3D culture of primary cells isolated from fresh tissue has already been studied in various types of cancers, like breast cancer [57], gastrointestinal cancer [58] and organs of heart and liver [57, 59]. Compared to 2D cell culture, 3D culture could better mimic the in situ tumor and normal tissues microenvironment of cell-cell and cell-extracellular matrix (ECM) interactions. Up to now, a number of methods were tested to establish 3D spheres and most of them are using polymers as scaffolds, including collagen, hyaluronic, Matrigel and alginate [60, 61].

WNT endogenous inhibitors are separated into three types: membrane inhibitors like Secreted frizzled-related protein (sFRP1-5), Dickkopf-related protein (DKK1-4), cytosolic inhibitors like tissue inhibitors of metalloproteinase (TIMP1-3), and nuclear inhibitors including Transducin-like enhancer protein (TLE2,4) and Transcription

factor (TCF3,4) [62]. The involvement of these WNT inhibitors has already been reported in connection with thymic involution and senescence and various other tumors [54] [63] [64]. For example, TCF4 has been found to have multiple roles in the regulation of WNT pathway in different tumors, which could work as tumor repressor in colon cancers, breast cancer and other cancers [63] [65] [66].

Nevertheless, the entire spectrum of interacting signaling mechanisms that regulate thymic involution is still far from being understood [52]. Among the known required stimuli that help to maintain the functional integrity of the thymic stroma are Notch, BMP, NF-κB and WNT signaling as well as WNT inhibition, and there is some evidence that decreasing WNT signaling could contribute to thymic involution in the human thymus [14] [15] [62] [67] [68]. By contrast, it has remained unclear, whether WNT pathways, including WNT4 signaling, play a role in human TETs. WNT pathways have been investigated in various tissues and diseases for long time, but a comprehensive expression analysis of all WNT ligands and corresponding Frizzled receptors in human normal thymus and TETs has not been reported until now [69] [70] [71] [72]. In addition, endogenous WNT ligand expression in TETs and their function in the pathogenesis of TETs is largely unknown. Thus, comparison of expression of WNT ligands among different TETs as well as between TETs and normal thymuses across different age groups might have the potential to achieve a more direct and comprehensive idea of the role of WNT ligands in TETs.

1.4 Aims of the study

Against the open questions just described in relation to the role of WNT signaling in thymic epithelial tumors, the current project had the following three main objectives:

i) To determine the in situ mRNA expression levels of 15 WNT ligands, corresponding frizzled receptors (FZD1-9) and WNT endogenous inhibitors at different cellular levels (membrane, cytosolic and nuclear) in various

histological types of thymic epithelial tumors compared to non-neoplastic thymectomy specimens;

ii) To determine the impact of increased and decreased WNT4 secretion in various ex vivo derived thymoma (type AB and B3) derived primary thymoma epithelial cells (pTECs) on various cellular functions;

iii) To clarify whether activation of the non-canonical WNT4/PCP/JNK pathway in ex vivo established thymoma AB and B3 primary epithelial cells has an impact on the NF-κB and AKT signaling pathway.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 General Chemical and reagents

Reagents for protein associated experiments, including RIPA buffer (PierceTM Thermo scientific, USA), protease inhibitor and phosphatase inhibitor cocktail (ProteoBlock; Fermentas), Bradford assay reagent (Coomassie Plus, Thermo scientific, USA) and protein molecule weight ladder (Precision Plus protein dual color standards) was purchased from Bio-Rad company (USA). Stacking and separation gels were prepared with 30% acrylamide (Bio-Rad company, USA), pH8.8 0.5M Tris (Roth), pH6.8 1.5mM Tris (Roth), 10% SDS, 10% ammonium persulfate (APS) and Tetramethylethylendiamin (TEMED) (Thermo Fisher Scientific). NuPAGE MES SDS running buffer (20x), NuPAGE transfer buffer (20x) and filter paper, nitrocellulose membrane and SuperSignal West Dura Substrate were bought from Thermo fisher Scientific company. Commercial human recombinant WNT4 was bought from R&D system company. NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833)used for nuclear protein extraction was bought from Thermo fisher. RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) and Fast SYBR Green master mix used for PCR amplification measurement was bought from Applied Biosystems (Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) used for cell proliferation assay was purchased from Roth (Nr.4022.2). The Porcupine inhibitor IWP-3 for WNT secretion was obtained from Sigma-Aldrich (St. Louis, MO). The AKT and IKK inhibitors MK2206 and TPCA-1 (Selleckchem and Tocris, Germany) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis) with a final stock concentration of 10 mM, 15mM and 25 mM, respectively. Commercial human recombinant WNT4 was bought from R&D system company. Reagents applied for immunohistochemistry including peroxidase blocking solution (Aligent S202386), antibody diluent (Zytomed ZUCO-25-500) and specific secondary antibody (Dako

REAL ™ EnVision ™ /HRP, Rabbit/Mouse (ENV)). General reagents like Trichloroacetic acid and acetone were bought from Merck (Darmstadt, Germany) and Carl Roth company. Matrigel matrix (5ml) was bought from Corning company.

2.1.2 Antibodies

Antibodies to β-catenin (Cell Signaling Technology, D10A8), WNT4 (9HCLC, Thermo Fisher), GAPDH (EPR16891, Abcam, USA), phospho-AKT (s473, D9E, Cell signaling Technology) and NF-κB/p65 (D14E12, Cell Signaling Technology), phospho-IKKα/β (S176/180, 16A6), phospho-JUN antibodies (Sigma-Aldrich, St. Louis, MO), RAC1/2/3 antibody (Cell signaling Technology) and β-actin (Santa Cruz) were used for western blot analysis or immunohistochemistry. Peroxidase-conjugated secondary antibodies (Anti-Rabbit/Mouse) were from Cell signaling Technology Company.

2.1.3 Cell culture reagents

The common reagents used for cell lines culture including Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco^{TM} Thermofisher Scientific), which contains glucose, L-glutamine and pyruvate. Besides, other reagents like fetal bovine serum (FBS), penicillin-streptomycin solution (10,000 U/ml), and trypsin-EDTA (0.05% and 0.25%) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid (HEPES, 1M) were also bought from thermo fisher company. Phosphate Buffered Saline (PBS) was from Sigma-Aldrich (Dublin, Ireland) company. The Liberase™ TL (Thermolysin Low) used for primary cells isolation from tissues was bought from Roche, Germany. The special tissue plastic dishes were purchased from Becton & Dickinson, Heidelberg, Germany

2.1.4 Mammalian Cell lines

Cell lines including the thymic carcinoma cell line 1889c, the immortalized keratinocyte cell line HaCaT and human embryonic kidney cells HEK293T. WNT4-

overexpressing thymic epithelial cells (WNT4-TEP1) [73] were kindly provided for by Dr.Christian Kvell from Szentagothai Research Center, University of Pecs, Hungary. Primary thymic epithelial cells (pTECs) were isolated from fresh TETs through tissues cutting into small pieces and liberase II digestion.

2.1.5 Kits and columns

RevertAid First Strand cDNA Synthesis Kit	ThermoFisher, Lithuania
MicroAmp Fast 96-Well Reaction Plate	Thermo Fisher, China
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific, Germany
Amicon centrifugal filter	Sigma-Aldrich, St. Louis

ELISA assays for WNT4 measurement were purchased from Diagnostic System Laboratories (Webster, TX).

2.1.6 shRNA transfection

Two *WNT4* shRNA interference plasmids were constructed with the pU6-shRNA Vector targeting the following sequences GAAGAGGAAACTTAACCAC, GCAGACAAACCAAGAATGC. Type AB and B3 thymoma derived pTECs and TC cell line 1889c were transfected with *WNT4*-shRNA and control shRNA for 48h using lipofectamine 2000.

2.1.7 Instruments

Cell culture equipment including a laminar flow hood and a 37° C CO^2 water-jacketed incubator.

Centrifuger 5415 R	Eppendorf, Hamburg
Infinite M200 NanoQuant Microreader	Tecan, Austria
DMIRB Inverted Leica Modulation Contrast Microscope	Leica, Germany
Biorad Power PAC 300	Bio-Rad, Munich
Chemismart 5100 Fusion SL	Peqlab, Erlangen, Germany
Thermomixer compact	Eppendorf, Hamburg
Step One plus Real Time PCR System	Applied Biosystems, Germany

2.2 Methods

2.2.1 Sample collection

Characteristics of the patients and 86 biopsies of thymomas, thymic squamous cell carcinomas (TQSCCs) and normal thymuses are summarized in Table 1. All these tissues were collected from several hospitals, including Universitätsklinikum Mannheim, Robert-Bosch-Krankenhaus (Stuttgart), Thoraxklinik-Heidelberg, and Universitätsklinikum Regensburg and the histopathological features of all cases were centrally reviewed by Prof. Alexander Marx. Normal thymus samples were obtained from 6 children and 15 adults aged from 28 to 82 years after different cardiac surgery treatment like congenital heart disease or ischemic cardiopathy. Thymomas of different subtypes in WHO criteria and TQSCC [7] samples were collected and classified in to stage I-IV according to Masaoka-Koga method [74]. I will use TC instead of TQSCC in the thesis because our findings of carcinoma tissues may not apply to the 5% other carcinomas that are not TSQCCs.

The clinical data of the presence of myasthenia gravis complication was also was also taken into account in this study. Our study was approved by Mannheim local Ethics Committee (approval #2009-290N-MA/2010) and all involved patients signed the informed consents.

Table 1. Characteristics of the thymoma and thymic carcinoma [7] patients and tissues, and of adult and pediatric control non-neopalstic thymuses (NT) from cardiac surgery patients studied for WNT ligands, frizzled receptors and WNT inhibitors. Thymoma type A, AB, B1 B2, and B3 (WHO classification); TC: thymic carcinoma; MG+ (%): percentage of patients with Myasthenia gravis; stage, according to Masaoka-Koga [74]; CT: childhood thymuses; NT: non-neoplastic (adult) thymuses.

Diagnosis	N	Age range (y)	Sex (m:f)	Stage (I-IV)	MG+ (%)
Type A	9	36-81	4:5	I (n=4) II (n=5)	0
Type AB	25	26-77	15:10	I (n=18) II (n=7)	9 (40.9%)
Type B2	18	21-81	10:8	I (n=3) II (n=6) III (n=5) IV (n=4)	8 (44.4%)
Type B3	19	41-76	8:11	I (n=5) II (n=4) III (n=8) IV (n=2)	4 (21.05%)
TC	11	32-74	6:5	I (n=5) II (n=3) III (n=1) IV (n=2)	0
CT	6	0-10	4:2	---	---
NT	15	28-82	6:9	---	---

2.2.2 Primary thymic epithelial cells (pTECs) and cell lines

pTECs were prepared and cultured as described [13, 75]. Shortly, cell suspensions were prepared by liberase II digestion of tissue fragments, and grown at 37°C in a 5% CO2 incubator on uncoated 10cm tissue plastic dishes with RPMI 1640 with 25mM HEPES,

200mM L-Glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal bovine serum (FBS). The medium of pTECs culture was changed every 3-4 days depending on the cell growing. Epithelial cell content of the primary cell cultures was determined by anti-EpCam expression of immunofluorescence (clone 4G10 Abcam, Heidelberg, Germany) and flow cytometry [13]. The thymic carcinoma cell line 1889c was also cultured in the above RPMI 1640 medium as well as WNT4-overexpressed thymic epithelial cells (WNT4-TEP1) while DMEM replaced RPMI in cultures of the immortalized keratinocyte cell line, HaCaT and the 293T human embryonic kidney cells, HEK293.

Capsule tissues were removed from thymoma and normal thymuses and cut into small pieces in RPMI 1640 supplemented with Pen/Strep, HEPES, and 10% FBS, the thymocytes were released by crushing the pieces of tissue and passing through a 100 µm cell strainer in the medium and isolated using Ficoll density gradient centrifugation. Thymocytes were counted and shock frozen for later RNA isolation and designed cell culture with conditioned medium.

2.2.3 Conditioned medium collection

The protocol of conditioned medium collection has been performed in primary thymic epithelial cells derived from thymomas and normal thymuses. Supernatants were obtained from pTECs cultured within RPMI 1640 with 4.5 g/L, 25 mM HEPES, 200 mM L-Glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal bovine serum for 96h. All these supernatants were collected after centrifuging at 2000xg for 15min, and then stored at -20°C freezer. In order to minimize the effect of repeated freeze-thaw cycles, the collected medium was divided into aliquots. pTECs were cultured with normal culture medium or conditioned medium enriched media or 200 ng/ml recombinant human WNT4 (R&D system, 6076) supplement medium.

2.2.4 pTECs cultures in collected conditioned medium

Conditioned medium (CM) of supernatants from pTECs were thawed at 37°C and added to triplicate wells of cultured primary thymic epithelial normal and tumor cells at 25% v/v. pTECs were cultured in this collected medium and passaged as usual normal cell culture. Different passages of CM stimulated pTECs were harvested for cell proliferation assay and for western blot analysis.

Frozen preserved thymocytes derived from thymoma and normal thymus were also cultured in conditioned medium at 15% (v:v) for 10 days with the density of 10^5 cells/ml in uncoated round-bottom 96-well plate. The cell viability assay of these cultured thymocytes was detected every two days during the 10 days' culture and these suspension cells were also checked with flow cytometry to know the thymocytes what we are dealing with are mostly immature cells or expanded mature T cells.

2.2.5 Three-dimensional (3D) spheroid and organoid-like culture of pTECs

pTECs were isolated from fresh thymoma tissues as described in 2.2.1, then these cells were cultured in two different ways, one is 3D multicellular spheroid (MCS) model culturing 5000 pTECs/well 4 days in 48-well ultra-low attachment plates (ULA), the other is 3D organoid-like cell culture model which pTECs were embedded in 50ul Matrigel (Corning) as a scaffold. MCS are scaffold-free spheroid aggregates of in vitro cells that could mimic in vivo microenvironment [76]. Organoids are also using 3D culture of stem cells or tissues to establish in vitro tissue construct to mimic in vivo functions[77].

Fresh isolated primary thymoma epithelial cells were either grown in ultralow attachment plates (Corning, Germany) at 5.10^4 to 10^5 cell /ml in RPMI 1640 with 2 g/L glucose, 25 mM HEPES, 200 mM L-Glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% calf serum (Sigma Aldrich, Germany) or embedded in 200µl Matrigel (corning, Germany), dropped in prewarmed 6 Well plates in several 50µl Matrigel drops. The plates were inverted and stored by 37°C for 40min then fed with the MEBM medium (Lonza) supplemented with B27 supplement (Gibco), 0.5 µg/ml

hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 4 μg/ml heparin (Sigma), 20 ng/ml bFGF (Invitrogen), and 20 ng/ml EGF (Sigma) [78]. Multicellular Spheroids (MCS) and organoid-like were collected on day 7-12 of culture for RNA and proteins isolation. Imaging of 3D cultured was performed using an inverse microscope Zeiss Axio Observer Z1.

2.2.6 Cell viability and proliferation assay

Proliferation assays were performed on pTECs cultured with stimulation and control cells with the density of 10^4 cells per well for triplicate in a 96-well flat-bottom plate based on mitochondrial dehydrogenase activity as surrogate proliferation marker. After incubation with applied stimulus for desired time (usually 12-72 hours), MTT (dissolved in PBS at final concentration of 5mg/ml) was added to each well and incubated for 4 hours at 37°C when purple formazan crystals are observed in cells under microscope. Then DMSO was added to the wells after discarding the MTT mixture in the plate. After 30 minutes to 1 hour incubation until the colored crystals were dissolved, the 96-well plate was measured on a Tecan Infinite 200 microreader (Austria) with a wavelength of 560 nm and a reference wavelength of 670 nm. For suspension cells like thymocytes, the MTT reagent was added to the cultured cells with medium and incubated for 4 hours, then the mixture was centrifuged and the pellet was dissolved with DMSO. The viability of stimulated pTECs and thymocytes was calculated as the average of triplicate wells in independent experiments.

2.2.7 Transient transfection of cell line with plasmid DNA

Two WNT4 shRNA interference plasmids were constructed using the (pU6-shRNA Vector) targeting the following sequences GAAGAGGAAACTTAACCAC, GCAGACAAACCAAGAATGC. AB and B3 thymoma derived pTECs and 1889c cell lines were transfected by WNT4-shRNA and control shRNA for 48h using lipofectamine 2000 (Fischer Scientific, Germany). First, shRNA Plasmid DNA solution and diluted shRNA Plasmid Transfection Reagent were mixed gently and incubated 15-

45 minutes at room temperature. At the same time, cells which were cultured at 50-70% confluency in 6-well plate, were washed twice with 2 ml of shRNA Transfection Medium. Then add 200µl mixture of shRNA Plasmid DNA and shRNA Plasmid Transfection Reagent to well with 0.8 ml shRNA Plasmid Transfection Medium inside. Following with 5-7 hours incubation at 37°C in a CO2 incubator and then incubate with 1ml of normal growth medium containing 2 times the normal serum and antibiotics concentration for 48 hours.

2.2.8 RNA isolation, cDNA synthesis and real-time qRT-PCR

Cultured cells were washed with ice cold PBS and then lysed directly in the culture dish by adding 1 ml of TRIzol reagent (Invitrogen) and scraping with cell scraper. Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Collect these samples in new 1.5mL Eppendorf tubes Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 minutes. Centrifuge the samples at 12,000xg for 15 minutes at 4°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase, which contains RNA. Transfer upper aqueous phase into fresh tube. Precipitate the RNA from the aqueous phase by mixing with 0.5 ml isopropanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at room temperature for 10 minutes and centrifuge at 12,000xg for 10 minutes at 4°C. Remove the supernatant completely and wash the RNA pellet once with 1 ml 75% ethanol per 1 ml of TRIZOL Reagent. Mix the samples by vortexing and centrifuge at 7,500 x g for 5 minutes at 4°C. Air-dry RNA pellet for 5 minutes. Dissolve the pellet in 20µl DEPC-treated water and measure sample concentration in TeCan micro-reader machine with OD at 260 nm and 280 nm.

Total RNA was then reverse-transcribed with Thermo Scientific RevertAid RT Kit in our study, which used the both random and oligo dt primers scheme for initiating cDNA

synthesis. All the kit components were thawed on ice and the volumes of the different components (4µL 5xReaction Buffer, 2µL 10 mM dNTP Mix, 1µL RT Random Primers, 1µL ReverseAid RT (200 U/µL), 1µL RiboLock RNase Inhibitor (20 U/µL), Nuclease-free water) needed to prepare the required number of reactions were calculated. Then 500ng-1µg of RNA sample were added to each tube with certain volume reverse transcription reactions making the total volume to 20µL, loaded into PCR reaction tubes and transferred to a thermal cycler. The procedure included cycles of 5 min at 25°C, followed by 60 min at 42°C and terminate the reaction by heating at 70°C for 5 min. Then the reverse transcription reactions were stored in a -20°C freezer for further applications.

For relative mRNA quantification of genes analysis, we performed the qRT-PCR with the STEP one plus TaqMan PCR System using FAST SYBR Green master mix. For each patient sample, 1000µg total RNA was used for reverse transcription into cDNA. Then 10µL cDNA was applied to amplify each target gene. For the reactions (triplicates), 5µL of FAST SYBR Green master mix, 1µL of primer (Life Technology), 2µL of nuclease-free water and 2µL (5ng/reaction) of template cDNA from 10ug RNA were added into 96-well PCR plates (Life Technologies). The amplification profile included initial steps of 2 min at 50°C and 10 min at 95°C, then 40 cycles of 15 seconds (sec) at 95°C and 1 min 60°C. The fold change in expression was calculated using the ΔΔCt method with *GAPDH* and *Cytokeratin* 19 (*CK19*) as an internal control. Primer sequences are available in Table 2. The PCR data was analyzed with STEP One Plus software tool version 2.0.2 (Invitrogen, Germany).

To investigate the endogenous profiles of WNT ligands and FZD receptors gene expression 53 thymomas (9 A, 21AB, 13 B2, 10B3) and 10 TCs and 21 non-plastic thymuses (6 childhood and 15 normal aged ones) in vivo, total RNA was isolated from frozen preserved whole tissue samples (Table 1), reverse transcribed, and quantified using qRT-PCR. During the measurement, targeted genes expression was normalized

to the expression of housekeeping gene *CK19* as a surrogate marker of epithelial cell content for internal normalization.

Table 2: List of primer sequences for Real time PCR

Genes	Sequences 5‘-3‘
GAPDH	fwd GTCAGTGGTGGACCTGACCT rev TGCTGTAGCCAAATTCGTTG
CYTOKERATIN 19 (CK19)	fwd AATCCACCTCCACACTGACC rev TTTGAGACGGAACAGGCTCT
WNT1	fwd CGGCGTTTATCTTCGCTATC rev GCCTCGTTGTTGTGAAGGTT
WNT2	fwd GTGGATGCAAAGGAAAGGAA rev AGCCAGCATGTCCTGAGAGT
WNT3a	fwd GGAGAAGCGGAAGGAAAAATG rev GCACGTCGTAGATGCGAATACA
WNT4	fwd ACCTGGAAGTCATGGACTCG rev TCAGAGCATCCTGACCACTG
WNT5a	fwd GACACCCCATGGCACTTG rev AGGGCTCCTACGAGAGTG
WNT5b	fwd GTGCAGAGACCCGAGATGTT rev GTCTCTCGGCTGCCTATCTG
WNT7a	fwd GCTGCCTGGGCCACCTCTTTCTC rev GGAGGGTCCTTTTCCTCGGGT
WNT7b	fwd TCAACGAGTGCCAGTACCAG rev CCCTCGGCTTGGTTGTAGTA

WNT8b	fwd TCCCAGAAAAACTGAGGAAACTG rev AACCTCTGCCTCTAGGAACCAA
WNT9a	fwd GGGTGTGAAGGTGATCAAGG rev GCAAGCATCTGAAGCACAAG
WNT9b	Fwd TGCACCTGTGATGACTCTCC Rev CTGATACGCCATGGCACTTA
WNT10a	fwd GGCAACCCGTCAGTCTGTCT rev CATTCCCCACCTCCCATCT
WNT10b	fwd GAAAACCTGAAGCGGAAATG rev GGGTCTCGCTCACAGAAGTC
WNT11	fwd GGCTTGTGCTTTGCCTTCA rev TTTGATGTCCTGCCCTCCTT
WNT13	Fwd TGCCAAGGAGAAGAGCCTTAAG rev GTGCGACCACAGCGGTTATT
WNT15	fwd CAGGTGCTGAAACTGCGCTAT rev GCCCAAGGCCTCATTGGT
FZD1	fwd CAGCACTGACCAATGCCAAT rev CACCTTGTGAGCCGACCAA
FZD2	fwd TTTCTGGGCGAGCGTGAT rev AAACGCGTCTCCTCCTGTGA
FZD3	fwd GCTCGGTCATCAAGCAACAG rev ACGGTGTAGAGCACGGTCAAC
FZD4	fwd GGCGGCATGTGTCTTTCAGT rev GAATTTGCTGCAGTTCAGACTCT
FZD5	fwd CGCGAGCACAACCACATC

	rev AGAAGTAGACCAGGAGGAAGACG
FZD6	fwd CTGGGTTGGAAGCAAAAAGA
	rev CCATGGATTTGGAAATGACC
FZD7	fwd CAACGGCCTGATGTACTTTAAGG
	rev CATGTCCACCAGGTAGGTGAGA
FZD8	fwd TCTTGTCGCTCACATGGTTC
	rev GGTGCCGATGAAGAGGTAGA
FZD9	fwd CTTCTCCACCGCCTTCAC
	rev GAAACTACTGCCCAGCACC
FZD10	fwd AGCATCCCCAGAAAACTCAC
	rev AACACAACCAAGAAAAGCACC
VANGL1	fwd TTACCTCCGATCCTGTGGAG
	rev AACAAAAGGGCACGAAACAC
VANGL2	fwd CTCGGAGAGGAAAACAGCAC
	rev CAGCCGCTTAATGTGAGTGA
SFRP2	fwd GCCTCGATGACCTAGACGAG
	rev GATGCAAAGGTCGTTGTCCT
SFRP5	fwd TGGAGCCCAGAAAAAGAAGA
	rev GCAGGGGTAGGAGAACATGA
DKK1	fwd ATTCCAACGCTATCAAGA
	rev CCAAGGTGCTATGATCAT
DKK4	fwd AGCTCTGGTCCTGGACTTCA
	rev CAACCCACGACATGTAGCAC
TIMP1	fwd AATTCCGACCTCGTCATCAG
	rev TGCAGTTTTCCAGCAATGAG

TIMP2	fwd AAAGCGGTCAGTGAGAAGGA rev' CTTCTTTCCTCCAACGTCCA
TIMP3	fwd CTGACAGGTCGCGTCTATGA rev GGCGTAGTGTTTGGACTGGT
TLE2	fwd CCAGCGAGAAGACGGAAATG rev CAGACGCTTCACAATCTCCG
TLE4	fwd GTTTCCGAGGTGCTGAGAAG rev TAATCGGGGCATCTTTCTTG
TCF3	fwd CATCTGCATCCTCCTTCTCC rev GAGTAGATCGAGGCCAGTGC
TCF4	fwd ATGCTTCCATGTCCAGGTTC rev CACTCTGGGACGATTCCTGT

2.2.9 Protein isolation and measurement

Cultured cells were trypsinized and washed with PBS prior to resuspension in 300ul-500ul lysis buffer (10ul Protease and phosphatase inhibitors/ 1ml RIPA buffer) in a pre-cooled microcentrifuge tube. cells were maintained constant agitation for 30 min on ice and then centrifuged 20 min at 12,000 rpm in 4°C microcentrifuge. Finally, the supernatant was aspirated in a fresh tube and the pellet was discarded.

Cytosolic and nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents. Cells were harvested and washed twice with 1x PBS at 500xg for 5min, then certain ice-cold CER I reagent was added to the pellet and the mixture was vortexed vigorously at highest speed. After incubation on ice for 10 min, ice-cold CER II was added and centrifuge briefly for 16000xg for 5 min. Then the supernatant (cytoplasmic extract) was transferred into a new tube and the insoluble fraction was suspended with ice-cold NER followed by vortex every 10min and incubation on ice

for a total of 40 min. The supernatant (nuclear protein) was collected with centrifuge at 16000xg for 10 min. After the protein isolation, samples were frozen at -80°C.

The collected protein was then measured through Bradford assay in the Tecan machine, which 2ul protein sample with 18ul RIPA buffer was prepared and 5ul of the mixture was pipetted in each well (triplicate wells) with 250ul Bradford reagent (protect from light) in a 96-well flat plate and Bovine serum albumin (BSA) is a frequently used protein standard. For the use of western blot, the protein samples need to be denatured with 5ul Loading buffer (2-mercaptoethanol/sample buffer (1:9)) to each sample and boiling at 95°C for 5 min.

2.2.10 Proteins from cells supernatant and Enzyme-linked immunosorbent assay (ELISA)

WNT4 protein secretion was measured in cultured pTECs supernatants derived from different 4 days old thymoma and 7 days old NT samples using ELISA method. These supernatant samples were collected from 18 thymomas (8 AB, 3 B2, 7 B3) derived pTECs cultured for 4 days, from 6 samples of B3 thymomas derived pTECs cultured for 10 to 20 days which were also called senescent pTECs (sen) and from 3 NT derived pTECs cultured for 7 days. In addition, supernatant from WNT4-overexpressing murine thymic epithelial cells (WNT4-TECs) were used as positive control group (WNT4-TEP1), which could secrete WNT4 protein in the supernatant due to the transfection of human WNT4 expression vector [79]. To quantify these WNT4 protein secretion, the supernatants either passing the supernatants through the YM10 membrane filters for concentrating sample or processed as following protocol: Trichloroacetic acid 20% (TCA, Merck, Darmstadt, Germany) was added to cell culture supernatant at 1:1 (v.v). The supernatants were incubated on ice for 20 min and then centrifuged at 10,000 x g for 5 min. The protein pellets were washed twice with 0.5 ml of cold 100% acetone, air dried, resuspended in protein loading buffer (2x Laemmli buffer) and stored at -80°C for further protein analysis.

ELISA assays to detect WNT4 were performed after the manufacturer´s instructions. Briefly, the monoclonal antibody for WNT4 was firstly pre-coated onto a 96-well microplate. Standards and samples were then pipetted into the wells which could bind to the immobilized WNT4 antibody. After washing with 1xPBS, an enzyme-linked polyclonal antibody specific for primary WNT4 antibody was added to the wells and the substrate solution is further added to the wells to develop color, which is proportional to the amount of WNT4 bound in the wells. Then the color development is stopped with stop solution and the intensity of the color is measured in microplate reader (Tecan Infinite M200 NanoQuant). All protein lysate samples from cells and supernatants were assayed in triplicates. The mean of the three values was used for further statistical evaluation.

After 3-day treatment of pTECs with IWP3 inhibitor, supernatants were collected from these treated short term cultured pTECs from 7 thymomas (3AB, 1B2, and 4B3), 6 senescent long-term cultured pTECs from 6 thymomas (3AB and 3B3) and 3 NT derived pTECs and used for WNT4 secretion quantification using ELISA method.

2.2.11 Immunoprecipitation and Western Blot

WNT4 quantification were analyzed in different supernatant samples through immunoprecipitation and western blot in two B3 thymoma and one AB thymoma cases of supernatants collected after short-term (4-day, 4D) pTECs culture and long-term (10- or 12-day, 10D, 12D) senescent pTECs culture either with or without treatment of the WNT secretion inhibitor, IWP3. Supernatants from WNT4-TEP1 cells were collected and served as positive control. WNT4 immunoprecipitation was performed after concentration of cell culture supernatants for macromolecular components purification. For this purpose, the samples were processed as followings: 5X 1ml of Trichloroacetic acid 20% (TCA, Merck, Darmstadt, Germany) was added to 1ml of cell culture supernatants at 1:1 (v.v), incubated on ice for 20 min and then centrifuged at 10,000 g for 5 min. The protein pellets were washed twice with 0.5 ml of cold 100% acetone (Carl Roth), air dried and resuspended in either IP Lysis buffer (Pierce, Thermo Fisher

Scientific, Germany) or protein loading buffer (2x Laemmli buffer) and stored at -80°C for further protein analysis.

Precipitated protein was followed by western blot using standard protocols as described previously [80]: Briefly 20 µg of protein lysates extracted from cultured cells were loaded onto SDS-PAGE gels and separated by 10% (w/v) SDS-polyacrylamide electrophoresis, followed by protein transfer onto nitrocellulose membranes. The membranes were blocked for 1 h in blocking buffer (5% (w/v) nonfat milk in 1xTris-buffered saline/0.1% Tween® 20 Detergent (TBST)) and incubated with different primary monoclonal antibodies like WNT4, ß-actin and other antibodies at 4°C overnight, and washed with 1xTBST 3 times/10 min and re-probed with the horseradish peroxidase-conjugated secondary antibody. Protein binding results was visualized with chemiluminescence detection kit and read out with the Chemismart 5100.

2.2.12 Immunohistochemistry of patients' tissues

Protein expression of β-catenin was performed in 6 type B3 thymomas and 9 TCs samples by immunohistochemistry using a rabbit monoclonal antibody against human β-catenin. Formalin-fixed paraffin-embedded sections (1µm) from whole tissue were deparaffinized in three Xylene baths and rehydrated in graded ethanol concentrations. Antigen retrieval was performed with epitope retrieval solution, pH 6.0, for 40 minutes under microwave and then allowed to cool down at room temperature for other 30 minutes. Endogenous peroxidase was blocked with peroxidase blocking solution for 7 minutes. Sections were then incubated with a 1:50 dilution in Antibody Diluent of the β-catenin antibody. Negative control were only incubated with PBS. After rinsing with 1xTBS two times, the samples were applied with goat anti-mouse and goat anti-rabbit secondary antibody, conjugated with peroxidase for 30 minutes (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV)). The staining was visualized with 3, 3'-diaminobenzidine (DAB) as chromogen and samples were counterstained

with hematoxylin, dehydrated and finally mounted. Sections of desmoid tumors with CTNNB1 mutations and nuclear β-catenin protein expression served as positive control.

2.2.13 Data and Statistical Analysis

All statistical analyses were performed with GraphPad Prism V6.0 software (GraphPad Software Inc, La Jolla, USA). qRT-PCR data were analyzed with the STEP One Plus software tool. One-way ANOVA test was used with a confidence level of 95% ($p<0.05$ was considered significant) when comparing WNT ligands and frizzled receptors gene expression levels between thymomas and normal thymuses and childhood thymuses. WNT inhibitors gene expression in different groups of TETs and normal thymuses were also compared through one-way ANOVA test. A subsequent Tukey's multiple comparisons test was used to compare variances with a confidence level of 95% ($p<0.05$ was considered as significant).

3. RESULTS

3.1 Expression of WNTs is heterogeneous in normal and neoplastic thymic tissues

WNT ligands were differentially expressed in childhood thymuses, adult normal thymuses and TETs are shown in Figure 1. In particular, most WNT genes were found to be expressed heterogeneously in TETs compared to non-neoplastic thymuses, however without a trend in one direction. Accordingly, a significant higher expression of some WNT genes was observed among type B3 thymomas as well as TCs compared to normal thymuses ($p<0.0001$ and $p=0.0015$) and childhood thymuses ($p<0.0001$ and $p=0.049$) (Fig2A, B, F and G). However, typical genes relevant for WNT non-canonical pathways, such as *WNT4*, *WNT5a* and *WNT7a* showed higher expression in childhood thymuses (CT) compared to normal adult thymuses and indolent thymoma subtypes A and AB. Especially *WNT4* showed a significant decrease expression in aged thymuses compared to childhood thymuses. In addition, some WNT genes were found to be expressed at relatively higher levels in non-neoplastic thymus tissues (NT) compared to childhood thymuses, like *WNT2* and *WNT5b*, whereas *WNT11* had the highest expressed mRNA levels in NT (Fig2A and B). Furthermore, expression of some WNT genes (*WNT5a* and *WNT11*) appears to be functionally relevant in childhood thymuses and NT, since they showed lower expression in TETs (Fig 2). When comparing the distribution pattern of these genes among TETs, a correlation between WNTs gene expression profiles and the histotypes according to the WHO classification were observed. For example, type A thymoma showed elevated expression in canonical pathway gene, like *WNT1* and *WNT3a*, while expression levels of these genes were lower in type AB and B2 thymomas (Fig2C, D, E). In contrast, some WNT genes like *WNT2*, which is poorly expressed in type A thymoma, was shown at significantly enhanced levels in type AB and B2 thymomas (Fig2C, D, E). Therefore, it appears that type AB thymomas combine features of type A and B2 thymomas, which may be associated with the cellular composition of type AB thymomas that share spindle cell features with type A thymomas and many immature thymocytes with type B2

thymomas (Fig2D). In B2 thymoma WNT genes were generally expressed at lower levels than in other thymomas and normal thymuses, except for *WNT2*, which showed the highest expression in type B2 thymomas (Fig 2E).

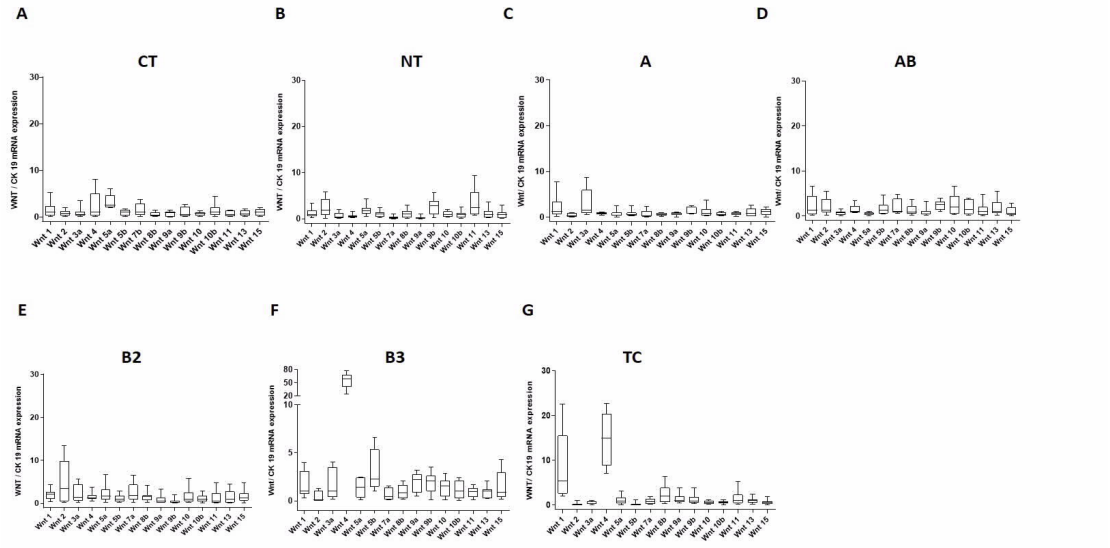


Figure 2: WNT ligands mRNA expression analysis.

mRNA expression levels of 15 WNT ligands measured by qRT-PCR in 21 non-neoplastic thymuses and 63 thymic epithelial tumors: A: Childhood thymuses (CT) (n=6), B: Non-neoplastic thymuses (NT) (n=15). C: type A thymoma (n=9). D: type AB thymoma (n= 21). E: type B2 thymoma (n=13). F: type B3 thymoma (n=10) and G: thymic carcinoma (n=10). Cytokeratin 19 was used as reference to take the highly variable content of non-neoplastic thymocytes in thymoma tissues and WNT-producing thymic epithelial cells in the various thymomas into account.

3.2 Type B3 thymomas and TCs show increased *WNT4* expression compared to type A and AB thymomas and non-neoplastic thymuses in vivo

From the characterization of endogenous profiles of all WNT genes in TETs and NTs ex vivo, significantly higher expression levels of *WNT4* mRNA were found in the most aggressive TETs, i.e. type B3 thymomas (55.32 +/-6.17, n=10, p=0.002) and TCs (23.58 +/-2.60 (n= 10), $p < 0.0001$) compared to other investigated TETs and NTs (Fig3 and Table 3). Furthermore, *WNT4* mRNA was more highly expressed in childhood thymuses (CT) (2.43 +/-1.02, age 0-10 years, n=6) than in the non-neoplastic adult thymuses (NT) (0.54 +/-0.08, age 18 - 78, n=15, p=0.007) (Fig3 and Table 3). The result that *WNT4* expression decreases in NT is in consistent with previously published data that suggest the decline of *WNT4* is a critical regulatory mechanism during thymic

involution and senescence [81]. Lower expression levels of *WNT4* gene were also found in type A thymomas (0.83 +/-0.10, n=9) while slight increase in type AB (1.53 +/-0.30, n=21, p<0.0001) and type B2 (1.51 +/-0.23, n=13, p=0.0016) thymomas were observed when compared to NT (Fig3 and Table3). The *WNT1* gene might also play an important role in thymic tumor development with higher expression levels in TCs compared to childhood thymuses and NTs (p<0.5, p<0.001) (Fig3A). By contrast, *WNT5b* showed relatively lower gene expression in TCs compared to NTs and thymoma samples (Fig3C).

WNT4 gene expression were analyzed in 21 normal thymuses with three different age groups, including decade I with 1 to 10 years old childhood thymuses (n=6), decade II with 18 to 31 years old NT (n=5) and decade III with 35 to 76 years old NT (n=10) (Fig4A). *WNT4* mRNA was highly expressed in the childhood thymus group compared to the two adult groups. Interestingly, while *WNT4* mRNA expression levels decreased during aging in normal thymuses, which was not the case in TETs (Fig4B and C). Accordingly, *WNT4* gene expression levels were negatively correlated with age in non-neoplastic thymuses (Pearson test: r=0.6718, p=0.0167 (Fig4B), while no significant correlation was found in type B3 thymomas and TCs (Fig 4C).

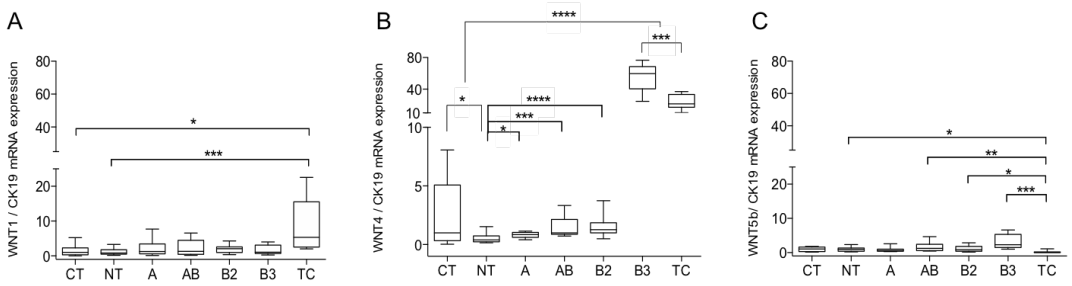


Figure 3 : *WNT1*, *WNT4* and *WNT5b* gene expression in normal thymuses and different thymic tumor subtypes.

Gene expression of WNT1 and WNT4 measured by qRT-PCR in all thymic tissues are shown in box plots using GraphPad software. A: *WNT1* shows higher expression in TC compared to childhood thymuses (CT) and non-neoplastic thymuses (NT); B: *WNT4* shows highest gene expression in malignant type B3 thymomas and TCs compared to childhood thymuses and NT samples. C: *WNT5b* shows lower gene expression in TCs than NT. **p=0.0070, *** p<0.001, **** p<0.0001. *Cytokeratin 19* was used as reference to take only the epithelial cell content into account.

A

TETs vs. childhood thymuses	p-values	Significance
A	0.0494	*
AB	<0.0001	****
B2	0.007	***
B3	0.0021	**
TC	<0.0001	****

B

TETs vs. adult normal thymuses	p-values	Significance
A	0.5270	ns
AB	<0.0001	****
B2	0.0016	**
B3	0.0021	****
TC	<0.0001	****

Table 3: Statistical data of *WNT4* gene in TETs compared to childhood thymus and normal thymus.

Expression of the *WNT4* gene in thymic epithelial tumors (TETs), including thymomas and thymic carcinomas, in comparison to normal childhood thymuses (A, n=6) and non-neoplastic adult thymuses (B, n=15) as represented in Fig2 (two-tailed student t-test).

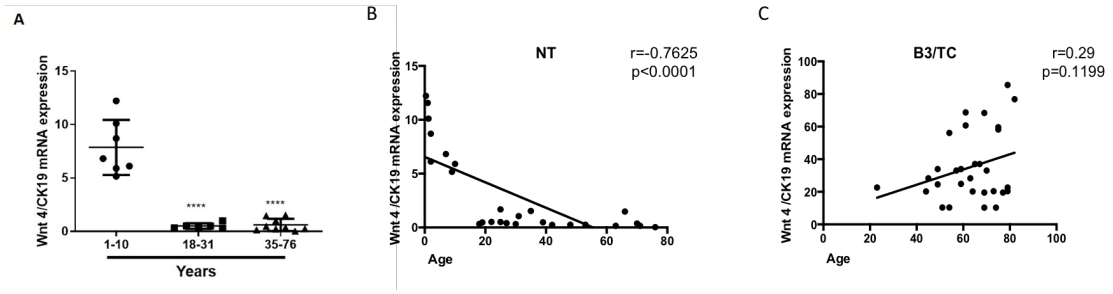


Figure 4: *WNT4* mRNA expression analysis in normal thymuses and the correlation between gene expression levels and age in non-neoplastic thymuses (NT) and thymic epithelial tumors.

WNT4 was measured by qRT-PCR in 24 NT samples of different ages: Decade 1 with 1 to 10 year-old childhood thymuses (n=6), decade 2 with 18 to 31 year-old NT (n=5), decade 3 with 35 to 76 year-old NT (n= 10) (A). Correlation between *WNT4* expression levels and different ages in NT (B) and in type B3 thymomas (B3) and thymic carcinomas [7] (C). Pearson's correlation (r) values are indicated within each graph. *WNT4* levels were normalized to expression levels of *Cytokeratin 19* as surrogate marker of epithelial cell content (**** p<0.0001).

3.3 Type B3 thymomas and TCs show increased frizzled receptor 6 (*FZD6*) expression

In order to have a more comprehensive understanding of the WNT signaling pathway in TETs, levels of WNT related receptors such as frizzled receptors and LRP receptors, were also investigated to get a clue to differences between thymomas and NTs using qRT-PCR method. FZD receptor mRNA levels were normalized to *CK19* to take the

highly variable content of non-neoplastic thymocytes in thymoma tissues into account. (Fig5).

Expression of FZD receptors in terms of mRNA was heterogonous and exhibited high variability even among cases of the same histotype. In childhood thymuses, none of these receptors (*FZD1-8*, *FZD10*) showed any significant difference in expression levels compared to *FZD9* with relatively low expression. By contrast, the fold-change of *FZD6* were highest in the aggressive type B2 and B3 thymomas and TCs (Fig6B), while *FZD1* was significantly increase compared to NT only in type B3 thymomas and TCs (Fig6A). All investigated FZD receptors showed lower mRNA levels in non-neoplastic thymuses and indolent thymomas compared to aggressive type B3 thymomas and TCs. In addition, *FZD2*, *FZD9* and *FZD10* were expressed at low levels in all thymic tissues. *FZD6* mRNA expression in NTs and different TETs subtypes is summarized in Figure 6.

While *WNT4* gene expression was higher in childhood thymuses than NT (see above), mRNA levels of its receptor, *FZD6* was higher in the adult NT group compared to the childhood thymuses group. Therefore, the expression levels of *FZD6* receptor and *WNT4* genes showed a negative correlation in B3 thymomas (Pearson test: r=0.6718, p=0.0167), while there's no significant correlation in NT samples. (Fig6, Fig7).

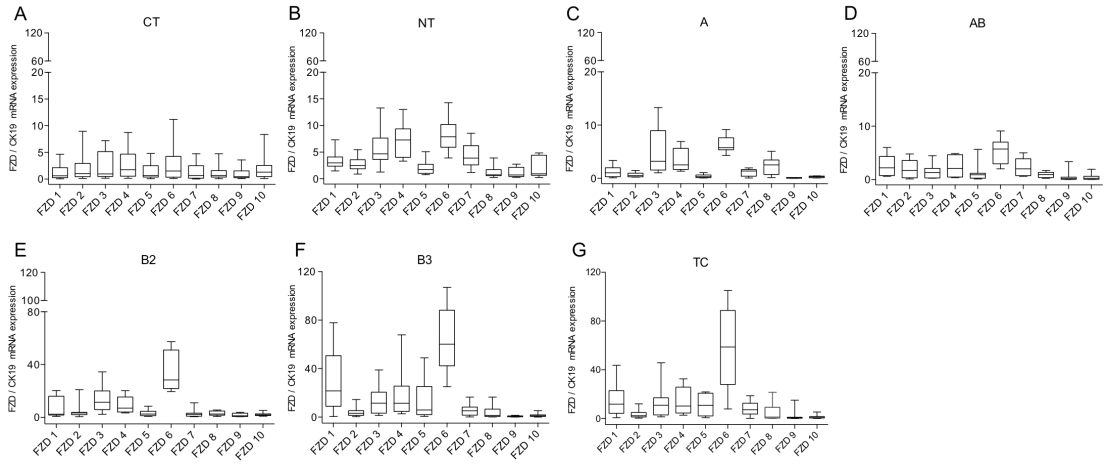
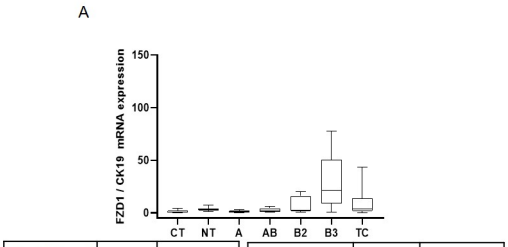


Figure 5: Frizzled receptor expression in normal thymuses and thymic tumors.

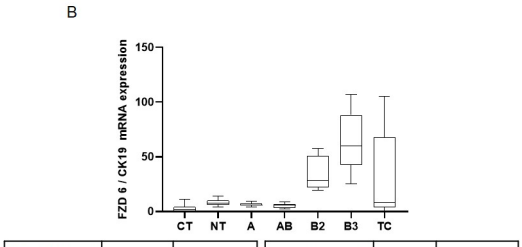
Total RNAs from frozen whole tissue samples were screened by qRT-PCR for frizzled receptor expression levels. After normalization to *CK19* expression, the relative expression of the frizzled receptors compared to the lowest expressed *FZD9* were depicted as box plots for each subtype. (A)1 to

10 year-old childhood thymuses (CT, n=6). B: adult non-neoplastic thymuses (NT, n=15). C: thymoma type A (n=9). D: thymoma type AB (n=23). E: thymoma type B2 (n=18). F: thymoma type 3 (n=18) and G: thymic carcinoma [7] (n=11). *Cytokeratin 19* was used as endogenous control of epithelial cell content.



Vs childhood thymuses	p-values	Significance
A	0.0001	ns
AB	0.6999	ns
B2	0.0689	ns
B3	0.0001	***
TC	0,999	****

Vs adult normal thymuses	p-values	Significance
A	0.999	ns
AB	0.9747	ns
B2	0.0915	ns
B3	<0.0001	****
TC	0.0059	**



Vs childhood thymuses	p-values	Significance
A	0.7567	ns
AB	0.8283	ns
B2	<0.0001	****
B3	<0.0001	****
TC	<0.0001	****

Vs adult normal thymuses	p-values	Significance
A	0.9915	ns
AB	0.8743	ns
B2	<o.0001	****
B3	<0.0001	****
TC	<0.0001	****

Figure 6: Representative examples of *FZD1* and *FZD6* mRNA quantification in normal thymuses and different thymic tumor subtypes.

Gene expression of *FZD1* and *FZD6* measured in all thymic tissues were shown in box plots with GraphPad software. A: *FZD1* shows higher expression in B3 thymoma and TC compared to childhood thymuses (CT) and adult normal thymuses (NT); B: *FZD6* shows higher gene expression in B2 thymoma and malignant B3 thymoma, TC compared to childhood thymuses and NT samples. The tables represent the statistical p-values and the significance. ns: no significance. *Cytokeratin 19* mRNA levels as endogenous control of epithelial cell contents were used for normalization.

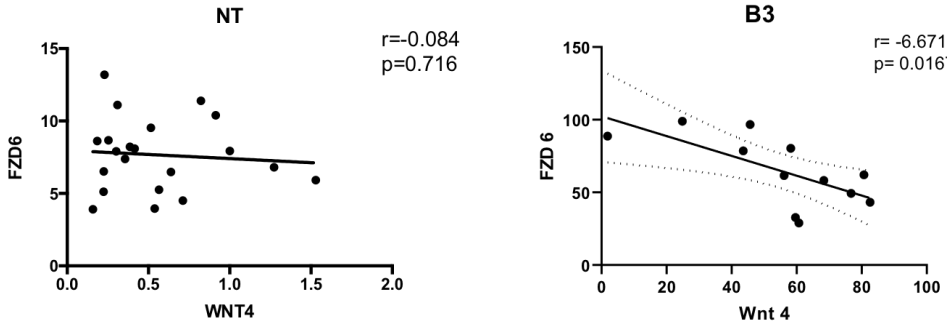


Figure 7: Correlation between expression levels of Frizzled receptor 6 and WNT4 in normal thymus and type B3 thymoma.

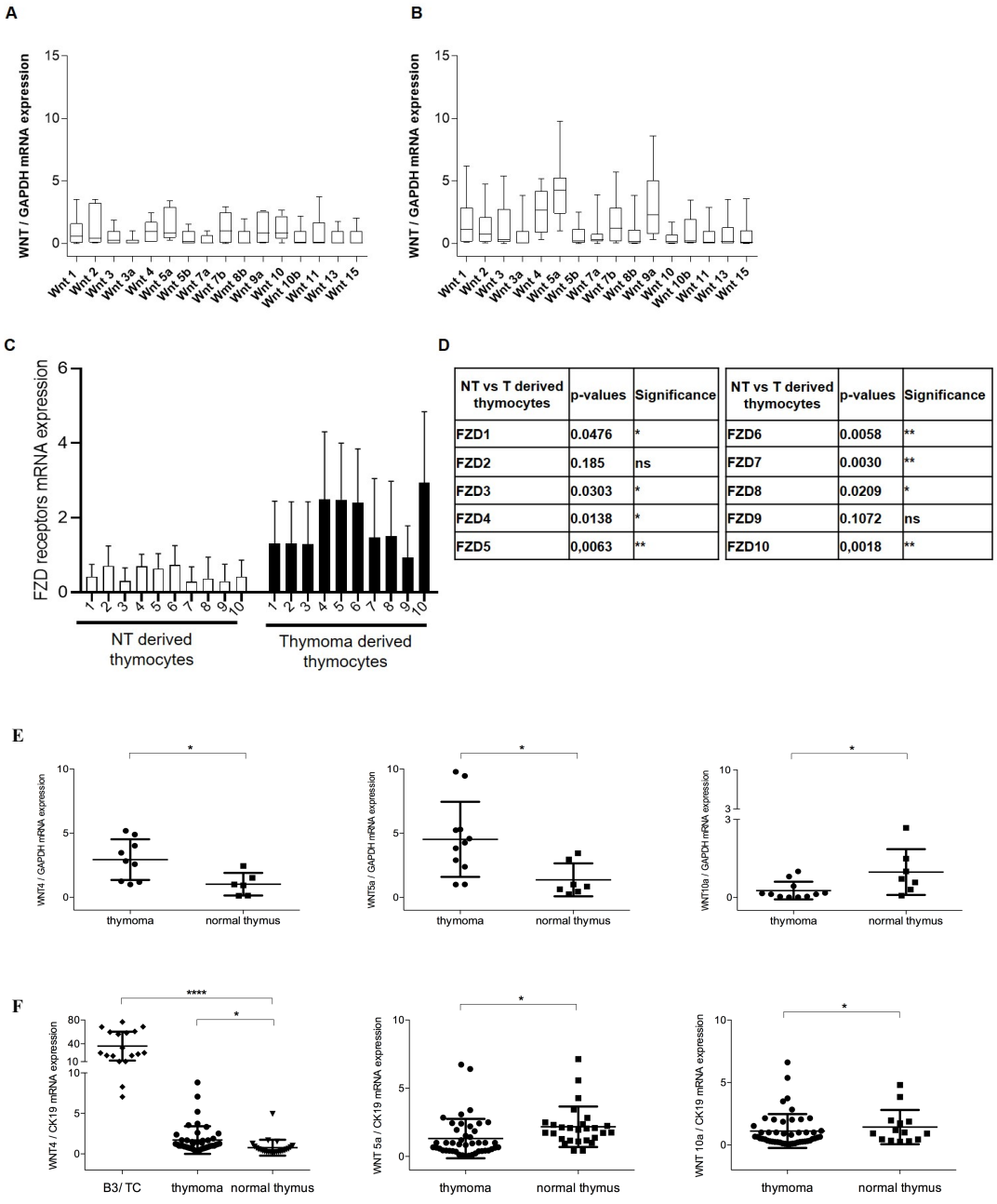
mRNA expression values of *FZD6* and *WNT4* in normal thymuses and B3 thymoma tissues measured by qRT-PCR. Pearson's correlation (r) values are indicated within the graph. *Cytokeratin 19* mRNA levels as surrogate markers of epithelial cell contents were used for normalization.

3.4 Expression of WNT ligands and frizzled receptors in thymocytes derived from thymic tumors

Considering the fact that WNT signaling pathway is also tightly involved in T cell development, expression of 17 endogenous WNT ligands and 10 FZD receptors were

quantified in thymocytes that were isolated from normal thymuses (n=8) and thymomas (N=11; AB, n=5; B2, n=3; B3, n=3) by density gradient centrifugation and qRT-PCR method (Fig 8A, B and C). At the mRNA level, three WNT ligands were found to have significantly different expression levels in thymocytes derived from NT as compared to thymocytes derived from various thymomas: *WNT4* (p=0.0193) and *WNT5a* (p=0.0169) expression levels were significantly higher in thymocytes derived from thymomas compared to NT thymocytes, while *WNT10a* levels were significantly lower (p=0.0264) in thymoma-derived thymocytes compared to NT samples. All FZD receptors showed significant higher expression in thymoma derived thymocytes compared to NT thymocytes, except *FZD2* and *FZD9* (Fig 8C, D).

Compared to the corresponding WNT ligand expression in frozen preserved whole thymic tissues, these three WNT genes showed different expression in thymocytes (Fig8E). The expression of *WNT4* in thymocytes as well as in whole tissue extracts showed significantly higher levels in thymoma compared to NT (p=0.0193, p=0.0154) (Fig 8E, F). By contrast, *WNT5a* was significantly (p=0.0169) more highly expressed in thymoma derived thymocytes (4.52 +/-2.92, n=11) compared to NT derived thymocytes (1.37 +/- 1.28, n=7) (Fig 8E). *WNT5a* showed lower expression levels in in whole tissue extracts thymomas (1.31+/-1.44, n=43) compared to NT (2.18+/-.48, n=21) (Fig 8F). Finally, *WNT10a* levels showed lower level expression of thymoma thymocytes than NTs (p=0.0264) as well as lower expression in thymoma whole tissue extracts (p=0.0333) to normal thymuses (Fig 8E, F).



NT vs T derived thymocytes	p-values	Significance
FZD1	0.0476	*
FZD2	0.185	ns
FZD3	0.0303	*
FZD4	0.0138	*
FZD5	0,0063	**

NT vs T derived thymocytes	p-values	Significance
FZD6	0.0058	**
FZD7	0.0030	**
FZD8	0.0209	*
FZD9	0.1072	ns
FZD10	0,0018	**

Figure 8: Expression levels of WNT ligands and WNT frizzled receptors in thymocytes isolated from thymic tumors and non-plastic thymuses (NT).

Expression of 17 WNT ligands and FZD receptors measured by qRT-PCR in thymocytes from NT (n=8) (A) and type AB, B2 and B3 thymomas (n=11) (B). (C): Expression analysis of FZD receptors in thymocytes derived from NT (n=8) and thymoma (n=11, AB, B2 and B3) derived (D): P-values and significances derived from the comparison of all mRNA expression levels of each FZD receptor in the NT group versus the thymoma group (Student t-test). (E): Scatter plot depicting significantly different expression of *WNT4*, *WNT5a* and *WNT10a* genes in thymocytes of NT and thymoma for. (F) Scatter plot depicting the expression levels of the respective WNT genes in whole tissue extracts. Statistical significance was assessed using the unpaired two-tailed t-test. Thymocytes were isolated from fresh

whole tissues by density gradient centrifugation. *GAPDH* was used as endogen reference for qRT-PCR analysis. ns: no significance.

3.5 Increased nuclear gene expression of the WNT inhibitors and accumulation cytosolic β-catenin in type B3 thymomas and TCs

Expression levels of selected endogenous WNT inhibitors acting on all three levels of the WNT signaling pathway (membrane, cytosol and nuclear) were investigated in our different snap-frozen cohorts of childhood thymuses, NTs, and TETs by qRT-PCR. WNT inhibitors were selected through literature mining which the schematic graph of WNT pathway and its endogenous inhibitors (Figure 9) [62]. Besides, expression of WNT inhibitors genes in TETs was also investigated in the Cancer Genome Atlas (TCGA) "Thymoma" database [82](Fig 10). The heatmap of the TCGA data shows that the membrane inhibitors, *SFRP2/5* and *DKK3/4*, the cytosolic inhibitors, *TIMP1/2/3* and the nuclear inhibitors *TLE4* and *TCF3* were more strongly expressed in indolent than aggressive TETs, while *TLE2* showed no differential expression (Fig10).

In our cohorts (7 normal thymuses and 71 TETs, including 11 TCs), mRNA levels of the membrane inhibitor *SFRP2* showed higher expression in B2 thymomas compared to NT ($p<0.0001$) and *SFRP5* is also representative in type B2 thymoma but not significant. Besides, *DKK4* levels were higher in type AB thymomas compared to other TETs and NTs. The results were different from those of the TCGA (Fig10, 11). All cytosolic inhibitors, *TIMP1*, 2 and 3 were expressed at low levels and without significant difference between TET histological subtypes (Fig11). As for nuclear WNT inhibitors, *TLE2* and 4, as well as *TCF4,* were significantly increased in type B3 thymomas and TCs as compared to NT ($p<0.01$), while *TCF3* showed no differential expression between NTs and TETs (except for AB thymomas) (Fig11). Of note, the higher expression levels of nuclear WNT inhibitors, *TLE2*, *TLE4* and *TCF4* in type B3 thymomas and TCs were accompanied by increased expression levels of *WNT4* and its receptor, *FZD6* in B3 thymomas and TCs as reported above paragraph 3.2 and 3.3.

To get a clue, whether the observed expression of WNT ligands and WNT inhibitors might collectively induce activation of the canonical WNT pathway, we next analyzed the expression of β-catenin protein by immunohistochemistry in type B3 thymomas (n=6) and TCs (n=9) (Fig 12), using β-catenin-mutated desmoid fibromatosis as positive control for nuclear β-catenin staining (Fig12A and B). β-catenin expression was detected in the cytosol of 50% of type B3 thymomas (Fig 12C) and of all TCs (Fig12D), while nuclear staining was absent.

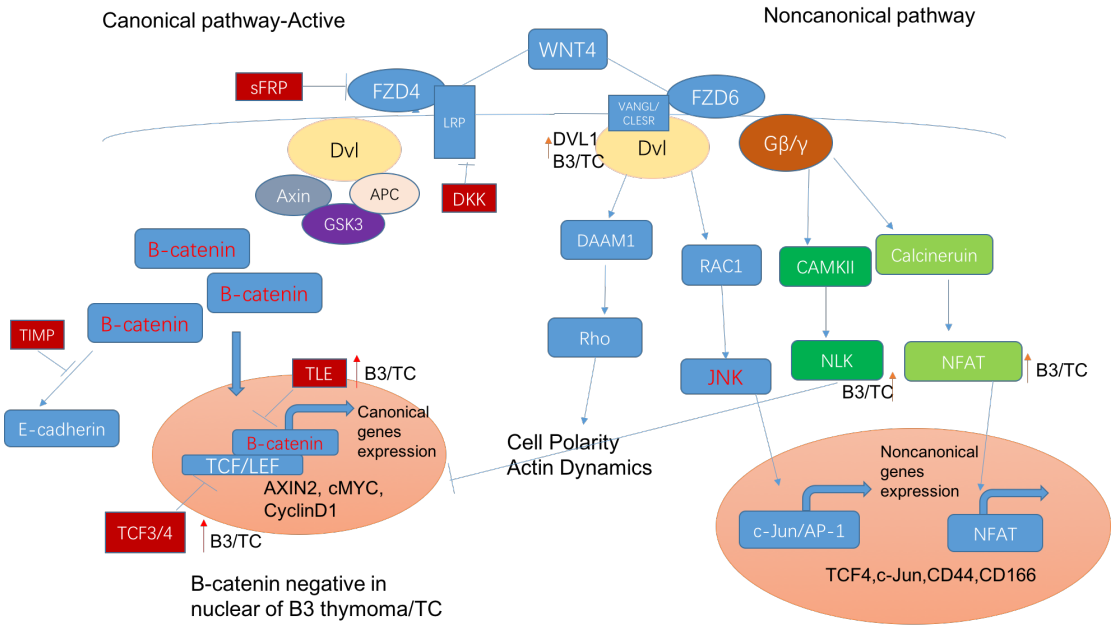


Figure 9: Scheme of the WNT pathway.

In this model of WNT4 induced signaling, canonical and non-canonical WNT pathways are both involved through activation of corresponding WNT receptors, *FZD4* and *FZD6*. Endogenous WNT inhibitors including membrane-associated inhibitors (sFRPs, DKKs), cytosolic inhibitors (TIMPs) and nuclear inhibitors (TLEs, TCFs) are marked in red. *TCF/LEF* gene family members are downstream targets of the canonical WNT pathway that depends on activated β-catenin. Downstream targets of the activated non-canonical WNT pathway are genes like *c-JUN* and *CD44* through JNK/cJUN and calcium signaling.

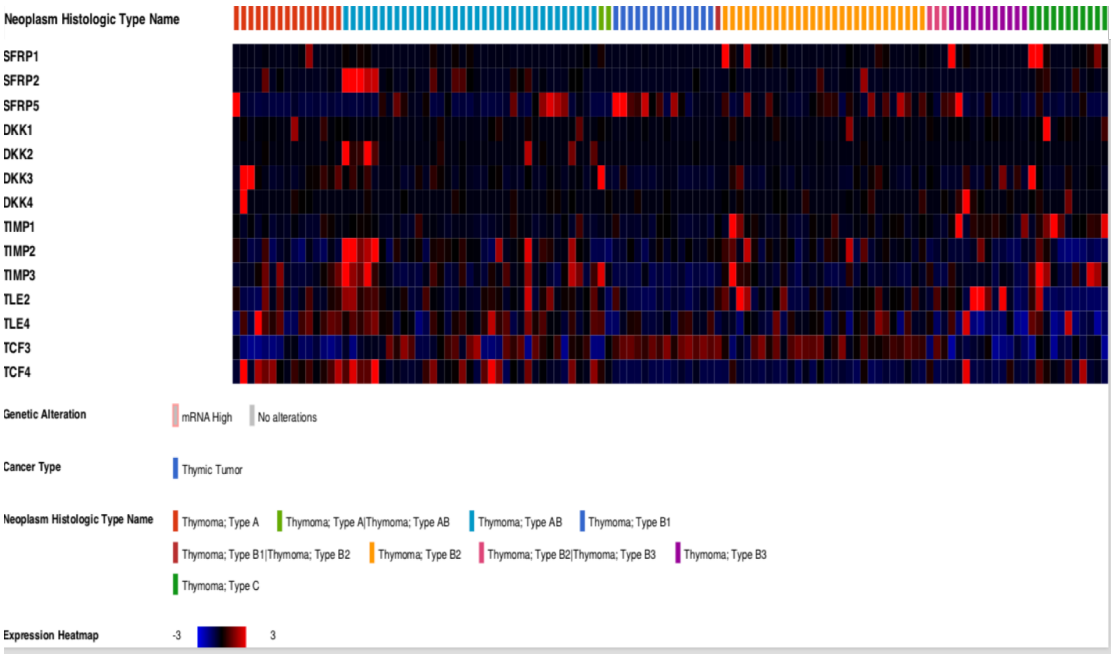


Figure 10: Heatmap of RNA expression levels of endogenous WNT inhibitors in thymic epithelial tumors of the TCGA cohort (n=115, TCGA PanCancer Atlas).

The heatmap shows expression data of type A, AB, B1, B2, B3 thymomas and thymic carcinomas ("Thymoma Type C"). Upregulated expression (Red), downregulated expression (Blue).

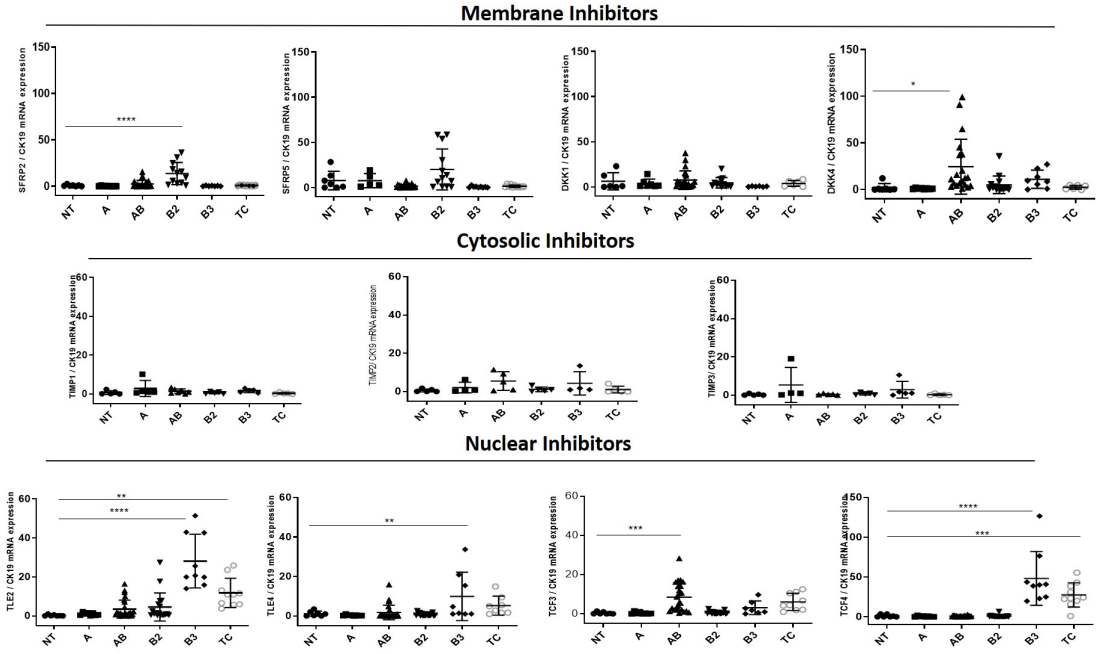


Figure 11: mRNA quantification of WNT inhibitors in our local (Mannheim) cohort of thymic epithelial tumors and non-neoplastic thymuses (NT).

Expression levels of mRNA encoding membrane-associated, cytosolic and nuclear WNT signaling inhibitors in thymomas (types A, AB, B2 B3, n=60) and thymic carcinoma (TC, n=11) as compared to NT (n=7) were measured by qRT-PCR. *Cytokeratin 19* mRNA levels as surrogate markers of

epithelial cell contents were used for normalization. ****=p<0.0001. ***: p=0.0007 and 0.0004, **: p= 0.0048 and 0.0034, *: p=0.0232

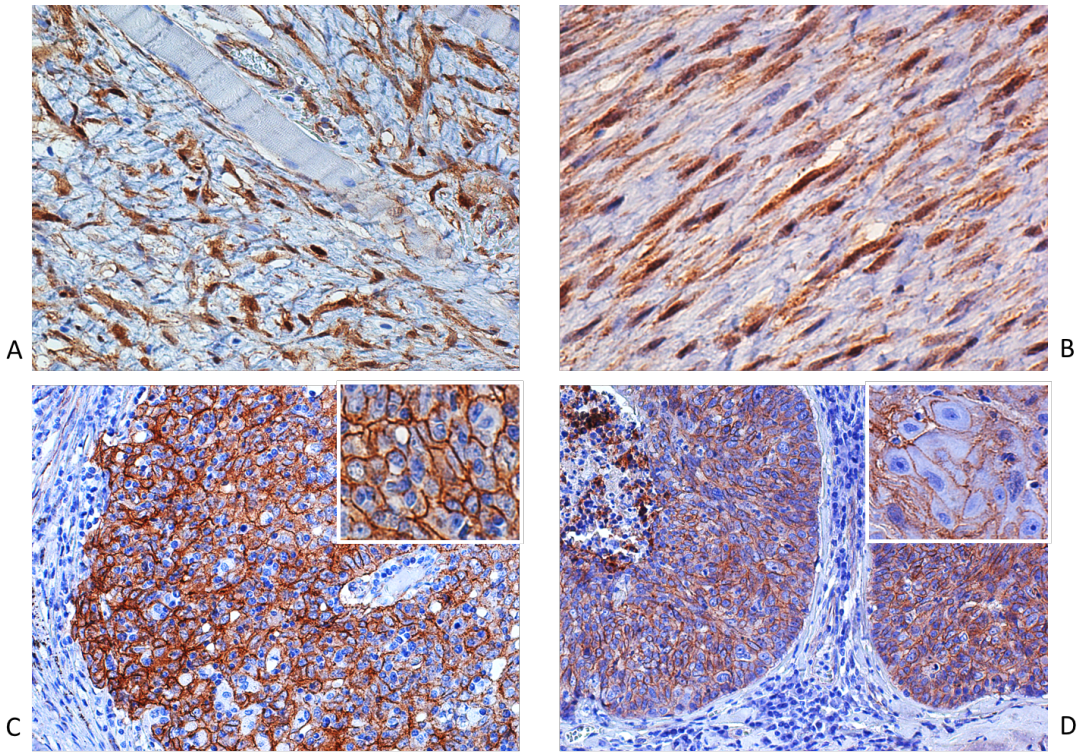


Figure 12: Immunohistochemistry of β-catenin expression in type B3 thymoma and thymic carcinoma.

(A), (B): Immunostaining of cytoplasmic and nuclear ß-catenin in desmoid fibromatosis (nuclear staining, positive control, A: x200, B: x400); C: Labeling of ß-catenin mainly on the cell membrane in a representative case of type B3 thymoma (x200; inset x400); D: ß-catenin immunostaining mainly of the cell membrane in a representative case of thymic squamous cell carcinoma (x200; inset 400). A-D: immunoperoxidase.

3.6 Increased WNT4 secretion in ex-vivo cultured thymoma derived pTECs

WNT4 protein was quantified by ELISA in conditioned media (CM) that were collected from different primary epithelial cell (pTECs) cultures of NT and thymomas after different culture periods. WNT4 protein secretion in CM after 4 days of pTEC culture (so-called fresh culture) of type B3 thymomas (n=7) was significantly higher than the secretion from fresh pTECs of NT (n=3), AB (n=8), and B2 (n=3) thymomas (****p=0.0001, ****p<0.0001 and ***p=0.0003) (Fig 13A). Secretion from fresh type B3 pTEC cultures was also significantly higher than from type B3 thymoma pTECs after 10-20 days in culture (****p<0.0001; n=6; so-called “senescent cultures”) (Fig 13A). After 3 days of treatment of ex vivo cultured pTECs with IWP3 (an inhibitor of

WNT secretion through blockade of the protein, porcupine o-acyltransferase, encoded by PORCN) [83], supernatants contained only 25% of the WNT4 that was released by control pTECs, i.e. in the absence of IWP3 (Fig 13A, B). This degree of blockade was comparable to the 70% blockade through IWP3 treatment of the WNT4-transfected (WNT4 overexpressing) TEP1 thymic epithelial control cell line, WNT4-TEP1 (Fig13A,B).

The results obtained by ELISA were verified by western blot (Fig13C and D). Immunoprecipitation and western blot results of WNT4 in supernatants collected from short term type AB and B3 thymoma pTECs cultures in the presence and absence of IWP3 are shown in Fig13D. Levels of WNT4 protein were higher in supernatants collected from type B3 thymoma pTECs compared to type AB thymoma pTECs and also WNT4 secretion in supernatants declined following the treatment of type AB and B3 thymoma-derived pTECs with IWP3 (Fig 13D).

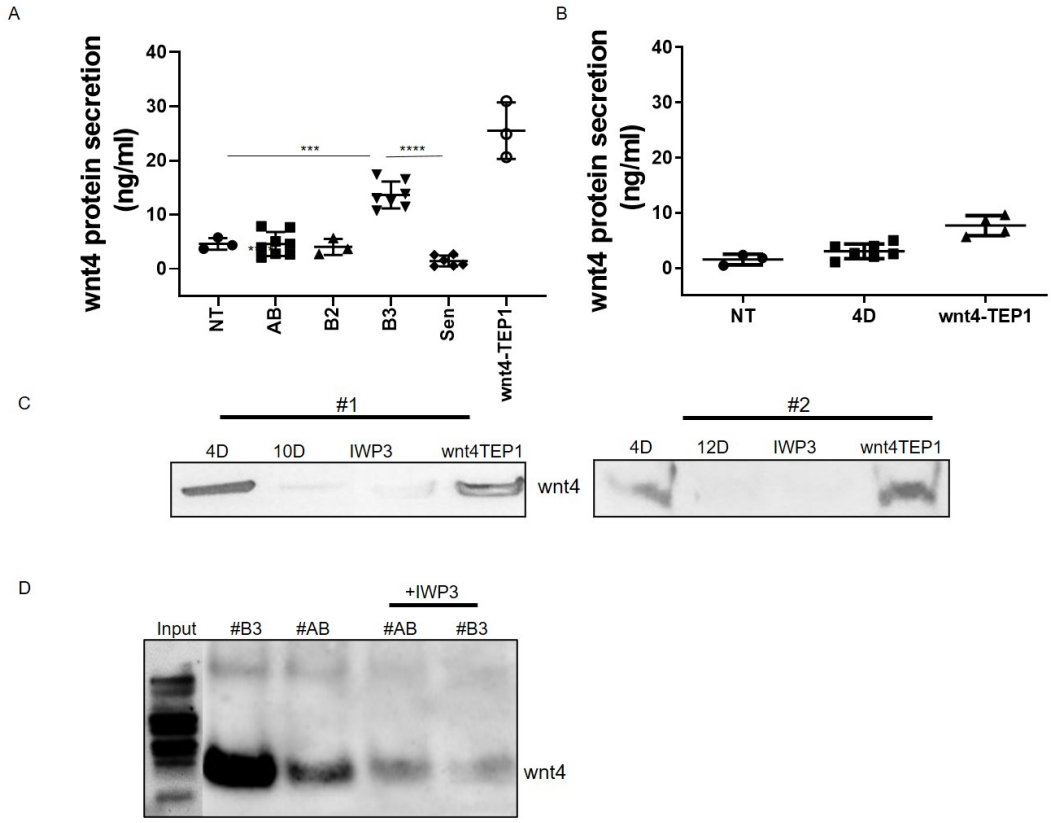


Figure 13: Analysis of WNT4 secretion of primary thymic epithelial cells (pTECs) derived from non-neoplastic thymuses (NT) and thymomas.

WNT4 measurement in short-term conditioned media (CM) collected from different NT and thymoma derived pTECs 7 days (NT) or 4 days (thymomas) after seeding. (A): ELISA-based measurement of

WNT4 in supernatants of fresh pTECs from 3 NT, 8 type AB, 3 type B2, and 7 type B3 thymomas as well as from 6 long-term, senescent cultures of 3 type AB and 3 type B3 thymoma-derived pTECs ("Sen"). WNT4-TEP1 are murine thymic epithelial cells transfected with human WNT4 expression vector (positive control). (B): After 3 days of IWP3 treatment, ELISA measurement of WNT4 in supernatants of pTECs from short-term (4-day, 4D) culture of 7 thymomas (3 type AB, 1 type B2, and 3 type B3 thymomas), 6 senescent long-term cultures of 3 type AB and 3 type B3 thymomas, and 3 NTs. IWP3 was used for WNT4 secretion inhibition. (C): WNT4 analysis by western blot in supernatants collected from 2 B3 thymomas (#1, #2) after short-term (4-day, 4D) and long-term (10- or 12-day, 10D, 12D) culture, either with or without treatment of the cultures with the WNT secretion inhibitor, IWP3. Supernatants from WNT4-TEP1 cells served as positive control. Protein was concentrated by precipitation using Trichloroacetic acid 20% 1:1. (D): Immunoprecipitation of WNT4 from supernatants collected from short term pTECs cultures of one type AB and type B3 thymoma with or without IWP3 treatment. D: days.

3.7 Cell culture medium enriched with conditioned medium from thymoma-derived pTECs maintained thymic epithelial cells and thymocytes via secreted WNT4

Culturing pTECs in RPMI medium with a 25% proportion of conditioned medium (CM) harvested from primary thymoma TEC cultures ('CM-enriched medium') allowed for cell growth and expansion of thymoma-derived pTECs (n=5, 2AB and 3B3) for 24 days (Fig 14A), while the CM-enriched medium maintained the viability of NT-derived pTECs (n=2) without significant expansion for 12 days (Fig 14B). It also maintained cell morphology of pTECs derived from thymoma (cell growing in D4, D9, D16 and D24) (Fig 14A) and pTECs derived from NT (cell growing in D7 and D12) (Fig 14B). In addition, pTECs stimulated with CM-enriched medium from cultured pTECs of thymoma and NT showed healthy morphology and higher cell numbers as judged by confluent growth as compared to control pTECs that were not stimulated with CM (Fig 14A and B). By contrast, cell numbers declined when pTECs were grown in standard RPMI/HEPS/FBS medium and showed signs of senescence, i.e. cells with enlarged size and poor proliferative activity. Metabolic activity (as a surrogate marker of cell numbers) of pTECs derived from AB and B3 thymoma and NT were measured with MTT assay after culturing with or without CM-enriched medium (Fig 14C and D). Using the MTT assay, it turned out that long-term cultures of pTECs derived from thymomas and NT was possible for up to 20 days if cultured in CM-enriched medium,

while pTECs derived from NTs stayed alive for only 6 days, if cultured with standard medium in the absence of CM. Finally, pTECs derived from type AB and type B3 thymomas proliferated strongly if cultured in CM-enriched medium, while controls grown in pure standard culture medium (RPMI, 10% FBS) did not.

CM-enriched medium maintained not only pTECs but also thymocytes isolated from type AB and type B3 thymomas. Quantification of cell numbers revealed that long-term cultures of thymoma-derived thymocytes was possible for 8 days if the cells were grown in CM-enriched medium, while thymocytes grown in standard culture medium survived for only 48h (Fig15). Furthermore, if CM from senescent thymoma pTECs (p3-p5) was used to generated a "senescent CM-enriched" medium, thymocyte survival was shorter than the period if grown in "fresh CM-enriched" medium, in which the CM stemmed from an early thymoma-derived pTEC culture (Fig15 A and B).

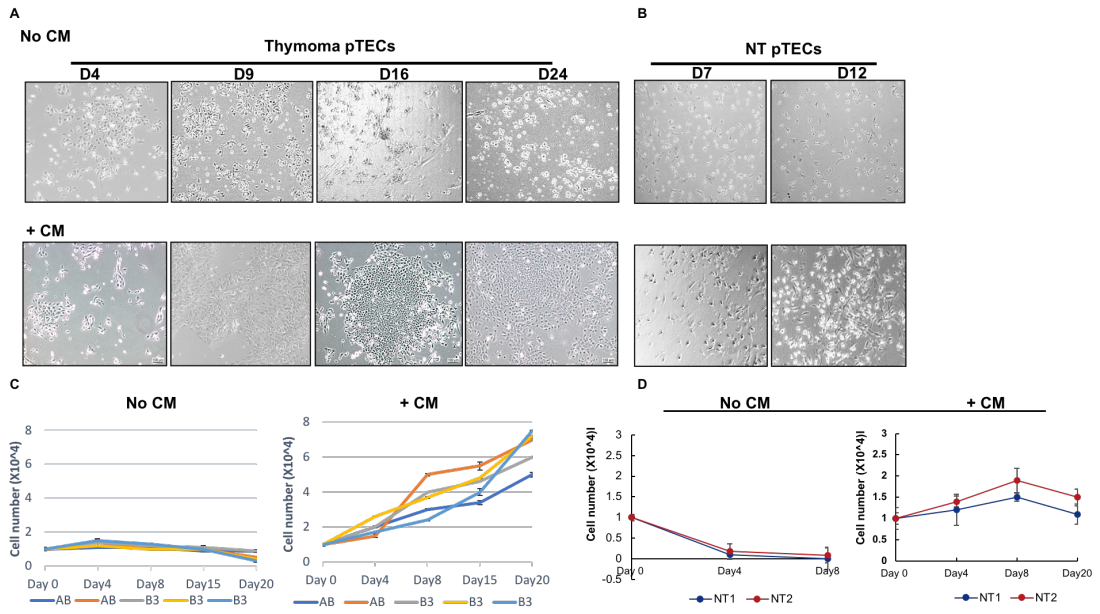


Figure 14: Effect of conditioned medium (CM) on primary thymic epithelial cells (pTECs) derived from thymoma and non-neoplastic thymus (NT).

Thymoma and NT-derived pTECs were grown in triplicate wells either with CM-enriched standard medium (in which the CM stemmed from fresh thymoma-derived pTECs) or pure standard medium (RPMI/HEPEs/ FBS) in 96-well plate. (A): representative images of type B3 thymoma- derived pTECs cultured from 4 to 24 days (B): representative images of NT-derived pTECs from 7 to 12 days. (C, D): Cell proliferation using MTT assay after culturing type AB and type B3 thymoma-derived pTECs (C)

and NT-derived pTECs (D) derived pTECs with and without CM. No CM: without fresh conditioned medium. Triplicate measurements of MTT were performed of two independent experiments. FBS: fetal bovine serum

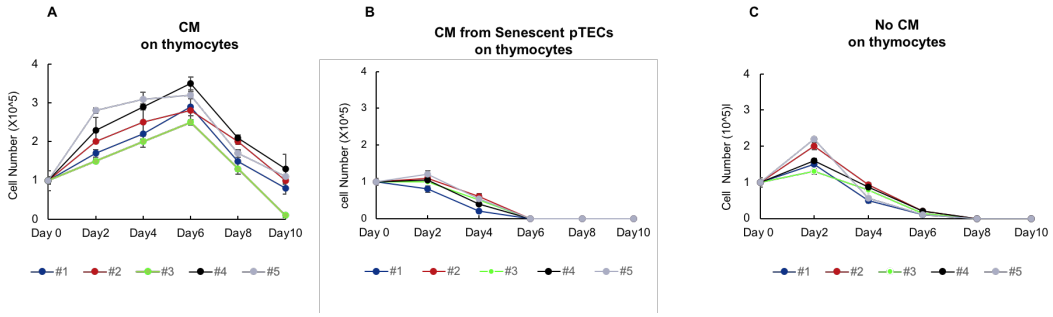


Figure 15: Effect of conditioned medium (CM) on thymocytes derived from type AB and type B3 thymomas.

Cell viability assessment using MTT assay after culturing type AB and type B3 thymoma-derived thymocytes (n=5, #1-#5, AB, n=2; B3,n=3) with two different CM-enriched media, both with a 25% proportion of CM, however, one from a short-term (fresh) primary thymoma epithelial cell (pTEC) culture, the other one from a long-term (senescent) pTEC culture) as compared to growth in pure standard RPMI/HEPES/FBS medium. The CM was obtained from the same subtype of cultured cells, i.e. the case type AB thymocytes was using CM-enriched medium obtained from type AB thymoma pTEC culture. (A) Analysis of thymocytes from type AB and type B3 thymomas grown in medium enriched with fresh CM. (B) Analysis of the same thymocytes but grown in medium supplemented with CM collected from senescent (long term) pTECs cultures. (C) Proliferation analysis of the same thymocytes but in pure standard medium (RPMI/ HEPEs/ FBS). CM: conditioned medium, No CM: without conditioned medium

3.8 WNT4-dependent autocrine loop drives WNT4 expression in 3D multicellular spheroid cell culture system

Having shown above 1) that neoplastic pTECs secreted WNT4 mainly during short term but not long-term culture; 2) that WNT4 expression declines spontaneously during pTEC culture in standard RPMI medium; and 3) that conditioned medium containing high WNT4 levels (Fig13) drives the proliferation of thymoma-derived pTECs in vitro, we wondered whether WNT4 itself in the short-term conditioned medium may drive its expression and secretion, i.e. whether a WNT4-dependent autocrine loop may be operative in pTECs. Indeed, culturing pTECS derived from AB and B3 thymoma in 3D multicellular spheroid in which tumor cells form compact clusters showed higher WNT4 mRNA expression and secretion than standard 2D cultures with dispersed cells (Fig 16D,E).

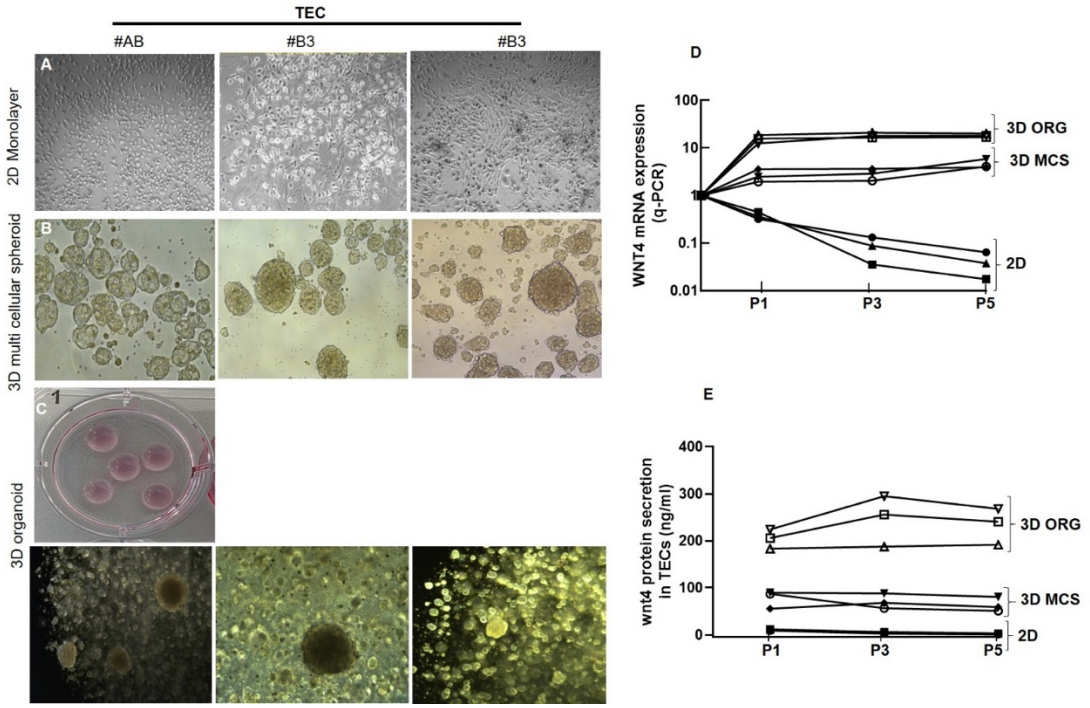


Figure 16: WNT4 expression and secretion in pTECs cultured in two different 3D cell culture models and standard 2D cell culture.

(A): Microscopic images of 2D monolayer cell cultures of pTECs (1 type AB and 2 type B3 thymomas). (B) Microscopic images of the same pTECs cultured as 3D multicellular spheroid (MCS) for 4 days using ULA plates. (C): Representative image of 3D cell culture model: TECs are embedded in 50µl Matrigel (Corning, USA) and microscopic images of the same pTECs in 3D cell culture model. (D): WNT4 mRNA expression analysis (qRT-PCR) of these three pTECs (1AB and 2B3) cultured in 2D and 3D (MCS and Spheres) for 3 passages (P1, P3 and P5). (E): WNT4 secretion quantification in cell culture supernatants in 3 different passages by ELISA. MCS: Multicellular spheres Images were performed using LEICA microscope and LEICA microscope imaging software with X200 for 2D and X100 for 3D cultures.

3.9 WNT4 activates the non-canonical WNT/JNK signaling in thymoma derived pTECs

As shown in Fig16, CM-enriched medium, as well as recombinant human WNT4 at 200ng/ml and 3D cell culture models (3D MCS and 3D like-organoid), prevented the time-dependent decline of WNT mRNA expression and WNT4 secretion in type AB and B3 thymoma-derived pTECs (Fig16D and E). Typical members of non-canonical WNT/JNK pathways, RAC1 and the downstream phosphorylated JNK (pJNK) proteins showed opposite expression change from p1-p3 between 2D (standard culture medium) and 3D models in both neoplastic pTECs, i.e. in standard culture medium RAC1 and

pJNK were declining from p1 to p3 while in 3D culture it's stable (Fig17A and B). Besides, both stimulants (CM and rec-WNT4) activated RAC1 and pJNK from p1 to p3 in type AB and B3 thymoma-derived pTECs compared to the standard culture medium without stimulants (RPMI-10% FBS) (Fig 17A and B). Increased WNT4, as well as RAC1 and pJNK expression, were found in both neoplastic pTECs cultured in 3D multicellular spheroid (MCS) and 3D like-organoid model (Fig 17).

Based on the above observations and the working hypothesis that CM-enriched medium and recombinant WNT4 may rescue the declining WNT4 expression observed in pTECs grown in 2D cultures, we applied different concentrations of recombinant WNT4 (50ng/ml, 100ng/ml and 200ng/ml) to long-term 2D cultures of type B3 and type AB thymoma-derived pTECs. We found slightly increased *WNT4* expression (Fig18 A, B) in late passages of both type AB and type B3 thymoma-derived pTECs with 200ng/ml recombinant WNT4 compared to the standard culture medium (RPMI/ HEPES/10%FBS). The WNT4 downstream gene *FOXN1* showed higher expression in 100ng/ml recombinant WNT4 treated B3 thymoma pTECs (Fig18C) and higher WNT pathway targeting gene *CyclinD1* expression in late passage with 200ng/ml recombinant WNT4 treated (Fig18D).

Furthermore, the JNK/JUN dependent genes, *FOXN1*, *MYC* and *CD44* showed their expression (particularly that of *CD44*) in pTECs were driven more strongly by growth in 3D cultures than by CM-enriched or recombinant WNT4-supplemented media in 2D cultures (Fig 19).

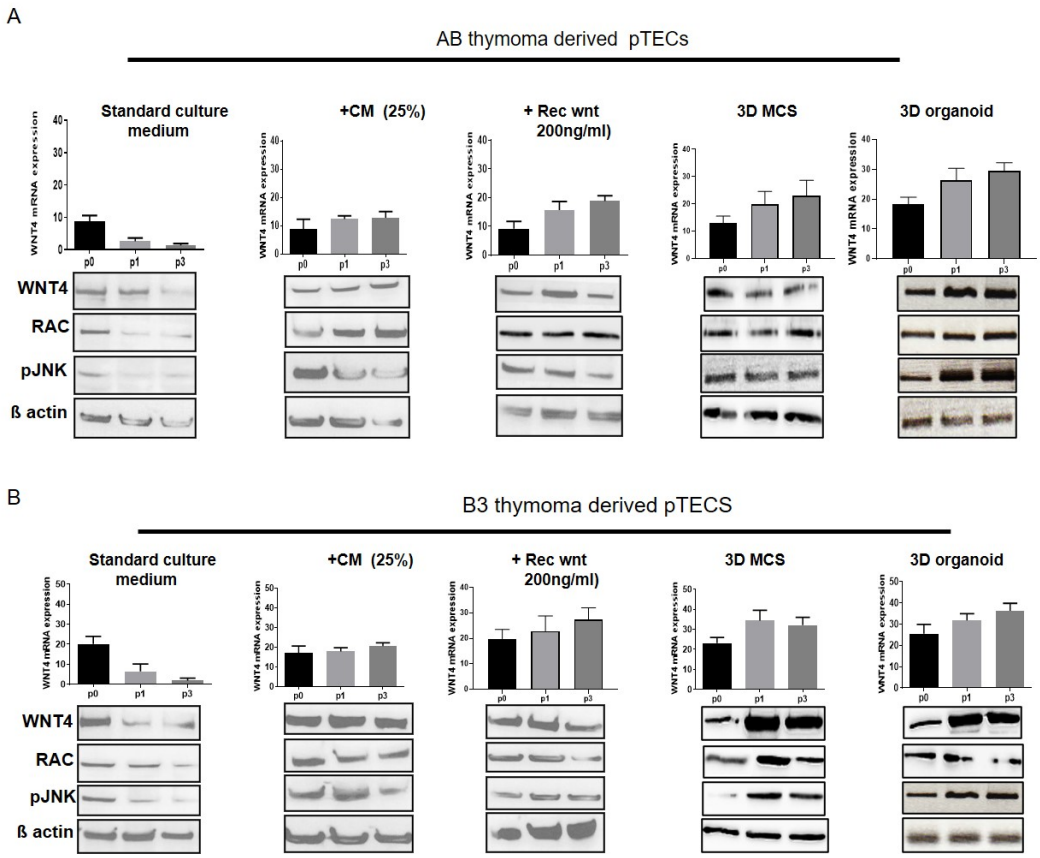


Figure 17: Maintenance and increase of WNT4 expression in long-term thymoma derived pTECs cultures by stimulation with CM-enriched medium and recombinant WNT4 and 3D culture models.

Slightly increased WNT4 and RAC1 and phospho-JNK (pJNK) protein expression in the late passage of neoplastic pTECs with 25% (v:v) conditioned medium (CM) as well as with 200ng/ml recombinant WNT4 protein (Rec WNT4) compared to the 2D standard medium (RPMI-10% FBS) culture and increased WNT4 expression in 3D cell culture models (MCS and 3D organoid-like) in AB thymoma-derived (A) and B3 thymoma-derived pTECs (B). RAC and its downstream protein pJNK were maintained in the late passage as well as in the early short-term 3D culture of both type AB and B3 thymoma-derived pTECs. ß-actin is used as a loading control for western blots.

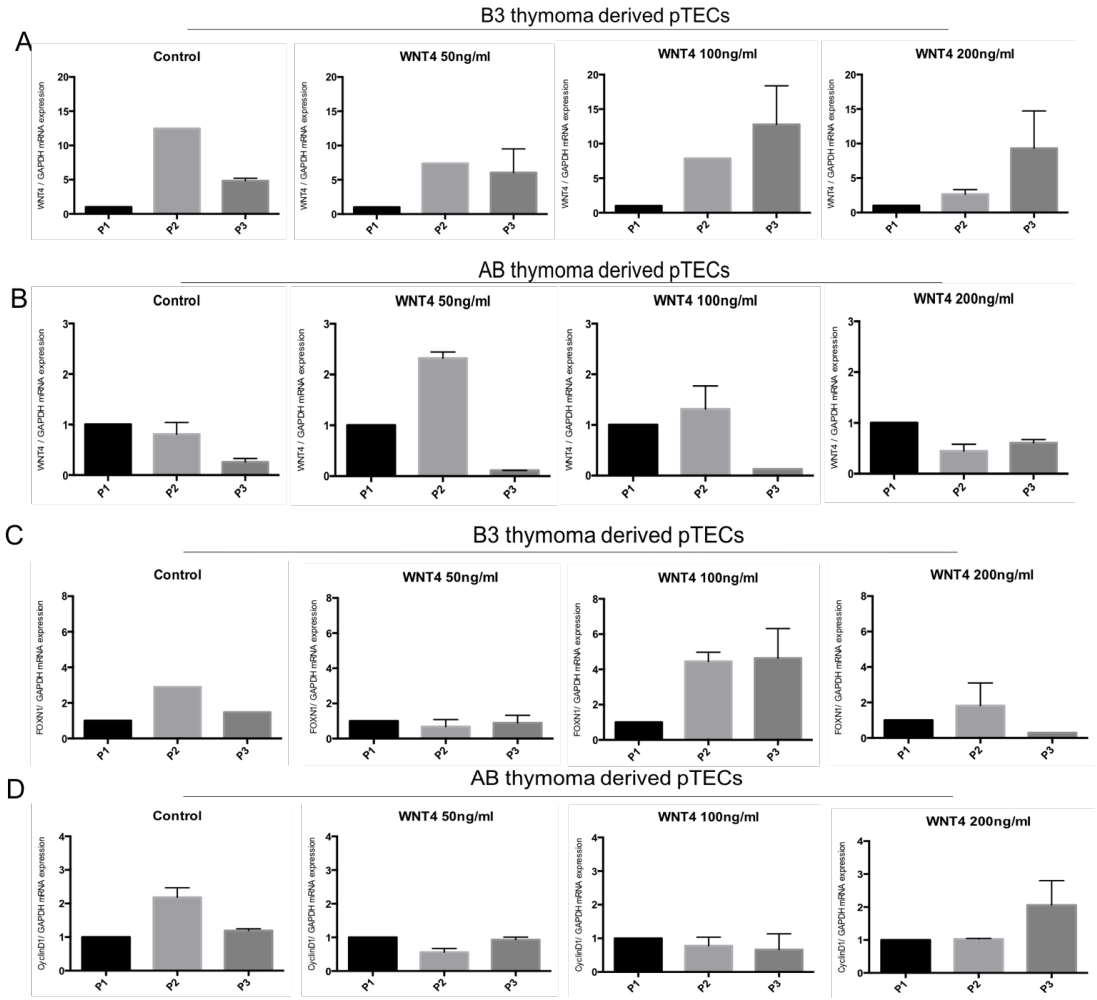
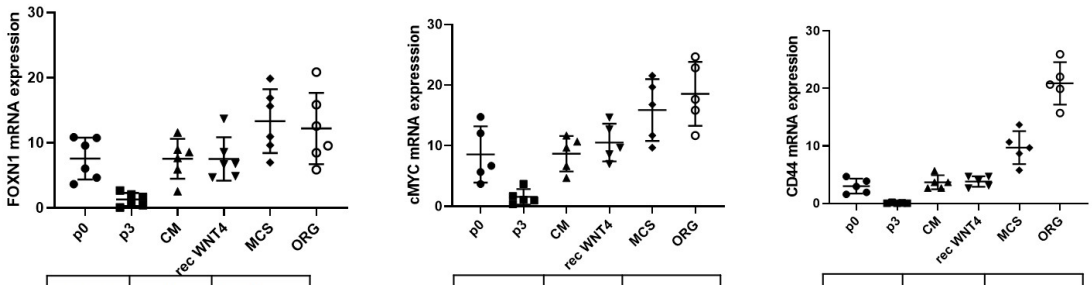


Figure 18: Maintenance and increase of *WNT4*, *FOXN1* and *CyclinD1* expression in long-term thymoma derived pTECs cultures by stimulation with recombinant WNT4.

Different concentrations of recombinant WNT4 (50ng/ml, 100ng/ml, 200ng/ml) were applied to type B3 thymoma-derived and type AB thymoma-derived pTECs in different passages. Slightly increased *WNT4* (A, B), *FOXN1*(C) and *CyclinD1* (D) expression in long term culture of the neoplastic pTECs were found in both type AB and B3 thymoma pTECs with 200ng/ml recombinant WNT4 compared to the standard culture medium (RPMI/ HEPES/10%FBS). Cells were passaged every three days. The experiments were based on triplicate parallel measurements and this is a representative plot of two dependent measurements. *GAPDH* is used as endogenous control for qRT-PCR.



Vs p3	p-values	Significance
p0	0.0297	*
CM	0.0311	*
Rec WNT	0.0313	*
MCS	<0.0001	****
ORG	0,0001	***

Vs p3	p-values	Significance
p0	0.0452	*
CM	0.0376	*
Rec WNT	0.0069	**
MCS	<0.0001	****
ORG	<0,0001	****

Vs p3	p-values	Significance
p0	0.1296	ns
CM	0.0453	*
Rec WNT	0.0362	*
MCS	<0.0001	****
ORG	<0.0001	****

Figure 19: mRNA quantification of the pJNK/JUN dependent genes, *FOXN1*, *MYC* and *CD44* in pTECs.

Expression of WNT/JNK pathway dependent genes, *FOXN1*, *MYC* and *CD44* were measured by qRT-PCR in pTECs (n=6: 2AB and 1B2 and 2B3) after short-term (p0) and long term (p3) culture, either with conditioned media (CM)-enriched or recombinant (rec) WNT4-enriched medium in 2D cultures, as well as culture as 3D multicellular spheroid (MCS) and 3D organoid-like (ORG) cultures. The tables represent the p-values and statistical significances from the comparison of all cultures against p3 senescent cultures using ANOVA test; ns: not significant.

3.10 WNT4 knock down has opposite effects on RAC1 and JNK expression in early type AB and B3 thymoma derived pTECs

Following the above observation that WNT4, RAC1 and pJNK levels decreased in parallel over time during long term culture of thymoma derived pTECs in normal culture medium, we next knocked down *WNT4* in early stage of pTECs (N=4, 2AB and 2B3) and the thymic carcinoma cell line, 1889c by *WNT4*-shRNA. *WNT4* knockdown led to a decline of RAC1 protein levels in type AB and type B3 thymoma-derived pTECs, while stimulation of parallel cultures with human recombinant WNT4 induced unchanged or slightly increased RAC1 levels (Fig20). Simultaneously, pJNK levels increased in pTECs of type B3 thymomas and 1889c cells while levels in type AB thymomas remained unchanged after *WNT4* knockdown. No clear difference was seen between stimulation with human recombinant WNT4 and with CM/WNT4-enriched supernatant. Taken together, the knockdown of *WNT4* in *early* pTECs does not mimic the spontaneous decline of WNT4, RAC1 and pJNK during long-term pTEC culture.

To elucidate the reason why pJNK levels are maintained or even increased in *early* pTECs and 1889c cells on *WNT4* knockdown, we next investigated expression levels of p-AKT and IKKα/β, since activation of the AKT and NF-kB pathway are known to induce JNK activation [84] [85]. As shown in Figure 20, such a mechanism could, in fact, be operative in *early* pTECs and 1889c cells, since *WNT4* knockdown strongly increased levels of pAKT and pIKK in all tested cells (N=5), and this was accompanied by either maintained (in type AB thymomas) or increased levels of pJNK (in B3 thymomas and 1889c cells). This suggested that JNK interacts with NF-κB and AKT pathways and, therefore, might be activated not only by RAC1 in response to WNT non-canonical signaling but also by IKKα/β and pAKT proteins as alternative mechanisms (Fig 20). Indeed, fresh CM-enriched medium and recombinant WNT4 protein did not have an obvious effect on IKKα/β and pAKT levels in neoplastic pTECs (n=4) except in one type B3 thymoma (case#1) and in the thymic carcinoma 1889c cell line, in which pAKT was induced either by rec-WNT4 (case#1) or CM-enriched medium (1889c cells) (Fig20).

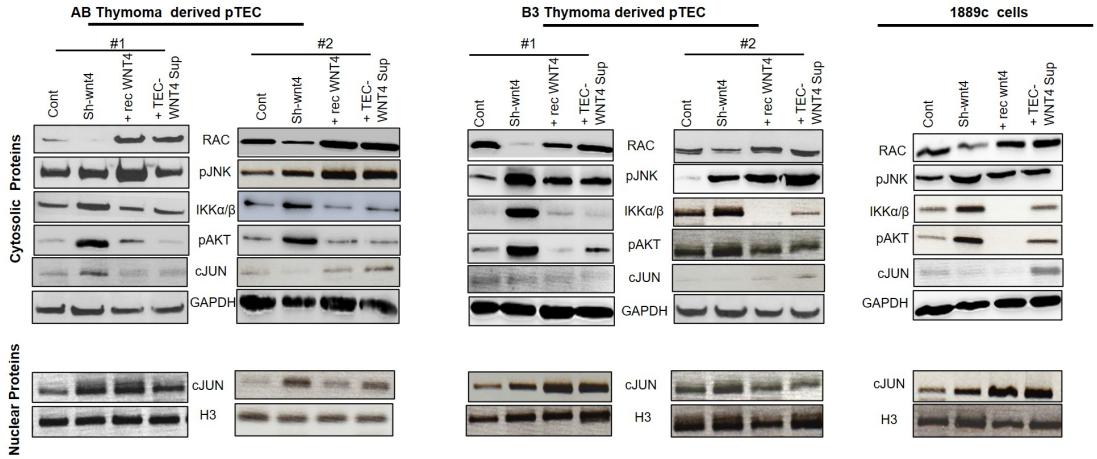


Figure 20: Downregulation RAC1 but not pJNK following *WNT4* knock down in early pTECs of type AB and B3 thymoma and 1889c cells.

Thymoma derived pTECs and cells of the thymic carcinoma cell line, 1889c were transfected using pU6WNT4-shRNA or stimulated with either 200ng/ml recombinant WNT4 or with the CM 25%(v:v). Proteins were extracted from the cultured cells and analyzed by western blot 48h after transfection and 4 days after stimulation with recombinant WNT4 and CM. (A): Protein analysis of RAC1, pJNK, IKKα/β, pAKT in 2 type AB thymoma derived pTECs. (B): Protein analysis of RAC1, pJNK, IKKα/β, pAKT in

2 type B3 thymoma derived pTECs and (C): Protein analysis of RAC1, pJNK, IKKα/β, pAKT in thymic carcinoma 1889c cell line. GAPDH and Histone 3 (H3) are used as loading controls for cytosolic and nuclear proteins, respectively.

3.11 JNK signaling is linked to NF-κB and AKT pathways in thymoma derived pTECs and thymic carcinoma 1889c cells

Since increased expression of IKKα/β and pAKT following *WNT4* knockdown was associated with WNT non-canonical JNK activation, the impact of pAKT and IKKα/β (i.e. NF-κB signalling) on JNK/JUN activation was further investigated. To this end, *WNT4* was knocked down in cells of the thymic carcinoma 1889c cell line and early type AB and B3 thymoma-derived pTECs (n=2 in each group) with *WNT4*-shRNA transfected in the early passage time, followed by treatment with the AKT-inhibitor, MK2206 and the NF-κB inhibitor, TPCA1. Cytosolic and nuclear proteins were isolated and analyzed by western blot for pJNK, pAKT, pJUN and p65 contents. As shown in Figure 21, both pJNK and pAKT were not detected in cytosolic extracts of neoplastic pTECs and cells of the 1889c cell line after the knockdown of *WNT4* and the additional simultaneous treatment with the two inhibitors. In addition, the proteins pJUN and p65 were absent in the nuclear fractions of type AB and type B3 thymoma-derived pTECs and 1889c TC cells. This suggests that nuclear translocation was prevented (Fig21). On the other hand, cytosolic expression of pJNK and pAKT proteins was maintained in both *WNT4* knockdown neoplastic pTECs and 1889c cells in the absence of drug treatment (Fig 20). This comparison of the two treatment methods suggested that i) IKKα/β and pAKT are involved in WNT4 signaling and ii) the non-canonical WNT/PCP/JNK pathway is associated with NF-κB and AKT signaling pathways.

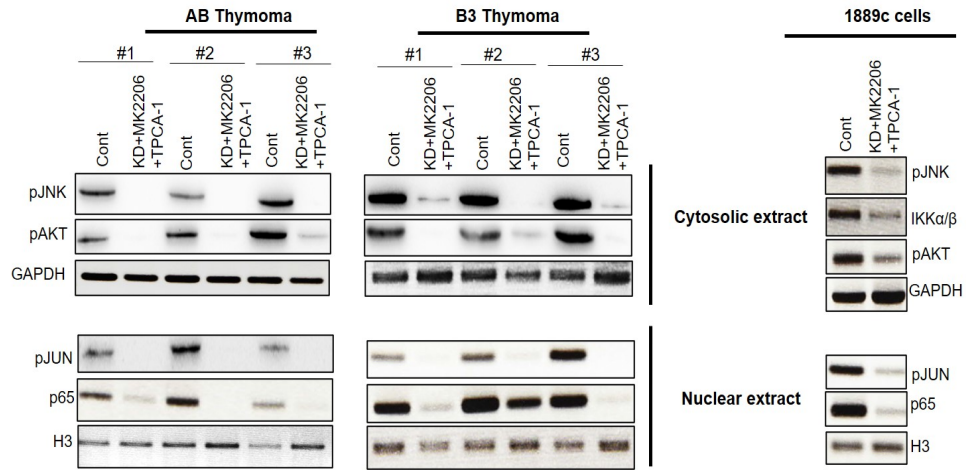


Figure 21: Inactivation of pJNK by the combined down-regulation of WNT4, pAKT and NF-κB pathways.

Type AB and type B3 thymoma-derived pTECs (n=3 in each group) and cells of the 1889c thymic carcinoma cell line were first transfected with pU6WNT4-shRNA and subsequently treated with a combination of MK2206 and TPCA1 that target pAKT and IKKα/β (NF-κB pathway), respectively. Cytosolic and nuclear proteins were isolated and pJNK, IKKα/β, pAKT, pJUN and p65 were analyzed by western blot. GAPDH and H3 were used as loading controls for cytosolic and nuclear proteins, respectively.

3.12 NF-κB, not pAKT inhibition induced non-canonical WNT4/PCP pathways

We focused further on the question of whether NF-κB or pAKT could activate non-canonical WNT4/PCP pathway, type AB and type B3 thymoma-derived pTECs were treated separately with pAKT inhibitor (MK2206, 2μM) and NF-κB inhibitor (EF24, 1μM). The control untreated cells were cultured in DMSO only.

Inhibition of NF-κB negatively affected the non-canonical WNT4/PCP pathway: Both WNT4 and FZD6 were decreased on mRNA and protein levels and their downstream proteins RAC1 and pJNK as well as the nuclear translocation of their target pJUN were declined (Fig 22). pAKT inhibition by MK2206 treatment showed no effect either on WNT4, FZD6 or RAC1 but on the downstream proteins pJNK as well as the nuclear translocation of its target pJUN.

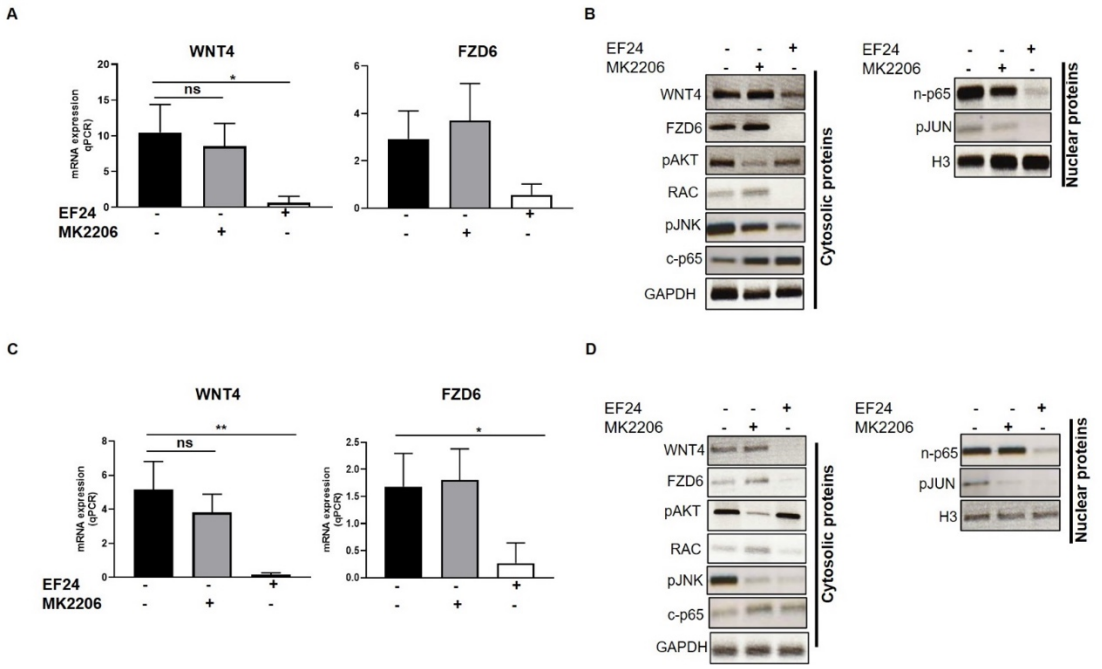


Figure 22: NF-κB activated non-canonical WNT4/PCP pathway.

pTECs derived from type AB (n=1)(C, D) and B3 (n=1)(A, B) thymoma were treated either with 1µM EF24 or 2µM MK2206 for 48h that target NF-κB (p65) and pAKT respectively. WNT4 and FZD6 were quantified using qRT-PCR (A, C). GAPDH was used as reference gene. Cytosolic and nuclear proteins were isolated then analyzed by WB for the following molecules: WNT4, FZD6, RAC1, pJNK, pAKT, p65and pJUN. GAPDH and H3 were used as loading control (B, D). c-p65: Cytosolic p65, n-p65: Nuclear p65. The experiments were based on triplicate parallel measurements and this is a representative plot of two dependent measurements.

4. DISCUSSION

With WNT pathways being investigated widely in human cancers, the important role of WNT signaling became more and more obvious. Basically, the WNT canonical/β-catenin pathway was first explored and found to be associated with a variety of cancers through activating TCF/LEF transcription factors. For example, researchers found that TCF4 activity was enhanced with mutations of *CTNNB1* (encoding β-catenin) resulting in increased cell proliferation and migration ability of colorectal cancer cells [86]. However, subsequent studies revealed that the development of many tumors cannot be accounted for by β-catenin-dependent pathways, i.e. canonical WNT/β-catenin signaling, leading to the discovery of the importance of WNT non-canonical pathways. The purpose of the current study was to explore the role of WNT pathways in thymic epithelial tumors (TETs). The WNT signaling pathways have already been investigated in a variety of tumors, like breast cancer, colon cancer, melanoma and, in a preliminary way, also thymic tumors[29] [32] [40] [87]. WNT ligands could activate the canonical WNT/β-catenin and non-canonical WNT/PCP and WNT/Ca^{2+} signaling pathways through binding to frizzled receptors and other co-receptors like LRP5/6, ROR2 and RYK. The role of each member of WNT ligands and FZD receptors are different in canonical and non-canonical WNT pathways in various tumors. For example, WNT1 and WNT3 are known canonical components while WNT5 and WNT11 are more relevant for non-canonical pathways. Huang T et found FZD6 could activate CaMKII/TAK1/NLK signaling in glioblastoma cells [88], whilst FZD6 was also demonstrated to activate the c-Jun N-terminal Kinase (JNK) in neuroblastoma with higher phosphorylated JNK expression in tumors with higher expression of FZD6 [89]. This current study is the first to comprehensively analyze the expression of all WNT ligands (WNT1-19) and frizzled receptors (FZD1-10) in whole tissues of normal thymuses and the major subtypes of TETs. This analysis also explored WNT signaling functions in primary thymic epithelial cells isolated from fresh thymic tissues by gene knockdown and drug treatment. We also found that nuclear β-catenin is absent in type

B3 thymomas and thymic carcinoma tissues and that WNT4 stimulation of pTECs derived from type AB and type B3 thymomas could increase the expression of *WNT4*, *FOXN1* and CyclinD1 gene in late cell culture passages. The main new findings of this thesis are i) Expression of *WNT4* and WNT frizzled receptor 6 (*FZD6*) is higher in type B3 thymomas and TCs compared to other thymomas and non-neoplastic thymuses, and the physiological decline of WNT4 expression with age is missing in TETs; (ii) WNT4 drives an autocrine WNT4 loop in long-term 3D-cultured but not 2D-cultured neoplastic pTECs; (iii) WNT4/JNK dependent non-canonical activation in thymic neoplastic pTECs and the autocrine WNT4 loop is dependent on the non-canonical WNT/PCP pathway in neoplastic pTECs and (iv) JNK activation in early neoplastic pTECs following acute blockade of non-canonical WNT4 signalling is stabilized through 'compensatory' activation of AKT and NF-κB pathways. We can conclude that WNT4 plays a critical role in the non-canonical WNT signaling pathway of TETs through activating WNT/PCP/JNK at the mRNA level and protein level.

Relevance of increased epithelial WNT4/FZD6 expression in TETs

The thymic epithelial architecture is thought to support the functional role of the thymus, recruitment of lymphoid progenitors, induction of T cell development and quality control of the emerging repertoire of somatically diversified antigen receptors for self-tolerance [90]. Several of the transcription factors [91] [92] [93] and signaling molecules [52], like E-cadherin and BMP could regulate thymus development [94] [95] [96] [97]. WNT-mediated signal transduction pathways have been recognized long time ago for their roles in regulating embryonic development [98]. Many studies have shown that WNT4 and the transcription factor Forkhead Box N1 (FoxN1) are among the key regulators of this signaling and play important roles in thymus development, architecture, function and senescence [96] [99] [100]. Thymic involution is characterized by WNT4 and FoxN1 regression during aging [48] [101] [87] and confirmed for NTs in the present investigation (Fig 4). However, WNT pathways has

been linked to different cancers and human disease processes in more and more studies [102] [103] [104].

Preliminary data with a single WHO type AB thymoma cell line suggested that increased WNT4 expression might play a role in thymoma development through non-canonical WNT signaling [87]. These findings inspired our working hypothesis that interference with thymic senescence through abnormal expression of WNT4 [55] [87] could contribute to oncogenesis across the spectrum of all TET histotypes, including TCs. Compared to Chen's published data [87] , the new findings here are the followings: the involvement of the WNT receptor FZD6, the importance of RAC1, AKT and NF-κB in this pathway, and the detection of a WNT4 dependent autocrine loop. Furthermore, we extended our study to the clinically most relevant type B3 thymomas and TCs compared to the indolent type AB thymomas, and also explored the role of WNT inhibitors in 2D and 3D culture systems. In contrast to our study, Kvell et al [97] reported that WNT4 could bind to FZD4 and showed that this receptor mainly drives the WNT canonical/β-catenin pathway. FZD6 was found in several literatures to activate WNT non-canonical pathway. By contrast, FZD6 that was identified as relevant in the current study was previously reported to activate the non-canonical WNT/Ca^{2+} pathway in glioblastoma [88] and the WNT/JNK pathway in neuroblastoma with higher expression of phosphorylated JNK in relation to higher expression levels of FZD6 in tumor cells [89]. Therefore, it is a key new finding here that WNT4 appears to be of particular relevance for the development of the most malignant TETs (type B3 thymomas and TCs), since they showed the most abnormally increased levels of WNT4 and FZD6 (Fig 2, 5).

Furthermore, increased WNT4 levels in TETs did not show any correlation with the patients' age, suggesting that physiological senescence signals regulating WNT4 expression in the normal thymus are offset in TETs. This conclusion is strongly underpinned by our in vitro experiments, since exogenous (e.g. conditioned medium-derived and recombinant) WNT4 was able to postpone senescence, maintain

proliferation and extend the survival of neoplastic pTECs in vitro (Fig15). Since treatment of pTECs with exogenous WNT4 induced the transcription of the *WNT4* gene (Fig18), and the same effect was seen spontaneously in 3D (but not 2D) cultures of neoplastic pTECs, it appears that an autocrine WNT4 loop is operative in aggressive TETs in vitro. Apparently, this abnormality is an intrinsic property of the tumor cells, since 3D cultures of neoplastic TECs showed stronger WNT4 gene expression and secretion for longer periods of time than 2D cultures of non-neoplastic pTECs. Similar phenomena have been reported previously in breast cancer (however with vascular endothelial growth factor A (VEGFA)[105]) and in lung and breast cancers (with IL6 as the growth factor [106]). Interestingly, WNT4 is known as a mediator and target of the autocrine loop that is driven in a subset of breast cancers by human growth hormone (hGH) [7].

The relevance of WNT4 for the biology of TETs is also suggested by our new finding that WNT4 as well as its downstream WNT non-canonical related proteins RAC1 and JNK declined in long term cultures of TECs and were restored either using conditioned Medium (CM) 25% (v./v.) or commercial recombinant WNT4 protein (Fig 20). Additionally, *FOXN1* and *CyclinD1*, targeting genes of WNT4 and non-canonical WNT signaling, were also increased in long-term cultures of thymoma derived pTECs when grown in media enriched with recombinant WNT4. These findings combined with the higher WNT4 secretion in type B3 thymoma derived primary cells compared to senescent and NT derived pTECs give strong hints to active the non-canonical WNT signaling in neoplastic pTECs (Fig 13).

We also investigated the expression of different WNT inhibitors. We hypothesized that they could affect TETs and might be decreased, because increased levels of WNT inhibitors are associated with TEC senescence [62] [101]. Surprisingly, however, levels of WNT inhibitors were - apparently paradoxically - increased in significant subsets of TETs. *DKK4,* an inhibitor of canonical WMT signaling at the membrane level, was highly expressed in some type AB thymomas (Fig 11), resembling the situation in some

other non-thymic tumors [107-109]. As shown previously, DKK4 inhibits canonical WNT signaling and downregulates expression of nuclear β-catenin, but still can promote tumor progression by activating non-canonical WNT signaling, such as the PCP/JNK pathway [110] [111]. Similarly, expression of the nuclear inhibitors, TLE2, TLE4 and TCF4 are increased in type B3 thymomas and TCs in this study. TLE2 and TLE4 belong to the Groucho/TLE family and could repress the canonical WNT signaling pathway based on the inhibited switch of WNT transcription factors from transcriptional repression of TLE to activation of TCF/LEF after β -catenin translocation. However, through WNT-driven ubiquitylation of the Groucho/TLE co-repressor complex can dissociate from TCF/LEF, allowing β-catenin/TCF/LEF assembly for initiation of a WNT-specific transcriptional program [112] [113]. TCF4 is known as a major nuclear mediator of canonical WNT signaling and a transcriptional repressor of ß-catenin target canonical genes and since type B3 thymomas showed cytosolic ß-catenin presence (Fig 12), this could be a hint that TCF4 could be a suppressor of ß-catenin and canonical WNT pathway in TETs. Besides, Angus-Hill et al. also described TCF4's tumor suppressor function in the canonical WNT pathway, while disruption of its activity contributes to colon tumorigenesis [63]. Taken together, these findings suggest that blockade of the canonical WNT pathway occurs at different levels in substantial subsets of different TET subtypes.

In line with this peculiarity of TETs, spontaneous and induced WNT4 expression in thymoma-derived pTECs was positively correlated with the expression of *FOXN1*, which was higher in CM and rec-WNT4 cultured pTECs compared to standard medium cultured pTECs (Fig17, Fig19). This suggests that WNT4 might drive expression of *FOXN1*, i.e. the master regulator of thymus development and maintenance in neoplastic pTECs in a similar way as in the normal thymus [114], including the rare quiescent but non-senescent TECs that presumably represent TEC progenitors in adult thymuses [115]. These cells can resume proliferation after acute insults (e.g. chemotherapy) to support thymus regeneration and form clusters in a particular niche at the cortico-

medullary junction, suggesting that cell-cell contact may be required to maintain their Wnt4^{high}Foxn1^{high} phenotype [115]. Since we find here that neoplastic pTECs in 3D culture maintain the *WNT4*$^{+}$*FOXN1*$^{+}$ phenotype of TETs much longer than in 2D cultures, we hypothesized that tumor cell-tumor cell and tumor cell-matrix interactions may be important to maintain the autocrine WNT4 loop, *FOXN1* expression and tumor cell survival of TETs in vivo. In support of a critical relevance of cell-cell contacts in this scenario, even the invasion front of TETs typically shows cohesive tumor cells, and the establishment of representative TET cell lines in 2D cultures has been exceptionally unsuccessful [116].

Which molecules mediate the clustering of the *WNT4*^{high}*FOXN1*^{high} presumed TEC progenitors in normal adult murine thymuses [115] and the cell-cell and cell-matrix interactions in human TETs is unknown. With regard to cell-matrix interactions, CD44 appears as a possible candidate, as it is preferentially expressed by neoplastic pTECs in 3D cultures (Fig 19) and in aggressive TETs in vivo [117]. Furthermore, CD44- together with WNT4- is a marker of cancer stem cells [118, 119]. As cell-cell interactions are concerned, identifying respective molecules in TETs should be a research priority, since interference with the 3D structure of neoplastic pTECs might elicit senescence through attenuation of the WNT4-FOXN1 axis, which could have therapeutic potential in TET patients. Overexpression of WNT4 and its corresponding, non-canonical frizzled receptor 6 activated the JNK-dependent non-canonical signaling in TETs. This finding and our new observation of the cytosolic presence and nuclear absence of β-catenin in type B3 thymoma and thymic carcinoma tissues (Fig 12) are at variance with previously published results [97, 120] These authors reported nuclear localization of β-catenin in TECs and suggested that β-catenin might drive thymoma progression. The reasons for this discrepancy are not clear but might be related to the lower specificity of historic β-catenin antibodies.

Link of NF-κB to WNT4 non-canonical pathway in thymoma derived pTECs

C-Jun N-terminal kinase (JNK) is not only a member of non-canonical WNT signaling but also of the mitogen-activated protein kinases (MAPKs) cascade, which plays vital roles in regulating diverse cell processes by activating downstream specific substrates such as c-JUN, ATF, NFAT, MYC, BCL-2, p53 and STAT to regulate cellular functions including proliferation, differentiation, migration, apoptosis and tumorigenesis [121]. JNK and AKT are positively correlated with each other and were reported to promote gastric cancer metastasis [85].

Some published work suggests that WNT/AKT-mTOR signaling is an important part in controlling cancer cell metabolism in a variety of tumor types. However, most of these studies focus on β-catenin-dependent oncogenic mechanisms [122] [123]. We reported for the first time that JNK persisted in WNT4 knockdown thymoma derived pTECs and cells of 1889c cell line, while RAC1 was repressed at the same time, and provide experimental evidence that increased pAKT and NF-κB activity might rescue JNK but not RAC1 expression (Fig 20). A few recent studies have shed light on the cross-regulation between NF-κB pathway and WNT/JNK pathway with impact on carcinogenesis [124]. The expression of non-canonical WNT5A protein is controlled by the NF-κB signaling pathway [125] and RelA (p65) also is involved in the down-regulation of the WNT/β-catenin pathway [126]. WNT/AKT, PI3K/AKT signaling pathways can modulate the downstream target gene transcriptional activation of the WNT signaling pathway, providing insight into a potential molecular mechanism for inflammation-induced carcinogenesis [127]. The kinases IKKα/β are critical activators of NF-κB pathway and our study showed JNK was rescued by IKKβ activation in *WNT4* knockdown thymic neoplastic cells (Fig 20). Vice versa, we elicited JNK downregulation in *WNT4* knockdown pTECs and in cells of the 1889c TC cell line after targeting AKT and IKKβ with pharmacologic inhibitors, MK2206 and TPCA-1 (Fig 22). Neither JUN nor p65 were translocated to the nucleus in *WNT4* shRNA transfected pTECs and 1889c cell line (Fig 20). In our study, both NF-κB and AKT activated JNK but only NF-κB inhibition WNT4 signaling (Fig 22). We found that WNT4 drived

thymoma oncogenesis and blocking WNT4 signaling might induce resistance mechanisms through AKT/NF-kB activation. These studies must be extended in order to more deeply understand how the "cross-talks" between WNT/JNK, NF-κB and AKT pathways in thymic cancers.

Translational Perspective

The clinically most aggressive TET subtypes, type B3 thymomas and TCs, show variable and combined increased mRNA expression of *WNT4*, its non-canonical receptor *FZD6* and downstream activation of JNK, which are in consistent with previous study [87] [104]. Therefore, patients with non-resectable, currently incurable TETs may benefit from clinical trials that test novel inhibitors of JNK [128]. Since strong expression of endogenous WNT inhibitors arose here as an unexpected new finding in many TETs, therapeutic targets of dickkopf family members (DKK1, DKK2, DKK3, and DKK4) appears as a potential novel therapeutic option as well [129].

New strategies targeting WNT signaling with small molecular inhibitors and antibodies, which are currently in clinical testing, could also be considered for testing in our preclinical 2D and 3D in vitro models of patient-derived pTECs, and, if successful, in patients with incurable TETs [130]. WNT secretion is also targetable by some inhibitors as exemplified by the inhibitor, IWP-2 that has been clinically tested, especially in patients with RNF43 mutated pancreatic cancers [131] [132], whereas PORCN inhibitors (WNT974 and ETC-159) have been applied to prevent mammary tumors and cancer stem cell survival [133] [134] [135]. However, in the face of the observed counter-regulatory mechanisms (including activation of AKT and NF-κB pathways) following the blockade of WNT4, it appears unlikely that any pharmacological monotherapy targeting WNT signaling would be sufficient to defeat TETs. Rather, combination therapies targeting WNT, AKT and NF-κB signaling together might have therapeutic potential for patients with aggressive TETs. Finally, in view of the observation that conditioned, WNT4-enriched cell culture medium prolonged the survival of thymocytes, it appears worthwhile to test [136], whether WNT signaling

might play a role in the context of thymoma-associated autoimmunity and in oncological strategies that apply immune checkpoint inhibitors.

5. SUMMARY

Thymic epithelial tumors (TETs) are rare malignant and incurable cancers if unresectable. Novel therapeutic options are needed, but targetable signaling pathways driving the pathogenesis of TETs are unknown. Therefore, this thesis **AIMED** to uncover the role of WNT pathways in TETs, using as **MATERIALS:** snap frozen TETs, normal thymic tissue; primary epithelial cells (pTECs) derived thereof; and the thymic carcinoma cell line, 1889c. **METHODS**: 2D and 3D cell cultures; molecular techniques, including qRT-PCR, western blots, shRNA knockdown technology; several functional in-vitro readouts; immunohistochemistry; statistical methods.

RESULTS: Among all WNT ligands and receptors tested, WNT4 and its receptor, FZD6 stood out as most significantly differentially expressed ligand/receptor pair in aggressive TETs (and their pTECs) as compared to non-neoplastic thymuses (NTs) and indolent thymomas. In contrast to rare historic studies, the non-canonical WNT4/PCP/JNK pathway was mainly activated in aggressive TETs, and previous reports about nuclear accumulation of β-catenin in TETs were not confirmed. Paradoxically, like in involuted thymuses, strong expression of endogenous canonical WNT inhibitors (TCF4) was common among TETs. A WNT4 autocrine loop was found to contribute to WNT/PCP noncanonical activation in aggressive TETs, promoting the proliferation and extended survival of respective pTECs. This loop depended on exogenous (e.g. recombinant) WNT4 in 2D cultures but was self-sustaining in 3D spheroids for weeks. Surprisingly, WNT4 knockdown induced cell death mainly in aggressive TETs and lead to decreased expression of RAC1 but not JNK, since expression and phosphorylation of the latter was rescued by ‘compensatory’ activation of NF-κB and AKT. Pharmacological inhibition of NF-κB and AKT reduced phosphorylation of JNK and RAC1 in neoplastic pTECs.

CONCLUSION: Activation of non-canonical WNT4 signaling, including operation of an autocrine WNT4 loop, and expression of the non-canonical WNT receptor, FZD6

are typical of aggressive TETs and likely linked to their oncogenesis, offering novel therapeutic perspectives.

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7. APPENDIX

7.1 List of figures

7.2 List of tables

8. CURRICULUM VITAE

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Education

10.2017-09.2020	Medical doctoral study of oncology, Medical Faculty of Mannheim, Heidelberg University, Germany
09.2016-09.2017	Translational Medical Research, Master of Science, Heidelberg University, Germany
09.2014-06.2016	Department of Respiratory, Master of clinical medicine, Anhui Medical University, China
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Publication

03.2016	*"Diagnostic and prognostic value of Tenascin-C and CEA in malignant pleural effusion." (Journal of Clinical Pulmonary Medicine, 2016)*
06.2017	*"Neoadjuvant chemotherapy followed by surgery versus upfront surgery in non-metastatic non-small cell lung cancer: systematic review and meta-analysis of randomized controlled trials" (Oncology, 2017)*

Experience

01.2017-09.2017	Master Thesis Department of Anesthesiology, Medical Faculty of Mannheim, Heidelberg University Supervisor: PD Dr. Holger A. Lindner Research Topic: "From data analysis to an in vitro model of sepsis: Analysis of transcriptional and phenotypic signatures in granulocytes"
10.2017-06.2021	**Medical doctor study** Department of Pathology, Medical Faculty in Mannheim, Heidelberg University Supervisor: Prof Dr. Marx Alexzander, Dr.ret.Djeda Belharrazem **Research Topic:** "Investigation of WNT pathways in thymic tumors"

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