Ph.D. thesis

The role of Wnt signalling in thymic senescence

Zoltán Varecza M.Sc.

Ph.D. Supervisor: Dr. Judit E. Pongrácz

Ph.D. Program Leader: Prof. Dr. Péter Németh

University of Pécs

Institute for Immunology and Biotechnology

Department of Medical Biotechnology

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1. Contents

2. Abbreviations

Abs-	Antibodies
ADRP-	Adipose differentiation-related protein
ANKRD-	Ankyrin Repeat Domain
AP-1-	Activator protein-1
APC-	Adenomatous polyposis coli
BMP-	Bone morphogenetic protein
CaMKII-	Ca-Calmodulin Kinase II
CTGF-	Connective tissue growth factor
DAG-	Diacylglycerol
DKK-	Dickkopf
DN1, 2, 3	Double negative
DP-	Double positive
Dvl-	Dishevelled
DX-	Dexamethasone
EpCAM1-	Epithelial cellular adhesion molecule
Fz-	Frizzled
GH-	Glucocorticoid hormone
GSK-3-	Glycogen synthase kinase 3
IER-	Immediate-early response
IGF-I-	Insulin like growth factor 1
JNK-	c-Jun N-terminal kinases
LAP2α-	Lamina associated polypeptide
LEF1-	Lymphoid Enhancer-binding Factor 1
LRP5/6-	Low density lipoprotein Related Protein 5 and 6
MHC-	Major Histocompatibility Complex
NFAT-	Nuclear Factor of Activating T- cells
NFκB-	Nuclear Factor kappa B
Nkd-1,Nkd-2-	Naked 1, 2
NLK-	Nemo-Like Kinase
pAb-	Polyclonal antibody
PCP-	Planar cell polarity pathway

PCR-	Polymerase chain reaction
PKC-	Protein kinase C
PLC-	Phospholipase C
PPARγ	Peroxisome proliferator-activated receptor
qRT-PCR-	Quantitative real-time polymerase chain reaction
RT-	Reverse transcription
SEMA-	Semaphorin domain
TAK1-	Transforming growth factor β–Activated Kinase 1
TCF1, TCF3-	T-cell Factor 1, 3
TCR-	T-cell receptor
TEC-	Thymic epithelial cell
TMB-	3,3’,5,5’-Tetra-Methyl-Benzidine

3. Introduction

3.1. Ageing in focus

3.1.1. Ageing and society

Ageing of the population is one of the most important challenges for the developed world to face over the next fifty years. The current demographic trends and consequent shrinkage of the active workforce will put enormous pressure on the financing of social protection and health systems, which likely to reduce living standards. Coupled with increased migration and emergence of novel infectious diseases, broad-scale provision of immunological protection by vaccination constitutes a strategic aim for longer and healthier lifespan.

3.1.2. Ageing of the immune system

Impaired immunological responsiveness in the elderly poses a major difficulty for achieving efficient immunization. The immunological competence of an individual is determined by the presence of mature lymphocytes formed in primary lymphoid organs, and specialized secondary lymphoid tissues performing diverse immune responses. Thus at systems level the maintenance of immunological equilibrium requires steady lymphocyte output, and controlled expansion coupled with continuous replacement. Lymphostromal interactions in both primary and secondary lymphoid tissues play essential roles in the development and function of lymphocyte subsets in adaptive immune responses. The thymic and lymphnode stromal microenvironments thus represent key elements in the development of the adaptive immune system. Consequently, impairment of the lymphoid microenvironment will ultimately lead to insufficient primary and secondary immune responses or to the decline of thymic selection, manifesting in late-onset autoimmune disorders, often observed in elderly. Self-tolerant cytotoxic and helper T-lymphocytes, the crucial regulator cells in adaptive immune responses, develop in the specialized epithelial network of the thymus. The thymus, however, gradually loses its capacity to support lymphopoiesis in a programmed involution process that results in a decline of *de novo* T-cell production.

3.1.3. Significance of thymic involution studies

In contrast to the extensive studies addressing haematopoietic cells, the in-depth analysis of determinants for stromal competence during immunological ageing is largely absent, despite its clear significance related to immunological responsiveness in the elderly. A more thorough understanding of molecular mechanisms responsible for stromal senescence of the thymus may lead in aged individuals for the restoration of immunological competence by a controlled reversion of the involuted thymic epithelium, and consequent strengthening capacity of peripheral lymphoid tissues to support T-cell dependent antibody-mediated immune responses.

3.2. T-cell development in the thymus

T-cell progenitors migrate to the thymus from the bone marrow where they undergo an extensive differentiation and selection process. After entering the thymus, thymocytes representing different stages of development occupy distinct regions of the thymus. The earliest $CD4^-CD8^-CD44^+CD25^-$ thymocyte progenitors, referred to as double negative 1 (DN1) cells are found near their site of entry at the cortico-medullary junction. The slightly more mature $CD4^-CD8^-CD44^+CD25^+$ (DN2) subset is found throughout the cortex, whereas $CD4^-CD8^-CD44^-CD25^+$ (DN3) subset is concentrated below the capsule. Following rearrangement of antigen receptor (TCR) genes (He and Kappes 2006) $CD4^+CD8^+$ (double positive or DP) thymocytes undergo positive (functional TCR) and negative (self-reactive TCR) selection in the cortex and medulla, to finally as $CD4^-CD8^+$ (cytotoxic) or $CD4^+CD8^-$ (helper) single positive (SP), mature, naïve T-cells leave the thymus for the periphery. Figure 1 shows a schematic diagram of T-cell development.

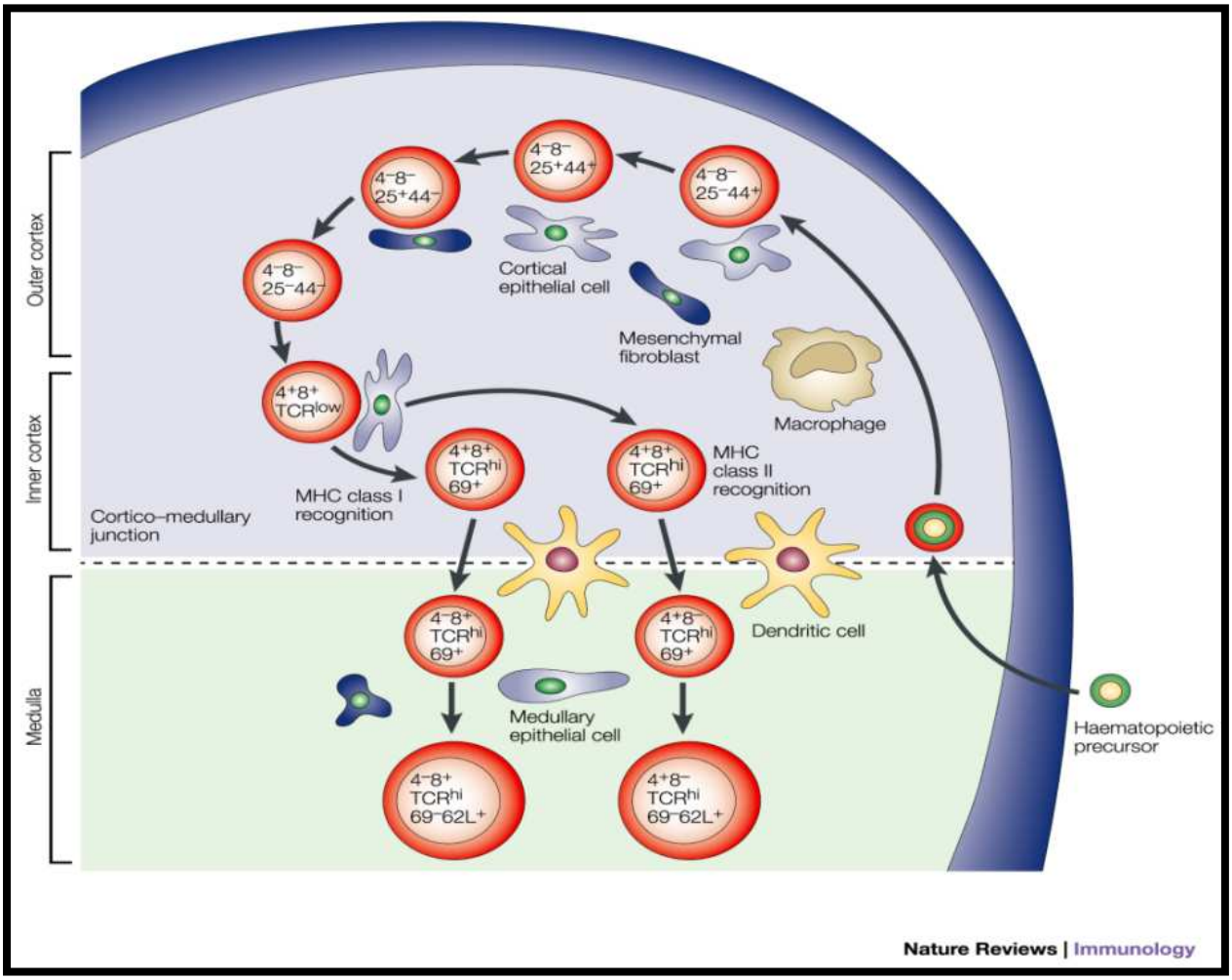


Figure 1. T-cell development in the thymus (Nature reviews: Immunology)

3.3. The role of thymic microenvironment in *de novo* T-cell production

Successful T-cell development requires the interaction of thymocytes with the thymic stroma, which creates the special thymic microenvironment for T-cell differentiation and selection. A large proportion of the thymic stroma consist of epithelial cells that develop from the epithelial thymic anlage from the third pharyngeal pouch around embryonic day 10-11 in the mouse (Manley 2000). Following several differentiation steps, including expression of FoxN1 -a member of the forkhead transcription factor family (Mandinova, Kolev et al. 2009)– that is essential for $Mts24^{+}$ epithelial progenitors (Bennett, Farley et al. 2002; Gill, Malin et al. 2002) to develop into various epithelial subsets (Dooley, Erickson et al. 2005) and to establish the special thymic epithelial cell phenotype (Manley 2000). FoxN1 expression in early stages of thymus organogenesis is regulated by secreted Wnt4 (Balciunaite, Keller et al. 2002) protein. The mature thymic epithelium consist of two major compartments, the cortex and the medulla, which apart from producing chemokines that attract haematopoietic stem cells to the thymus, also contribute the special thymic microenvironment. The

thymic epithelial network through release of cytokines (e.g. interleukin-7(Alves, Richard-Le Goff et al. 2009)), secretion of extracellular matrix components, and establishment of adhesive interactions (Crisa L, Cirulli V et al. 1996) (Schluns KS, Cook JE et al. 1997) regulate homing, intrathymic migration, and differentiation of developing T-lymphocytes. Thymocytes bearing diverse TCR repertoire are selected on MHC (major-histocompatibility-complex) and MHC bound-antigens presented to them by the thymic stroma, including epithelial cells. During T-cell development, which is characterised by the progression through phenotypically distinct stages (Lind, Prockop et al. 2001), thymocytes occupy spatially restricted domains of the mature thymus. T-cell precursors enter the thymus at the cortico-medullary junction (Blackburn and Manley 2004), then migrate to the subcapsular zone of the outer cortex, back through the cortex, then to the medulla, where they finally exit to the periphery (Blackburn and Manley 2004). Functional studies have shown, that the cortex is important in producing chemokines, which attract pro-thymocytes (Bleul and Boehm 2000) and are also essential for mediating positive selection (Anderson, Owen et al. 1994). Meanwhile the medullary epithelium has been implicated in driving the final stages of thymocyte maturation (Ge and Chen 2000) and has a crucial role in tolerance induction (Farr and Rudensky 1998; Derbinski, Schulte et al. 2001). Additionally, the thymic epithelium is also the source of other secreted and cell surface proteins that regulate T-cell development. These proteins include bone morphogenic protein (BMP) (Bleul and Boehm 2005), Notch (Valsecchi C 1997), and Wnt (Pongracz, Hare et al. 2003) family members.

3.4. Thymic involution during ageing

In comparison to other organs, ageing of the thymus is an accelerated process in all mammals. In humans, thymic senescence begins early, around late puberty and by 50 years of age 80% of the thymic stroma is converted to adipose tissue (Dixit 2010). As the thymic epithelium is replaced by adipose tissue, the whole process is called adipose involution (Marinova 2005). Due to decrease in thymic epithelial tissue mass, the thymus can no longer support the same output of T-cell production (Ribeiro and Perelson 2007). T-lymphocyte composition in the periphery therefore exhibits the dominance of memory T-lymphocytes resulting in impaired responses towards novel, particularly viral infections (Chidgey, Dudakov et al. 2007; Gui, Zhu et al. 2007; Grubeck-Loebenstein 2009). Since the thymic epithelium has a key role in deleting auto-reactive T-cell clones, functional impairment increases the chances of developing auto-immune disease (Hsu and Mountz 2003). One of the transcription factors, FoxN1 that is characteristic in thymus development is also affected by age. FoxN1 (Mandinova, Kolev et al. 2009) is not only essential for progenitor epithelial cells of the thymic rudiment to develop into various epithelial subsets (Dooley, Erickson

et al. 2005) but also to maintain TEC (Thymic Epithelial Cell) identity in the differentiated, adult thymus. Decreased levels of FoxN1 expression in the adult TECs result in accelerated thymic involution (Chen, Xiao et al. 2009; Cheng, Guo et al. 2010).

3.5. Trans-differentiation of fibroblasts into adipocytes

The nuclear lamina consists of a two-dimensional matrix of proteins located next to the inner nuclear membrane. The lamin family of proteins make up the matrix and are highly conserved in evolution. The family of lamin associated proteins (LAP) has several members with similar functions. Studies with fibroblast cells have revealed that fibroblast to pre-adipocyte transformation is strongly connected to LAP2α, the member of the LAP2 protein family (Dorner, Vlcek et al. 2006). While most splice variants associate with the nuclear envelope, LAP2α is involved in several nucleoplasmic activities including cell-cycle control and differentiation (Berger, Theodor et al. 1996; Hutchison, Alvarez-Reyes et al. 2001). LAP2α is synthesized in the cytoplasm and is then transported into the nucleus by a PKC-dependent mechanism (Dreger, Otto et al. 1999). The mere over-expression of LAP2α in fibroblasts is known to directly up-regulate PPARγ (Peroxisome proliferator-activated receptor γ) expression, an acknowledged marker and key transcription factor of pre-adipocyte differentiation (Dorner, Vlcek et al. 2006). In pre-adipocytes PPARγ expression is followed by an increase of ADRP expression (adipose differentiation-related protein) a known direct target gene of PPARγ. Although LAP2α over-expression alone initiates pre-adipocyte differentiation in fibroblasts, it is not sufficient to complete the adipocyte differentiation program in the absence of additional stimuli (Dorner, Vlcek et al. 2006).

3.6. Wnt signalling

3.6.1. Wnt molecules and pathways

The Wnt family of 19 secreted glycoproteins control a variety of developmental processes including cell fate specification, cell proliferation, cell polarity and cell migration. There are two main signalling pathways involved in the signal transduction process from the Wnt receptor (Frizzled) complex: the canonical or β-catenin dependent and the non-canonical pathway, which diverses into the planar cell polarity (PCP) or c-Jun-N-Terminal Kinase (JNK)/Activating Protein (AP1) dependent and the Ca^{++} or Protein kinase C (PKC)/Calmodulin Kinase (CaMKII)/Nuclear Factor of Activating T-cells (NFAT) dependent signalling pathways. Based on their ability to activate a particular Wnt pathway, Wnt molecules have been grouped as canonical (Wnt1, Wnt3, Wnt3a,

Wnt7a, Wnt7b, Wnt8) (Torres, Yang-Snyder et al. 1996) and non-canonical pathway activators (Wnt5a, Wnt4, Wnt11) (Torres, Yang-Snyder et al. 1996), although promiscuity is a feature of both ligands and receptors alike.

3.6.2. Canonical Wnt-pathway

The canonical or β-catenin/TCF dependent Wnt pathway is extensively investigated, and has been shown to be present in the thymus both in developing thymocytes (Ioannidis, Beermann et al. 2001; Staal 2001; Xu, Banerjee et al. 2003) as well as in the thymic epithelium (Balciunate, et al 2001, Pongracz et al 2003). Generally, in the absence of canonical Wnts, glycogen synthase kinase-3β (GSK-3β) is active and phosphorylates β-catenin in the scaffolding protein complex of adenomatous polyposis coli (APC) and axin (Ikeda 1998; Yamamoto 1999). The phosphorylated β-catenin is targeted for ubiquitination and 26S proteasome-mediated degradation, thereby decreasing the cytosolic level of β-catenin (Aberle 1997; Akiyama 2000). In the presence of Wnts, signals from the Wnt-Fz-LRP6 complex lead to the phosphorylation of three domains of Dishevelled (Dvl), a family of cytosolic signal transducer molecules (Noordermeer 1994). Activation of Dvl ultimately leads to phosphorylation and consequently inhibition of GSK-3β. Inhibition of GSK-3β results in stabilisation and finally cytosolic accumulation of β-catenin, which then translocates to the nucleus (Fig. 2), where is required to form active transcription complexes with members of the T-Cell Factor (LEF1, TCF1, TCF3, TCF4) transcription factor family (Staal and Clevers 2003) and transcription initiator p300 (Labalette, Renard et al. 2004).

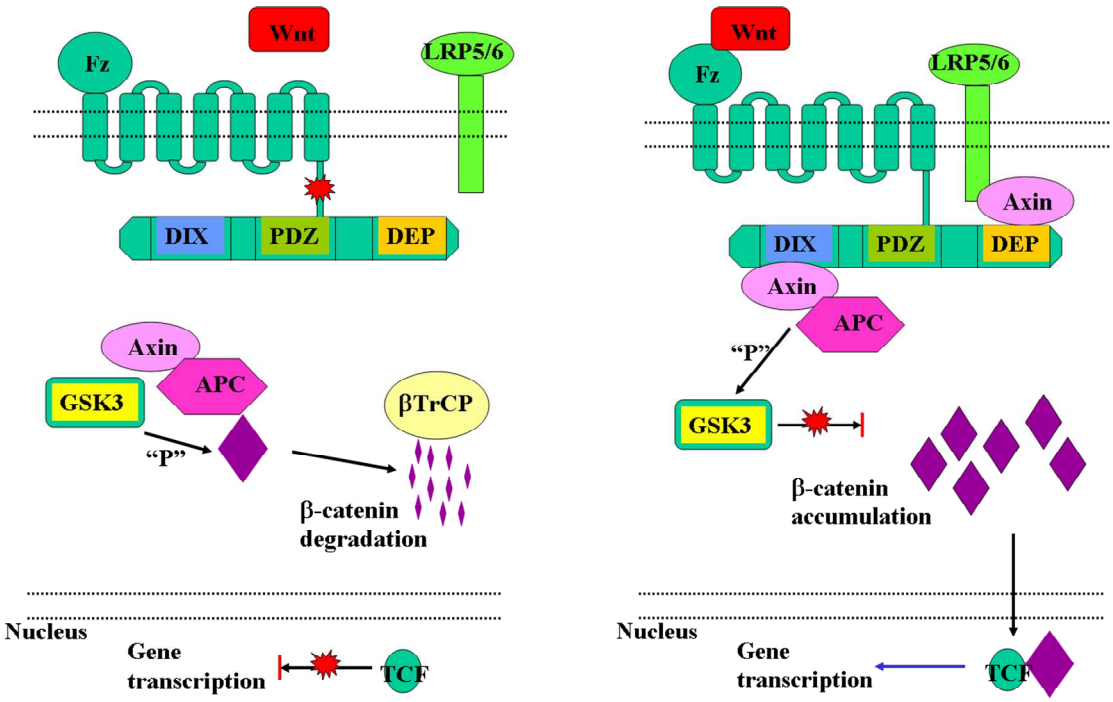


Figure 2. Canonical Wnt signalling (Pongracz&Stockley, 2006)

Successful assembly of the transcription complex leads to various target gene activation including cyclin-D1 (Shtutman, Zhurinsky et al. 1999; Tetsu and McCormick 1999), c-myc (He, Sparks et al. 1998), c-jun (Mann, Gelos et al. 1999), Fra-1 (Mann, Gelos et al. 1999), VEGFR (Zhang, Gaspard et al. 2001), etc. (Further target genes can be viewed at Nusse's Wnt website: http://www.stanford.edu/~rnusse/wntwindow.html).

3.6.3. Non-canonical Wnt-pathways

Generally, the two non-canonical signalling pathways are considered as regulators of canonical Wnt signalling and gene transcription. The two non-canonical Wnt pathways, the JNK/AP1 dependent, PCP (Polar cell polarity pathway) (Yamanaka, Moriguchi et al. 2002) and the PKC/CAMKII/NFAT dependent Ca^{++} pathway (Wang and Malbon 2003), just like the canonical Wnt pathway, become activated following the formation of Wnt-Fz-LRP6 complex (Fig. 3). Although the non-canonical pathways differ from the canonical pathway in their dependency on the type of G-proteins (Malbon, Wang et al. 2001), activation of Dvl, downstream of Frizzled, is critical for further signal transduction in both (Boutros, Paricio et al. 1998; Sheldahl, Slusarski et al. 2003). In further contrast to canonical Wnt signalling, phosphorylation of all three domains of Dvl, is not a requirement for transduction of non-canonical Wnt signals (Wharton Jr. 2001). Downstream of the cytosolic Dvl, the two non-canonical pathways activate different signalling cascades, which either involve JNK or PKC with CaMKII and trigger the transcription of different gene-sets. It has been proposed for non-canonical Wntsignalling receptors to be linked directly to heterotrimeric G-proteins that activate phospholipase-C (PLC) isoforms, which in turn stimulate inositol lipid (i.e. Ca^{++}/PKC) signalling. Growing evidence, however, indicates that G-proteins are functionally diverse and that many of their cellular actions are independent of inositol lipid signalling (Peavy, Hubbard et al. 2005), indicating high levels of complexity in both the PKC dependent and independent Wnt signalling cascades. The JNK dependent PCP pathway, partly shares target genes with the canonical pathway, including cyclin-D1 (Schwabe, Bradham et al. 2003) and matrix metalloproteinases (Nateri, Spencer-Dene et al. 2005). Certainly, canonical Wnt signals can be rechanneled into the JNK pathway *via* naturally occurring, intracellular molecular switches, like the Dvl inhibitors, Naked-s (Nkd-1, Nkd-2) (Yan, Wallingford et al. 2001) leading to AP1 rather than TCF activation. AP1 is not a single protein, but a complex of various smaller proteins, which can form homo- and heterodimers (cJun, JunB, JunD, cFos, FosB, Fra1, Fra2, ATF2, and CREB). The composition of the AP1 complex is a decisive factor in the activation of target genes therefore, regulation of AP1 composition is important. cJun and Fra1, two prominent members of the AP1 complex are targets of the canonical Wnt pathway (Mann, Gelos et al. 1999), indicating strong cross-regulation between the canonical and the non-canonical JNK dependent Wnt signalling cascades (Nateri, Spencer-Dene et al. 2005). While there are shared ligands (Rosso, Sussman et al. 2005; Wang, Shu et al. 2005) and target genes (Shtutman, Zhurinsky et al. 1999; Schwabe, Bradham et al. 2003) in the canonical and JNK dependent Wnt pathways, Ca^{++}/PKC dependent non-canonical signalling appears to be more independent of the other two pathways although cross-talk with both the β-catenin and the JNK pathways have been proposed (Kuhl, Geis et al. 2001).

Generally, Ca^{++} and PKC-dependent signals are frequently linked to AP1, NFκB and NFAT activation. Gene transcription, however, which is direct result of Ca^{++} dependent Wnt signalling has not been identified.

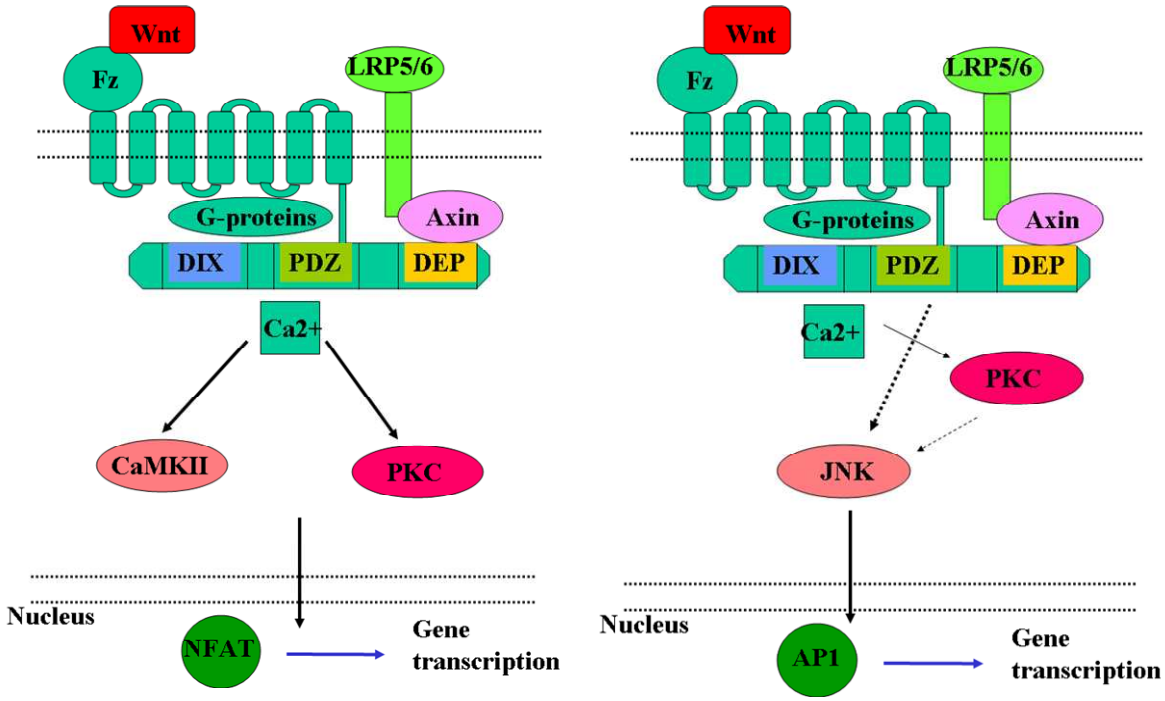


Figure 3. Non-canonical pathways of Wnt signalling (Pongracz&Stockley, 2006)

3.6.4. Inhibitory Wnt pathway

Besides the canonical and non-canonical Wnt pathways, inhibitory Fz pathways have also been described. Fz1 and Fz-6 are, for example, able to transduce inhibitory Wnt signals. While Fz1 inhibits Wnt signal transduction *via* a G-protein dependent manner (Roman-Roman, Shi et al. 2004) (Zilberberg, Yaniv et al. 2004), Fz-6 (Golan, Yaniv et al. 2004) inhibits Wnt dependent gene transcription by activating the Transforming growth factor β–activated kinase 1 (TAK1), a member of the MAPKKK family, and Nemo-Like Kinase (NLK) (Ishitani, Kishida et al. 2003; Smit, Baas et al. 2004) *via* a Ca^{++} dependent signalling cascade. NLK phosphorylates TCF that as a result cannot bind to β-catenin, consequently formation of the active transcription complex is inhibited (Smit, Baas et al. 2004) (Fig. 4).

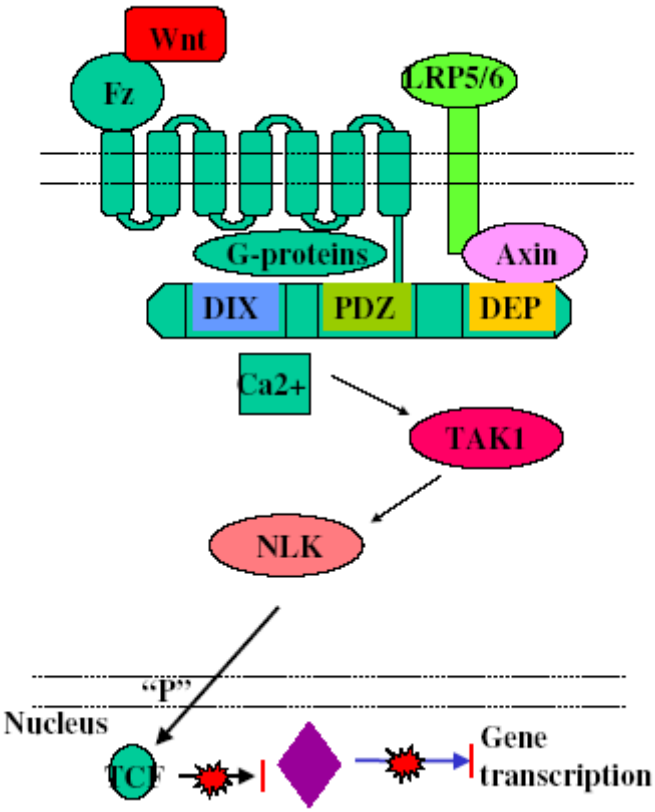


Figure 4. Inhibitory Wnt pathway (Pongracz&Stockley, 2006)

3.6.5. Wnts in ageing

As Wnts are important regulators of stem cell survival and differentiation, recent studies have started to investigate the Wnt family members in ageing. Most studies confirmed that drastically reduced Wnt levels can trigger ageing as tissue specific stem cells are depleted as a result of low Wnt signals. In contrast, the KLOTHO mouse, that carries a single gene mutation in KLOTHO, an endogenous Wnt antagonist also shows signs of accelerated ageing (Liu, Fergusson et al. 2007). It has been proposed that increased Wnt signalling leads to continuous stem cell proliferation which finally results in depletion of the stem cell pool causing accelerated ageing (Brack, Conboy et al. 2007).

3.6.6. Wnts in the thymus

The main source of Wnt glycoproteins in the thymus is the thymic epithelium, where 14 members of the Wnt family together with all 10 known Wnt receptors of the seven-loop transmembrane receptor family, Frizzleds (Fz) have been identified (Pongracz, Hare et al. 2003). That is a striking difference compared with thymocytes where developmentally regulated receptor expression is limited to Fz-5 and Fz-6 (Pongracz, Hare et al. 2003). The assembly of an active Wnt-Fz receptor complex also requires the presence of a co-receptor, the low density lipoprotein related protein 5 and 6 (LRP5/6) (Pinson 2000; Tamai 2000; Wehrli 2000), which is expressed both in thymocytes and thymic epithelial cells, indicating full ability in both cell types to respond to Wnt signals. Initial experiments, by manipulating the level of some Wnts and soluble Fz-s, have shown perturbation of T-cell development (Staal 2001; Mulroy 2002), highlighting the importance of Wnt dependent signalling for T-cell proliferation and differentiation, but not until data from Pongracz et al (Pongracz, Hare et al. 2003) revealed differential expression of Wnt ligands and receptors in thymic cell types, was it considered, that T-cell development may be influenced by indirect events triggered by Wnt signalling within the thymic epithelium. The canonical pathway has been shown to have an important role in thymocyte development regulating survival and differentiation (Ioannidis, Beermann et al. 2001; Staal 2001; Pongracz, Hare et al. 2003; Xu, Banerjee et al. 2003). In a thymic epithelial cell study, transgenic expression of cyclin-D1, one of the principal target genes of Wnt signalling, has lead to the expansion of the entire epithelial compartment (Klug, Crouch et al. 2000) suggesting that canonical Wnt signalling is involved in thymic epithelial cell proliferation, strengthening the argument, that thymic epithelial development is regulated by Wnts. So far, signalling studies have revealed, that Wnt4 can activate both the canonical (Lyons, Mueller et al. 2004) and the non-canonical (Torres, Yang-Snyder et al. 1996) (Chang, Sonoyama et al. 2007; Kim, Clark et al. 2009) Wnt-pathways.

3.7. PKC-s in the thymus

Members of the PKC family regulate a wide variety of cellular processes including proliferation (Clemens and Trayner 1992), differentiation (Clemens and Trayner 1992) and apoptotic death (Pongracz, Johnson et al. 1994; Pongracz, Tuffley et al. 1995). The PKC family comprises at least ten isoenzymes of serine/threonine protein kinases with a broad range of tissue distribution and differential cellular localisation (Dekker and Parker 1994). Based on their cofactor requirements for optimal catalytic activity, PKC-s are grouped into three main categories (Yamamoto, Takai et al. 1977; Saito, Kikkawa et al. 2002). While the classical (cPKC: α, βI-βII (splice variants) and γ), and

novel (nPKC: δI-III, ε, η, and θI-II) PKC-s bind diacylglycerol (DAG) that stimulates kinase activity, atypical (aPKC: ζ, PKMz (catalytic fragment of PKCζ), and ι/λ) PKC-s do not interact with DAG (Newton 2001). Ca^{++} is an additional requirement of cPKC-s but not for nPKC-s. Despite that most PKC-s are fully phosphorylated shortly after translation, they remain catalitically inactive due to the pseudosubstrate domain. Upon binding of lipid second messengers, like DAG and inositol triphosphate, two products of phospholipase-C (PLC) activity, the molecular conformation changes and the active centre of the enzymes become exposed and accessible for substrates. Catalytically active PKCs usually relocate from the cytosol to cellular or nuclear membranes (Giorgione, Hysell et al. 2003). Although PKC-s have been described as having a non-redundant role in signal transduction of various immune cell types including mature T-cells (Monks, Kupfer et al. 1997; Bi K, Tanaka Y et al. 2001) and developing thymocytes (Sun, Arendt et al. 2000; Michie, Soh et al. 2001), the expression of PKC family members and their function in the thymic epithelium remains obscure.

3.8. PKCs in Wnt signalling

Due to complexity of Wnt signalling as well as PKC activation and regulation, studies targeting PKC involvement in Wnt signalling are limited. Wnts of the Ca^{++} -dependent pathway have been demonstrated to trigger PLC activity that is responsible for the generation of lipid second messengers, like DAG and inositol phosphates (Cai Y, Stafford LJ et al. 2005). Recently, the orphan nuclear receptor RORα-mediated inhibition of canonical Wnt signalling has been identified (Lee, Kim et al.) to directly involve PKC. Inhibition of Wnt/β-catenin target genes is achieved by Wnt5a/PKCα-dependent phosphorylation of RORα on serine-35 residue. Additionally, Wnt-5a can mediate chondro-stimulatory effect of TGF-β3 through upregulation of PKCα and p38MAPK signalling (Jin, Park et al. 2006). While PKCδ has been identified as an essential activator of Dvl function (Kinoshita, Iioka et al. 2003) a main signal transducer from Fz-s, PKCζ is proposed to regulate GSK-3 phosphorylation and activity (Ossipova, Bardeesy et al. 2003). As GSK-3 mediated phosphorylation leads to proteosomal degradation of β-catenin (Moon, Bowerman et al. 2002), PKCζ modulates the activity of the canonical Wnt pathway (McManus, Sakamoto et al. 2005) (Fig. 5).

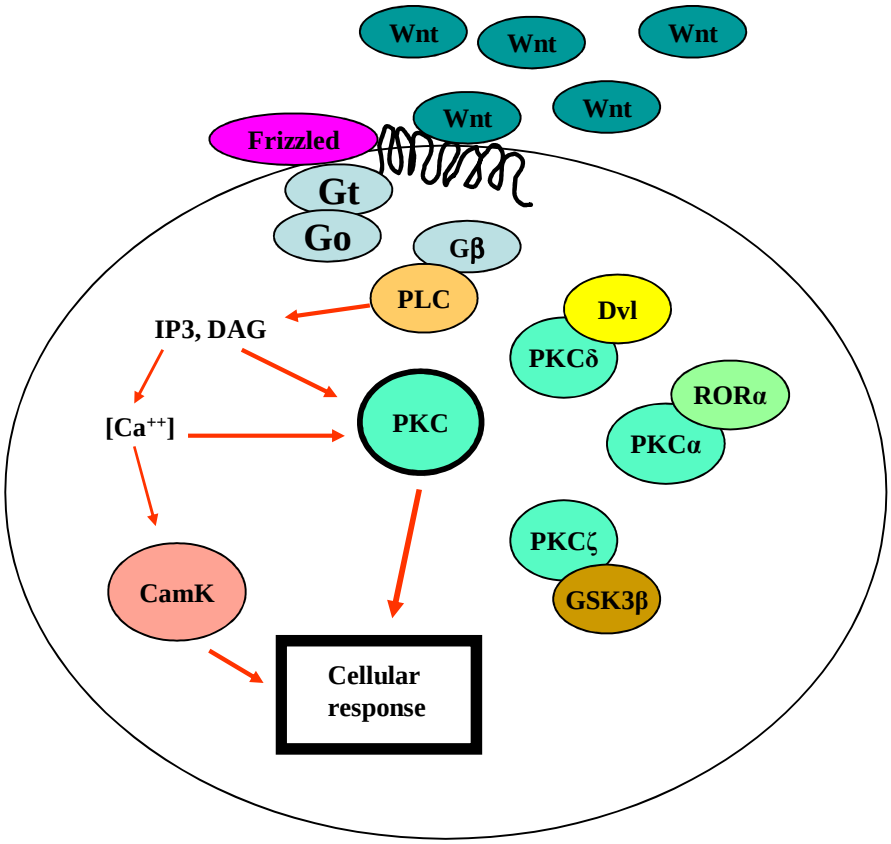


Figure 5. The role of PKC isoenzymes in signalling pathways

3.9. Steroids and ageing

Physiological steroids are implicated in the regulation of ageing. For example both surgical or chemical castration have been demonstrated to decrease the progression of ageing (Qiao, Chen et al. 2008) indicating that high steroid levels would accelerate the ageing process. Still, steroids used in therapy have not been fully investigated for their effects on ageing. Autoimmune diseases and haematological malignancies are treated by steroids, as they effectively promote apoptosis of leukaemia cells and trigger complex anti-inflammatory actions (Stahn, Lowenberg et al. 2007). Apart from triggering decreased expression of cytokines and MHC class II (MHC II) molecules, glucocorticoid (GC) analogues also induce apoptotic death of peripheral (Wust, van den Brandt et al. 2008) and developing T-cells. In mouse models, GCs cause massive thymocyte depletion, especially in the $CD4^{+}CD8^{+}$ (DP) thymocyte population, (Wiegers, Knoflach et al. 2001; Berki, Palinkas et al. 2002; Jondal, Pazirandeh et al. 2004) blocking *de novo* T-cell production. Experiments have also demonstrated that high-dose GCs induce a dramatic (Blomgren and

Andersson 1970) and apoptosis-associated (Boersma, Betel et al. 1979) involution of the thymus, and not only thymocytes but also TECs are seriously affected (Dardenne, Itoh et al. 1986). Recent reports (Fletcher, Lowen et al. 2009) have highlighted that TEC depletion appears reversible, and thymic epithelial stem cells play an important role in this process.

4. Aims of the study

I. To study the role of Wnt signalling in physiological thymic senescence:

1. Wnt4 induced signalling and gene expression patterns were investigated in the thymic epithelium. Identification of potential molecular targets of Wnt4 can reveal proliferation, differentiation –especially trans-differentiation - patterns within the thymic epithelial network.

2. As Wnt4 can activate both canonical and non-canonical Wnt signalling pathways and regulate TEC identity, identification of signalling elements and their role -especially PKCδ- in Wnt4 signalling can aid better understanding of regulatory mechanisms of thymic senescence.

II. To compare physiological and induced thymic senescence:

3. Comparison of molecular mechanisms of physiological and GC induced thymic senescence can reveal, whether molecular features of the two processes are shared or independent. It can also help to identify molecular targets to alleviate side effects of GC therapy and to identify potential therapeutic targets to avoid down-regulation of *de novo* T-cell production.

5. Materials and Methods

5.1. Antibodies

5.1.1. Western blot analysis

For western blot analysis rabbit polyclonal anti-PKCδ (C-17), goat polyclonal anti-Dvl (all from Santa Cruz) and rat monoclonal anti-Fz-6 (R&D Systems) were used as primary and donkey HRP-conjugated anti-rabbit, donkey HRP-conjugated anti-goat (Santa Cruz) and rabbit HRP-conjugated anti-rat as secondary antibodies.

5.1.2. Fluorescent microscopy

For fluorescent microscopic studies primary Abs were: anti-PKCδ (658-676) pAb (Calbiochem), anti-Dvl (C-19) (Santa Cruz), anti-PKCδ (C-17) antibody (Santa Cruz), anti-Fz-4 and anti-Fz-6 (R&D systems Inc.,), anti-Ly51-PE (clone 6C3), anti-EpCAM-FITC (clone G8.8, American Type Culture Collection (ATCC)), DAPI (Serva), ER-TR7-PE (kind gift from professor William van Ewijk, Laboratory for Lymphocyte Development, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and LipidTox Red (Invitrogen).
Secondary antibodies were Northern Lights donkey anti-goat IgG-NL493, Northern Lights donkey anti-goat IgG557, Northern Lights donkey anti-rabbit IgG-NL557, Northern Lights donkey anti-rat IgG-NL493 and anti-rat-Ig-PE secondary antibody (all from R&D Systems).

5.2. Animals

Balb/c mice were purchased from Charles River Hungary Breeding Ltd. The mice were kept under standardized conditions where tap water and food was provided *ad libitum*. Animals were allowed to age for 1, 3, 6, 9, 12, 18 months. During this time mice received human care according to the Guide for the Care and Use of Laboratory Animals published by the NIH (USA), and the experiment was approved by the Animal Research Review Committee of the University of Pecs, Medical School (BA02/2000-2/2006).

5.3. Cell cultures.

5.3.1 Cell lines

TEP1 (thymic epithelial)(Beardsley, Pierschbacher et al. 1983) (Tanaka, Mamalaki et al. 1993) and 293 and Phoenix (PHX) human kidney epithelial cell lines were cultured in DMEM supplemented with 10% FCS and 100 µg of penicillin and streptomycin (PAA).

5.3.2 Primary thymic epithelial cells

Balb/c mouse thymi were the source of primary cell material. Primary TECs were purified based on their expression of EpCAM1 cell surface marker using anti-EpCAM1-FITC Ab and magnetic cell sorter (Miltenyi Biotech). Thymic lobes were from adult Balb/c mice at 24h, 1 week, 1, 3, 6, 9, 12 or 18 month(s) of age, and from 1.5 year old GFP-transgenic BALB/c-mice. The GFP-transgenic BALB/c model was created using lentiviral transgenesis (Kvell, Czompoly et al.).

5.3.3 Dexamethasone treatment of cells and animals

TEP1 cell line was maintained and used for experiments as described in chapter 5.3.1., Wnt4 over-expressing TEP1 cell line was generated as described in section 5.4.1. Cell lines were treated with DX (Sigma, dissolved in DMSO until use) with a final concentration of 1 µM for 1 week or solvent, respectively. After incubation, the reaction was stopped by placing the tubes in liquid nitrogen (for Western blots) or with ice-cold PBS-azide (for microscopy) (Bartis, Boldizsar et al. 2006). 4 week-old BALB/c mice were used for the experiments. Animals received a single dose (20 mg/kg) Dexamethasone (DX, Oradexon, Organon) injection intraperitoneally (i.p.) in PBS, then were sacrificed 24 and 168 hours after injection (control animals received PBS). Another group of mice received PBS and DX for 3 months, respectively. There was also a group of mice receiving once high dose DX, then continuously low dose DX (2 mg/kg) in every second day for a month, to mimic the therapeutic regimen of autoimmune diseases (Buttgereit, da Silva et al. 2002; Buttgereit, Straub et al. 2004)

5.4 Manipulation of gene expression

5.4.1. Retroviral Constructs

Wnt4: The Wnt4 sequence was purchased and subcloned from an Origene (Origene) vector containing human full-length Wnt4 cDNA.

LAP2α: The full-length murine LAP2α cDNA containing plasmid was a kind gift of Dr. Simon Amos (Institute of Haematology, Chaim Sheba Medical Center, Tel-Hashomer, Israel)

PKCδ: PKCδ sequence in a pHACE vector was a kind gift of Dr. Jae-Won Soh, Professor of Biochemistry at Inha University, Korea.

The GFP (mock), LAP2α or Wnt4 over-expressing TEP1 cell lines were generated using retroviral vectors that were prepared as described previously (Kvell, Nguyen et al. 2005).
Wnt4 and PKCδ sequences were amplified and cloned into the MIGRI retroviral vector (a kind gift from W.S. Pear, Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA). Retrovirus was produced by transfecting the plasmid DNA into the Phoenix packageing cell line (American Type Cell Culture Collection) using Lipofectamine 2000 (Invitrogen).

5.4.2. Transient transfection of siRNA PKCδ

siRNA specific for PKCδ was purchased from Santa Cruz. TEP1 cells were grown to 80% confluency then siRNA and control siRNA was delivered using Lipofectamine according to manufacturer's recommendation. PKCδ mRNA levels were monitored by qRT-PCR prior Wnt treatment.

5.5. Detection of gene transcription

5.5.1. cDNA generation

cDNA was generated both from cell lines and primary cells by isolating total RNA either by TRI-reagent (MRC) or by using an RNA isolating kit (Macherey Nagel). DNA contamination was eliminated by a DNA digestion step using RNase free DNaseI. cDNA was made using the high capacity RNA to cDNA kit (Life Technologies Inc.). Reverse transcription of 0.5 µg of total RNA was performed in 50 µl total volume using random hexamer primers.

5.5.2. Standard Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted using Reddymix (ABgene) master mix solution and target sequence specific primer pairs as described previously (Moore, Anderson et al. 1993). Samples were matched for β-actin to ensure equal cDNA loading. Primer sequences used in PCR reactions are summarized in Table1 (p. 28.).

5.5.3. Microarray analysis

Microarray Sample Preparation, Hybridization, and Image Analysis - Starting with 100 ng of poly(A)+ RNA, one round of RNA amplification was performed with the MessageAmp aRNA amplification kit (Ambion) using a 4:1 amino-allyl UTP: UTP ratio for aRNA incorporation. For each sample, 8 µg of aRNA was coupled to N-hydroxysuccinimidyl esters of cyanine-3 or cyanine-5 (Amersham Biosciences). Following a clean up step, treated and untreated aRNA samples with opposing cyanine labels were combined, concentrated, and treated with a fragmentation reagent (Ambion) according to the manufacturer's protocol. For each slide, 4 µg of both treated and untreated cyanine-labelled aRNA samples were combined with a hybridization buffer (2.3x SSC, 18 mM HEPES, 0.2 mg/ml bovine serum albumin, 0.6 mg/ml poly(A), 0.2% SDS), heat-denatured for 3 min at 95 °C, and applied to microarrays under a LifterSlip coverslip (Erie Scientific). The slides were placed in a hybridization chamber (Dietech) and incubated in a 63 °C water bath for 16 h. Following hybridization, the slides were successively washed in 0.6x SSC with 0.025% SDS, 0.05x SSC, and water then dried. The microarrays were scanned with an Axon 4000B scanner and

adaptive spot segmentation performed with GenePix Pro software (version 5.0) (Axon Instruments). For each treated sample, three independent replicate microarray experiments were performed.

Microarray Data Analysis - Triplicate dye-swap, background-subtracted median intensity values were used as input to the LIMMA analysis package in Bioconductor (Gentleman RC, Carey VJ et al. 2004), and average LOESS-corrected $\log_2$ ratios were used to estimate differential gene expression. Microarray and microarray data analysis was performed by the Center for Genomics, University of Debrecen, Hungary.

5.5.4. Real-time qRT-PCR

Using SYBR Green PCR master mix in reagents and 100 nM sequence specific primers (Table 1) , PCR reactions were set up in the ABI Prism 7900HT sequence detection system.(95 °C incubation for 10 min, then 40 cycles (95 °C/15 s; 60 °C/1 min)). Threshold cycles (C_T) for three replicate reactions were determined using Sequence Detection System software (version 2.2.2), and relative transcript abundance was calculated following normalization with a β-actin PCR amplicon. Amplification of only a single species was verified by a dissociation curve for each reaction.

5.6. Cell sorting

TEP1 cells were infected with recombinant retroviruses containing MIG-WT- PKCδ-GFP or Wnt4-GFP and cells were sorted based on GFP expression by FACSVantage Cell Sorter (BD). Sorted cells were collected for mRNA and protein extraction, microarray, qRT-PCR and Western-blot analysis.

5.7. PKCδ activation assay

Cells were lysed in RIPA buffer (Sigma–Aldrich) supplemented with protease, and phosphatase inhibitors (Sigma–Aldrich) and immunoprecipitated with rabbit anti-PKCδ (658-676) pAb Lot# D28896 (Calbiochem) and protein-G resin (Sigma–Aldrich) overnight at 4^{o}C. The kinase assay was performed using the HTScan Kinase –assay Kit (Cell Signaling Technology Inc.) using a biotinylated substrate peptide in the presence of PKCδ diluted in kinase buffer (25 mM Tris-HCl pH 7.5 containing 10 mM $MgCl_2$, 0.1 µM Na3VO4, 5 µM β-glycerophosphate, 2 µM dithiothreitol (DTT)). Active PKCδ kinase GST fusion protein (162ng/µl (54ng/well) was supplied to the kit as positive control. PKCδ specific activity was quantified in a colorimetric ELISA Assay using 96-

well streptavidin-coated plates (Lot# 70850 (Institute of Isotopes, Budapest, Hungary, Soft Flow Hungary Kft. Pecs, Hungary). Phosphorylation level of biotinylated substrates from each kinase reaction mixes were measured using a rabbit anti-phospho-antibody (1:1000) detected by a HRP-labelled anti-rabbit (1:1000) secondary antibody (both antibodies provided with the kit) in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The optical density (absorbance) was read in an iEMS Reader MF V2.9 (Thermo Scientific) spectrophotometer using a bi-chromatic measurement system at 450nm and using 620 nm as reference.

5.8. Purification of proteins from cell membrane and cytosol

TEP1 cells were treated with Wnt4 containing SN-s (supernatants) for 5 minutes. Wnt4 treated cells as well as the Wnt4 over-expressing cell line were pelletted at 10000g for 1 min at 4 ^{o}C and resuspended in 500µl of ice cold buffer H (20 mM Tris pH 7,5 containing 0,25 M Sucrose, 10 mM DTT, 2 mM EDTA, 2 mM EGTA) supplemented with PMSF (1nM) and Leupeptin (100 µg/ml). Samples were sonicated on ice three times for 10 sec-s and pelletted (30 000g, 30min, 4^{o}C). Supernatants containing the cytosolic fraction were kept at -80 ^{o}C until used. Pellets were resuspended in 375 µl of ice cold buffer H supplemented with PMSF (1nM), Leupeptin (100 µg/ml) and 0,5% of Triton X-100 then vortexed. Samples were incubated on ice for 10 min then centrifuged at 30000g for 10min at 4^{o}C for collecting membrane proteins. Proteins of cytosolic and membrane fractions were separated on 10% SDS PAGE and PKCδ protein was detected using Western blotting.

5.9. Immuno-precipitation

TEP1 cells were lysed in RIPA-buffer (Sigma–Aldrich) supplemented with protease, and phosphatase inhibitors (Sigma–Aldrich) then anti-Fz-6 or anti-Fz-4 Abs and protein G resin (Sigma–Aldrich) were added to the protein mix and incubated overnight at 4^{o}C Resins were then pelleted, washed and prepared for western blotting.

5.10. Western blotting

TEP1 cells were collected (2000rpm, 5 min, 4^{o}C) and lysed in RIPA-buffer containing a Phosphatase Inhibitor Cocktail (Sigma-Aldrich) and RQ1 RNase free DNase (Promega).

After adding equal amount of 2× sample buffer (125mM Tris, 4% SDS, 10% glycerol, 0.006% Bromo-phenol-blue and 10% mercapto-ethanol), whole cell lysates were resolved in 10% SDS–PAGE. Gels were blotted onto nitrocellulose membranes using BioRad MiniProtean electrophoresis equipment (Bio-Rad), then blocked in TBS-T buffer (10mM Tris, pH 7.4, 100mM NaCl and 0.1% Tween 20) containing 3% fat-free dried milk. Membranes were then incubated in primary Ab (1:1000 dilution) for 2 h at room temperature, then washed three times in TBS-T and incubated in secondary Ab of HRP-conjugated donkey anti-rabbit Ig (1:1000 dilution). Proteins were visualised by enhanced chemiluminescence as described in the manufacturer's instructions (SuperSignal West Pico Chemiluminescent substrate (Pierce) and analysed by Fuji LAS4000 image station using different exposure times from 0.5 – 6 min for best quality.

5.11. Immuno-histochemistry

Frozen thymic sections (7-10 μm thick) were fixed in cold acetone or in 4% paraformaldehyde, then dried and blocked using 5% bovine serum albumin (BSA in PBS for 20 min) before staining with the appropriate antibodies for 30 min at RT. For histology fluorescent antibodies were used as described previously. The sections were analyzed by an Olympus Fluoview 300 confocal microscope with an Olympus Fluoview FV1000S-IX81 system or an Olympus BX61 microscope equipped with CCD-camera and AnalySIS software.

5.12. Statistical analysis

All experiments were performed minimum three separate occasions. Where appropriate, representative experiments or images are shown or Student t-test was performed and data are presented as mean ± 1 SD by error bars. Asterixes represent significant changes with ($*p<0.05$) unless otherwise mentioned.

Table 1. List of PCR primers

Gene	Forward primer	Reverse primer
β-actin	TGG CGC TTT TGA CTC AGG A	GGG AGG GTG AGG GAC TTC C
Wnt4 cloning primers	gaagatcttc ATGAGTCCCCGCTCGTGC	ccgctcgagcgg TCATCGGCACGTGTGCAA
Wnt4 PCR primers	CTC AAA GGC CTG ATC CAG AG	TCA CAG CCA CAC TTC TCC AG
CTGF	GGCCTCTTCTGCGATTTCG	CCATCTTTGGCAGTGCACACT
PKCδ	AGGCCGTGTTATCCAGATTG	CGGTTCATGGTTGGAAACTT
Fz-4	TCTGCTTCATCTCCACCACCTT	GCGCTCAGGGTAAGAAAACCT
Fz-6	GCGGCGTTTGCTTCGTT	CACAGAGGCAGAAGGACGAAGT
LAP2α	TGA ACT GCA GGC AGC TAA GA	TCA TAG CTA GAC TCT GAG G
Lamin1	TGA GTA CAA CCT GCG CTC AC	TGA CTA GGT TGT CCC CGA AG
PPARγ	CCC AAT GGT TGC TGA TTA CAA A	AAT AAT AAG GTG GAG ATG CAG GTT CT
ADRP	CGC CAT CGG ACA CTT CCT TA	GTG ATG GCA GGC GAC ATC T
E-cadherin	AAG TGA CCG ATG ATG ATG CC	CTT CAT TCA CGT CTA CCA CGT
N-cadherin	GTG GAG GCT TCT GGT GAA AT	CTG CTG GCT CGC TGC TT
FoxN1	Applied Biosystems TaqMan probe PN4351272 (Mm00477457_m1)	

6. Results

Summary of Results

I. Physiological thymic senescence

1. The mouse thymus undergoes morphological changes
2. Wnt4 is down-regulated while LAP2α is up-regulated during the process
3. Adipose involution is regulated by LAP2α, ADRP and PPARγ
4. Adipose involution is preceded by EMT marked by E-cadherin down-regulation
5. Wnt4 can reduce the expression of genes responsible for adipocyte-type trans-differentiation
6. Wnt4 receptors that transduce β-catenin pathway activator (Fz-4) and inhibitor (Fz-6) signals are up-regulated at early stages of senescence
7. PKCδ is activated by Wnt4 signals
8. PKCδ associates with both receptors but preferentially with Fz-6
9. Wnt4 target gene CTGF and one of its receptors, Fz8 are involved in a negative feedback loop regulating the canonical Wnt pathway

II. Steroid induced thymic senescence

1. Similarly to physiological senescence, Wnt4 is down-regulated while LAP2α is up-regulated during DX induced thymic involution

2. Wnt4 can protect against DX induced adipoid trans-differentiation

6.1. Physiological thymic senescence

6.1.1. Disintegration of the epithelial network

Senescence exhibits characteristic histological changes in both the human and mouse thymus (Oksanen 1971; Marinova 2005). In order to demonstrate this process in mice, thymic lobes of 1 month and 1 year old BALB/c mice were analyzed. In young adult mice, histology revealed strict segregation of epithelial cell compartments by staining for medullary ($EpCAM1^{++}$, $Ly51^{-}$) and cortical ($EpCAM1^{+}$, $Ly51^{++}$) epithelial cellular subsets. Thymic morphology shows high level of integrity just preceding puberty/early adulthood (Fig. 6). However, the highly organized structure disintegrates and becomes chaotic by the age of 1 year. By this age the previously shown strict cortico-medullary delineation becomes disintegrated, degenerative vacuoles appear surrounded by areas showing strong co-staining with both epithelial markers. There are also other large cellular areas that lack staining with either epithelial markers, a pattern completely absent at the young adult age. Staining of extracellular matrix components of fibroblast origin ($ER\text{-}TR7^{++}$) on cryostate thymic sections of 2 month and 9 month old BALB/c mice were performed to identify epithelial and mesenchymal elements in young adult and ageing thymic lobes. The above ages were selected to check additional time points and more precisely map the timeframe of thymic physiological senescence. The staining patterns are strikingly different at the two ages examined. In the 2 month old thymic tissue section, anti-EpCAM1 and anti-ER-TR7- show little tendency for co-localization. In stark contrast, by the age of 9 months anti-EpCAM1 and ER-TR7-staining show significant overlap within the thymic medulla, a phenomenon completely absent at earlier ages.

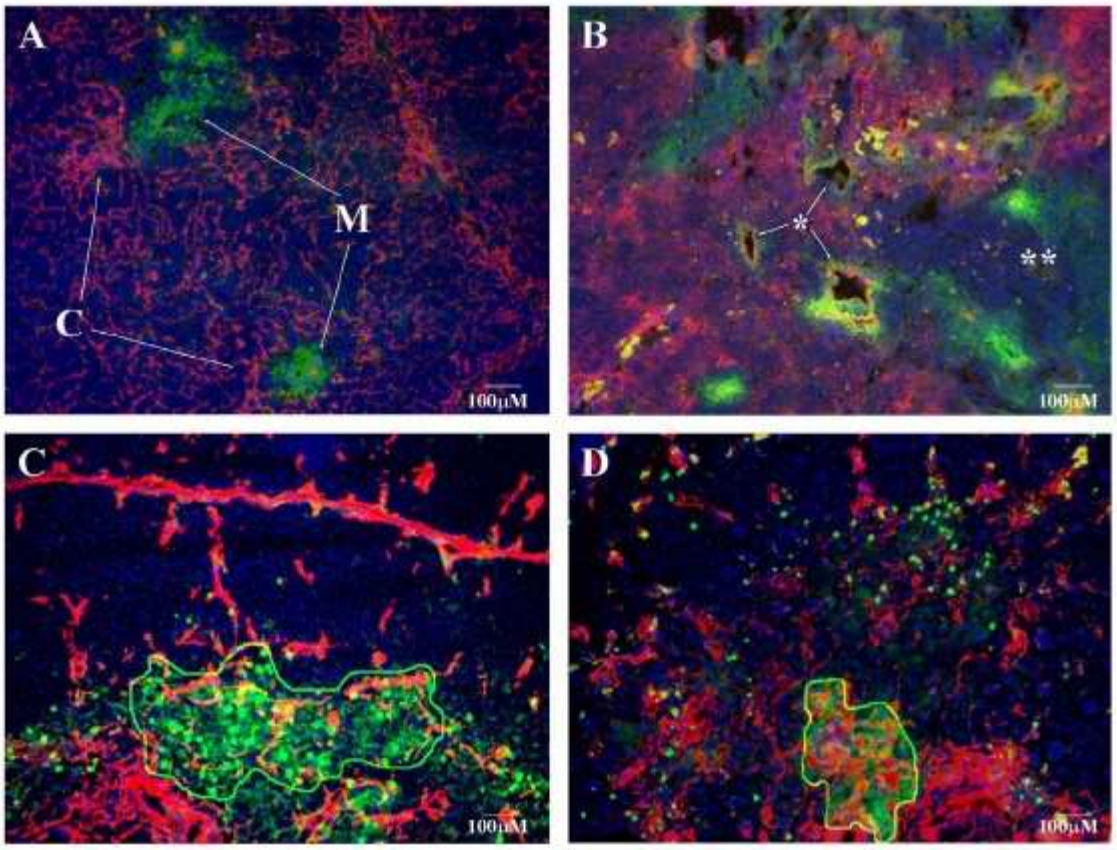


Figure 6. Disintegration of the epithelial network

Figure 6A demonstrates a cryostat section of 1 month, whereas figure 6B presents cryostat section of 1 year old BALB/c mouse thymus. Staining pattern: anti-EpCAM1-FITC (green), anti-Ly51-PE (red), DAPI (blue). 'M' marks medullary ($EpCAM1^{++}$, $Ly51^{-}$), while 'C' marks cortical ($EpCAM1^{+}$, $Ly51^{++}$) epithelial compartments on Figure 6A. Single asterisk (*) marks degenerative vacuoles, while double asterisk (**) mark the loss of epithelial staining on Figure 6B. Figure 6C (lower left) shows cryostate section of 2 month, whereas figure 6D (lower right) demonstrates cryostate section of 9 month old BALB/c mouse thymus. Staining pattern: anti-EpCAM1-FITC (green), anti-ER-TR7-PE (red), DAPI (blue). The $EpCAM1^{++}$ thymic medulla is outlined by continuous line on Figures 6C and 6D for easier visualization.

6.1.2. Adipose involution

To demonstrate how the disorganization of thymic epithelial network is followed by the emergence of adipocytes, thymic sections of 1.5 year old GFP-transgenic BALB/c mice were analyzed. This mouse strain develops and reproduces exactly like control BALB/c mice, and the thymic epithelial function and thymocyte maturation is indistinguishable from wild type controls (Kvell, Czompoly et al.). However, due to the ubiquitous and strong EF1 promoter-driven transgene transcription, bright GFP expression offers a native, green-coloured, cytoplasmic staining for all the cells in these mice. Thymic sections of senescent GFP-transgenic mice were co-stained with LipidTox Red to identify adipocytes. Histology shows the presence of relatively large, inflated cells in which the green-coloured (GFP-containing) cytoplasm is pushed to the periphery by red-staining neutral lipid deposits, a pattern characteristic of adipose cells (Fig. 7).

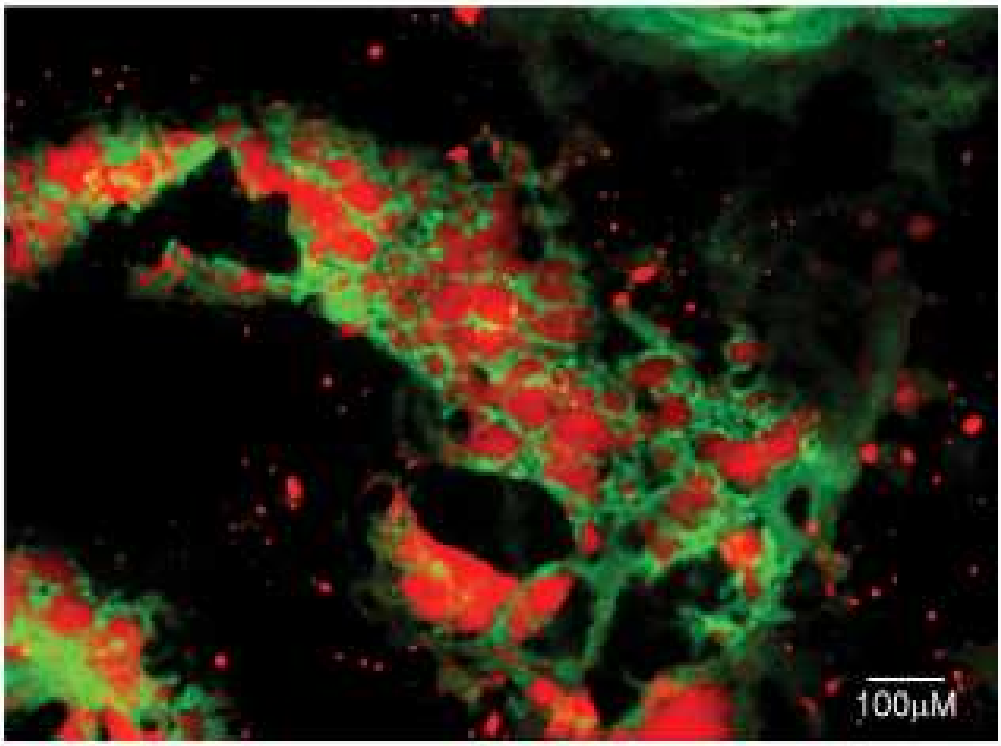


Figure 7. Adipose involution

Figure 7. shows adipose involution over cryostat section of 1.5 year old GFP-transgenic BALB/c mouse thymus. Staining pattern: GFP (green), LipidTox Red (red).

6.1.3. Gene expression changes in the thymic epithelium during ageing

To investigate the underlying molecular events of thymic epithelial senescence, gene expression changes were investigated in TECs purified from 1 month and 1 year old BALB/c mice based on EpCAM1 expression (MACS separation). Following cDNA synthesis, quantitative RT-PCR analysis was performed. Several genes including Wnt4, FoxN1, PPARγ, ADRP, lamin1 and LAP2α were tested. The expression of both Wnt4 and FoxN1 decreases in thymic epithelial cells. Highly decreased level (or total absence in some cases) of FoxN1 could be the consequence of strong Wnt4 down-regulation by the age of 1 year, indicating that TECs can down-regulate FoxN1 expression while maintaining that of epithelial cell surface markers like EpCAM1 (Balciunaite, Keller et al. 2002). At the same time, mRNA levels of pre-adipocyte differentiation markers PPARγ and ADRP rise with age in the same, EpCAM1-positive cell population. This finding is in harmony with histological data demonstrating the emergence of adipocytes in the thymic lobes of senescent mice. The expression of lamin1, a key component of the nuclear lamina remains unaffected during senescence in thymic epithelial cells; whereas, the expression of LAP2α increases significantly. This degree of dissociation between lamin1 and LAP2α expression is of note and suggests functional differences despite conventionally anticipated association of lamin1 and LAP2 molecular family members. The measured LAP2α up-regulation associated with age-related adipose involution is, however, in perfect agreement with other literature data suggesting the pre-adipocyte differentiation-promoting effect of LAP2α in fibroblasts (Dorner, Vlcek et al. 2006). The first report to show that such, normally fibroblast associated molecular changes occur in purified TECs was first performed in our laboratory.

As in the literature, epithelial mesenchymal transition (EMT) is associated with differential expression of E- (decrease) and N-cadherin (increase) (Seike, Mizutani et al. 2009). TECs were tested for these markers to investigate whether the first step towards pre-adipocyte differentiation is the EMT of epithelial cells. In purified TECs while E-cadherin mRNA levels significantly decreased, N-cadherin gene expression showed a slight increase during ageing, indicating that EMT might be the initial step in epithelial cell transition to become pre-adipocytes (Fig. 8).

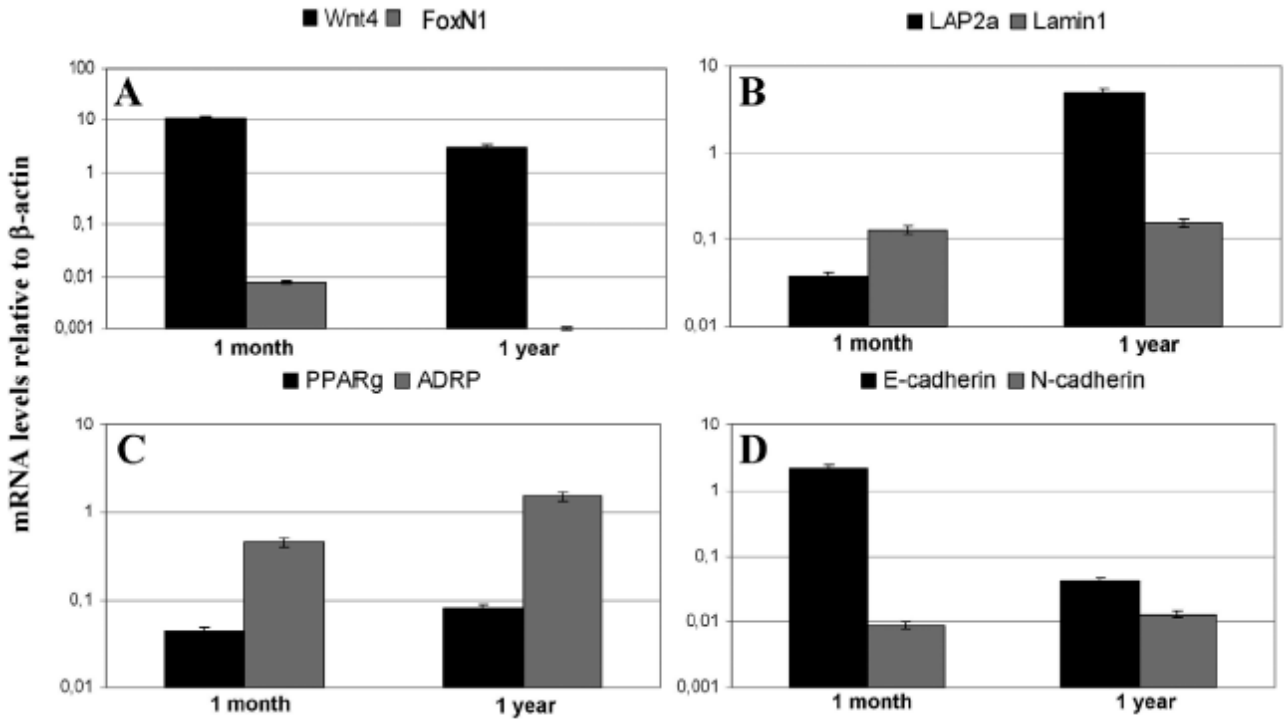


Figure 8. Molecular changes in thymic epithelium

Figures 8A–D demonstrate gene expression changes of MACS purified thymic epithelial cells measured by qRT-PCR. Please note that the Y-axis scale is logarithmic.

6.1.4. Studies of LAP2α and Wnt4 effects on TEC

To study the hypothesis that both LAP2α and Wnt4 play important but opposite roles in thymic senescence, stable LAP2α over-expressing or Wnt4-secreting transgenic TEP1 cell lines were established using lentiviral transgenesis. The use of a primary-derived model cell line provides the advantage of absolute purity, the complete lack of other cell types that could potentially affect the gene expression profile of epithelial cells (Beardsley, Pierschbacher et al. 1983). The established transgenic cell lines proliferated normally and did not show obvious signs of phenotypic changes (data not shown). In contrast to morphology, quantitative RT-PCR analysis revealed that LAP2α over-expression triggers an immense surge of PPARγ expression (Fig. 9). Such an increase in mRNA level suggests that this is not a plain quantitative, but rather a qualitative change. ADRP a direct target gene of PPARγ was also up-regulated although to a lesser extent. On the other hand in Wnt4-secreting TEP1 cells the mRNA level of both PPARγ and ADRP was decreased (Fig. 9). In the TEP1 cell line the expression of FoxN1 could not be addressed as it was very low and consequently undetectable (data not shown).

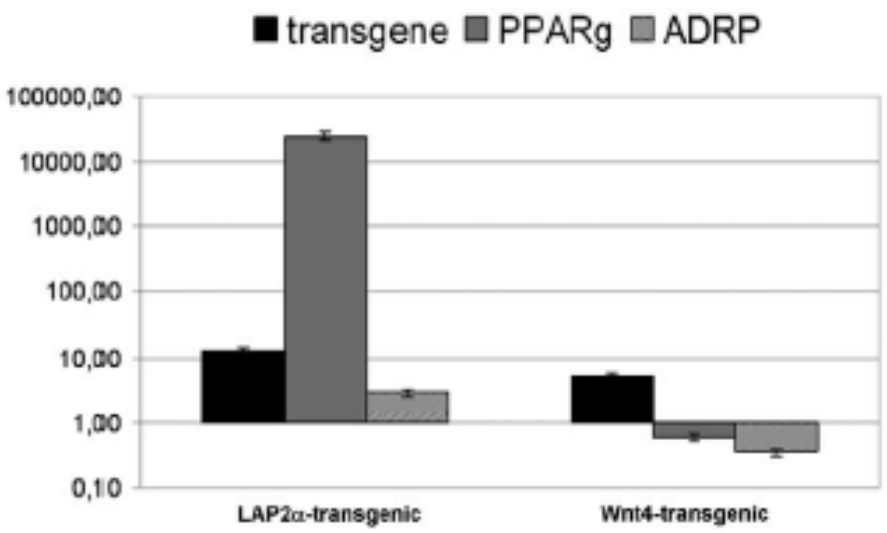


Figure 9. Gene expressions in stable LAP2α and Wnt4 over-expressing cell lines

Gene expression changes of LAP2α and Wnt4 over-expressing transgenic TEP1 cells measured by qRT-PCR. The Y-axis scale is logarithmic.

6.1.5. Fz-4 and Fz-6 levels are affected by age

Once the preventive role of Wnt4 was established in adipocyte-type trans-differentiation of TECs, receptor associated signalling studies have ensued to investigate what signal modifications can lead to Wnt4 effects. Initially, expression levels of Wnt4 receptors, Fz-4 and Fz-6 were analysed in thymi of 1 month and 9 month old Balb/c mice. qRT-PCR analysis of purified $EpCAM1^{+}$ TECs showed increased expression of both Fz-4 and Fz-6 mRNA (Fig. 10A and B) by 9 months of age. Immuno-histochemistry using Fz-4 and Fz-6 specific antibodies confirmed elevated levels of both receptor proteins (Fig. 11A-D). Additionally, differential expression pattern of Fz-4 and Fz-6 was also observed in the thymic medulla and cortex. While in the young thymus the medulla ($EpCAM1^{++}/Ly51^{-}$) was strongly stained for Fz-4 and Fz-6, the cortex ($EpCAM1^{+}/Ly51^{+}$) only faintly stained for these receptors. In the 9 month old thymus the medulla is less pronounced and in contrast to the young tissue, the whole section including the cortex became increasingly positive for both receptors.

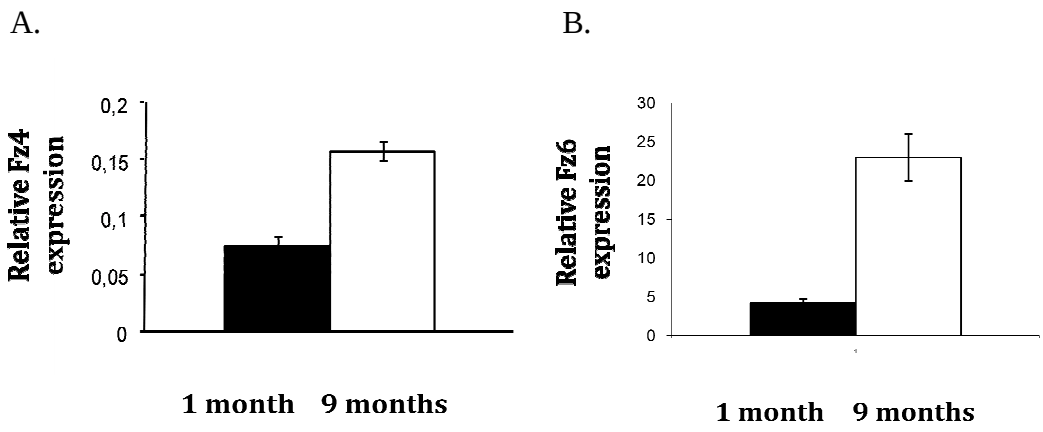


Figure 10. Fz-4 and Fz-6 expression during thymic senescence.

(A, B) QRT-PCR analysis of Fz-4 and Fz-6 expression in young (1month) and ageing (9 months) mouse thymic epithelium. Data was normalised to β-actin.

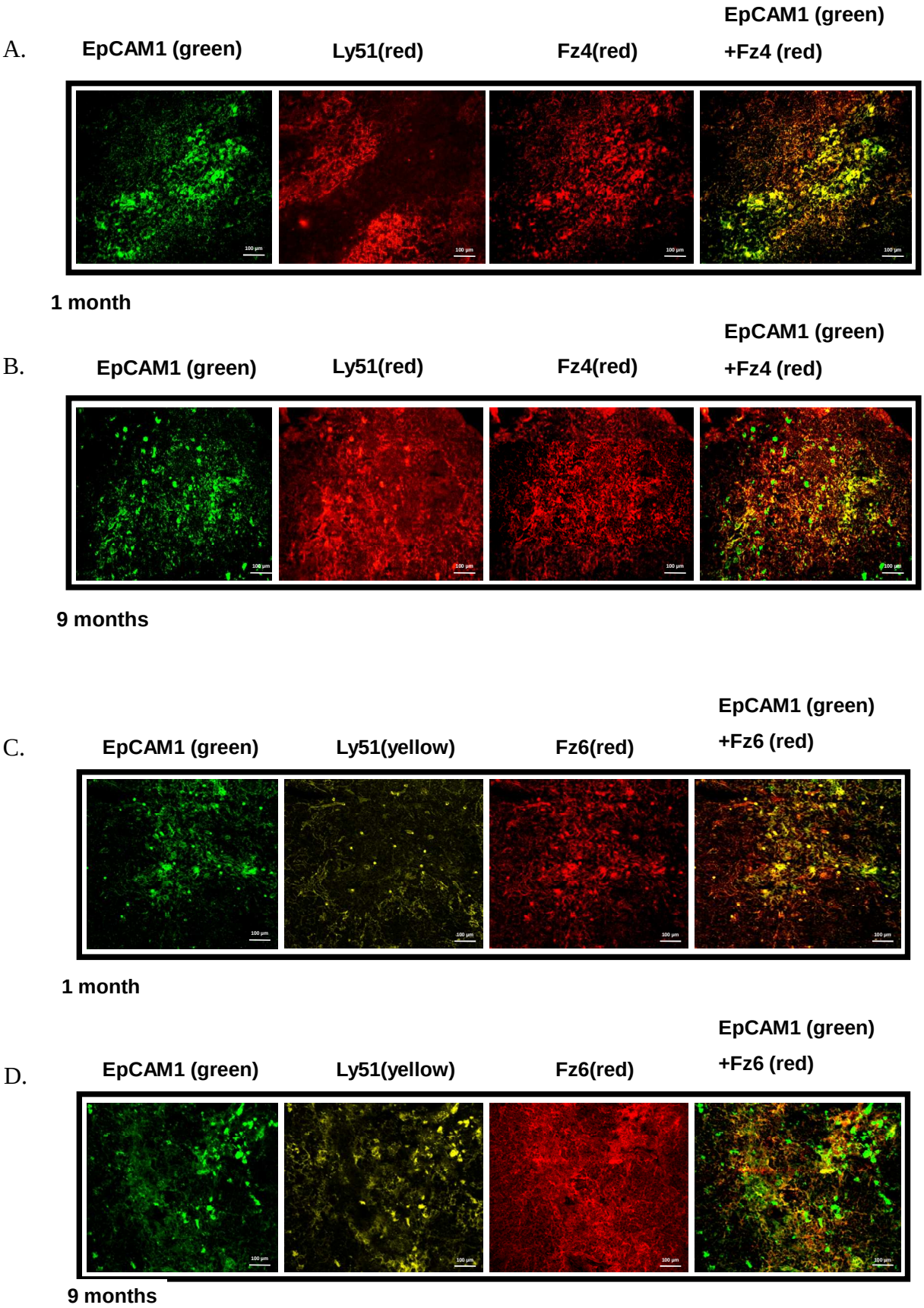


Figure 11. Changes in Frizzled receptor expression pattern during ageing

Figure 11. Changes in Frizzled receptor expression pattern during ageing (previous page)

Figure 11A-D shows the expression level and staining pattern of Fz-4 and Fz-6 assayed by histology using anti-Fz-4-NL663 and anti-Fz-6- NL663 antibodies, respectively. Thymic morphology was displayed *via* staining with anti-EpCAM1-FITC and anti-Ly51-PE TEC markers. Size marker is shown in the corner of the figure. Characteristic stainings are shown from a minimum of five repeats (bar=100μm).

6.1.6. Active receptor signalling is indicated by PKCδ translocation

As Wnt4 levels as well as its receptors are modulated during the ageing process, further studies were necessary to investigate active receptor signalling that is invariably associated with modified level of phosphorylation of receptor associated signalling molecules. As Fz-s associate with Dvls that are phosphorylated by the δ isoform of PKCs, PKCδ activity was investigated. Wnt4 overexpressing TEP1 cell line was established by retroviral transfection and validated by RT-PCR (Fig. 12A) and western blot analysis (Fig. 12B). Decreased level of β-catenin phosphorylation indicate stabilization of β-catenin that phenomenon is associated with canonical Wnt signalling. To test the involvement of PKCδ in Wnt4 signal transduction, increased Wnt4 levels were achieved by treatment using the supernatant of Wnt4-transgenic TEP1 cell line (Fig. 12C).

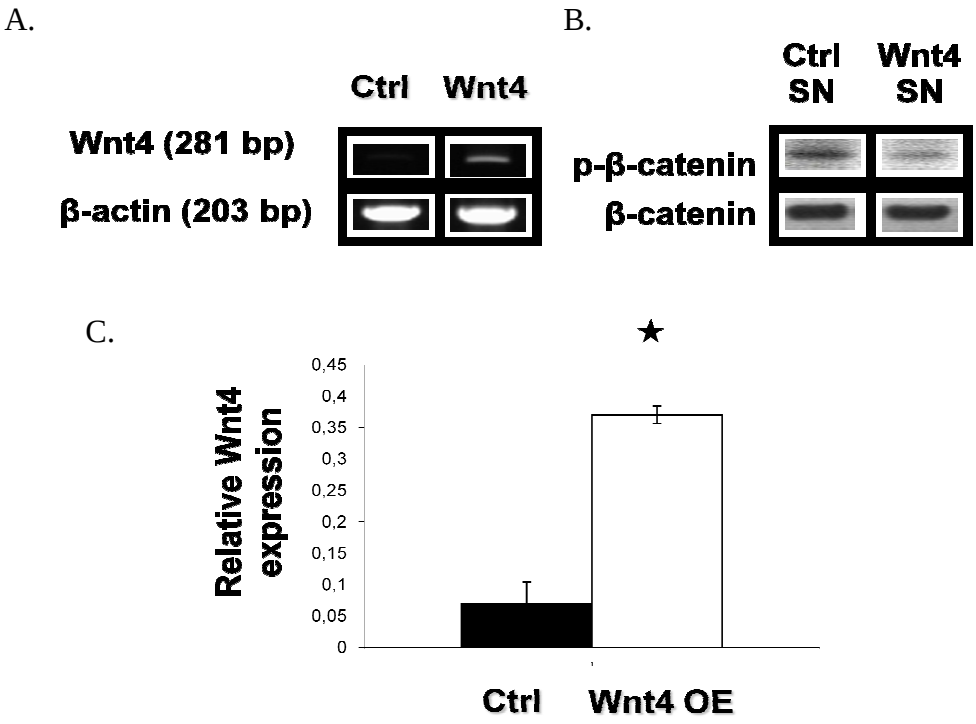


Figure 12. Generation of Wnt4 over-expressing TEP1 cell line by retroviral gene transfer

(A) RT-PCR analysis of Wnt4 over-expressing transgenic cells compared to mock (GFP) transfected TEP1 cells. (B) Verification of Wnt4 functionality by western blot analysis of p-β-catenin and total β-catenin levels. (C) qRT-PCR analysis of Wnt4 expression in Wnt4-GFP over-expressing compared to GFP transfected TEP1 cells. Statistically significant differences are marked by asterisks.

Wild type TEP1 cells were exposed to SNs of control (TEP1-GFP) and Wnt4 (TEP1-Wnt4-GFP) cells for 1 hr, then cytosolic and membrane fractions were isolated from cell lysates. Similarly to previous studies with Wnt-5a (Giorgione, Hysell et al. 2003), Western blot analysis revealed that within one hour of Wnt4 exposure PKCδ translocated into the membrane fraction (Fig. 13) where the cleavage products (Kanthasamy et al, 2006) characteristic of PKCδ activation were detected. Densitometric analysis of total and cleaved PKCδ demonstrated a 2 fold increase in PKCδ activation upon Wnt4 exposure. Additionally, increased membrane localisation of PKCδ was also detected (39 fold increase) in the Wnt4-overexpressing cell line (Fig. 13).

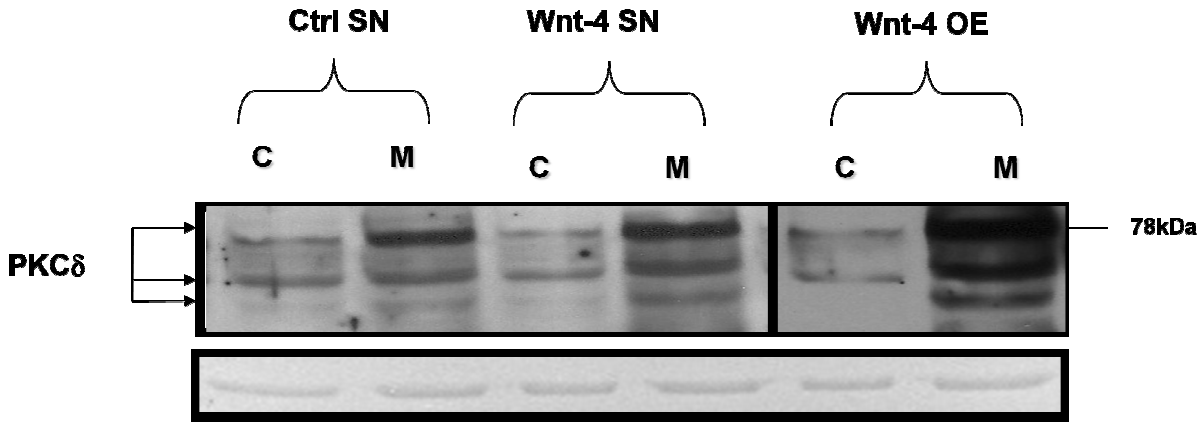


Figure 13. Intracellular translocation of PKCδ during active receptor signalling

Cytosolic and membrane proteins were separated from control, Wnt4 treated and Wnt4 overexpressing TEP1 cells. Western blot analysis demonstrated PKCδ translocation. Loading controls are shown below the Western blot as Ponceau red stained total protein. Representative blots are shown from three repeats.

As for Wnt4 specific receptor expression, both Fz-4 and Fz-6 levels increased with age, it was assumed that active receptor signalling might require more PKCδ during ageing. Indeed, apart from localisation of PKCδ to the membrane fraction (Fig. 13), up-regulation of PKCδ was also detected at both mRNA (Fig.14A) and protein level (Fig.14B and C) in the ageing thymi. Interestingly, a characteristic cortico-medullary PKCδ pattern has also emerged. In both young and ageing thymi PKCδ was preferentially expressed in the cortex ($EpCAM1^{+}/Ly51^{++}$) (Fig. 14B and C).

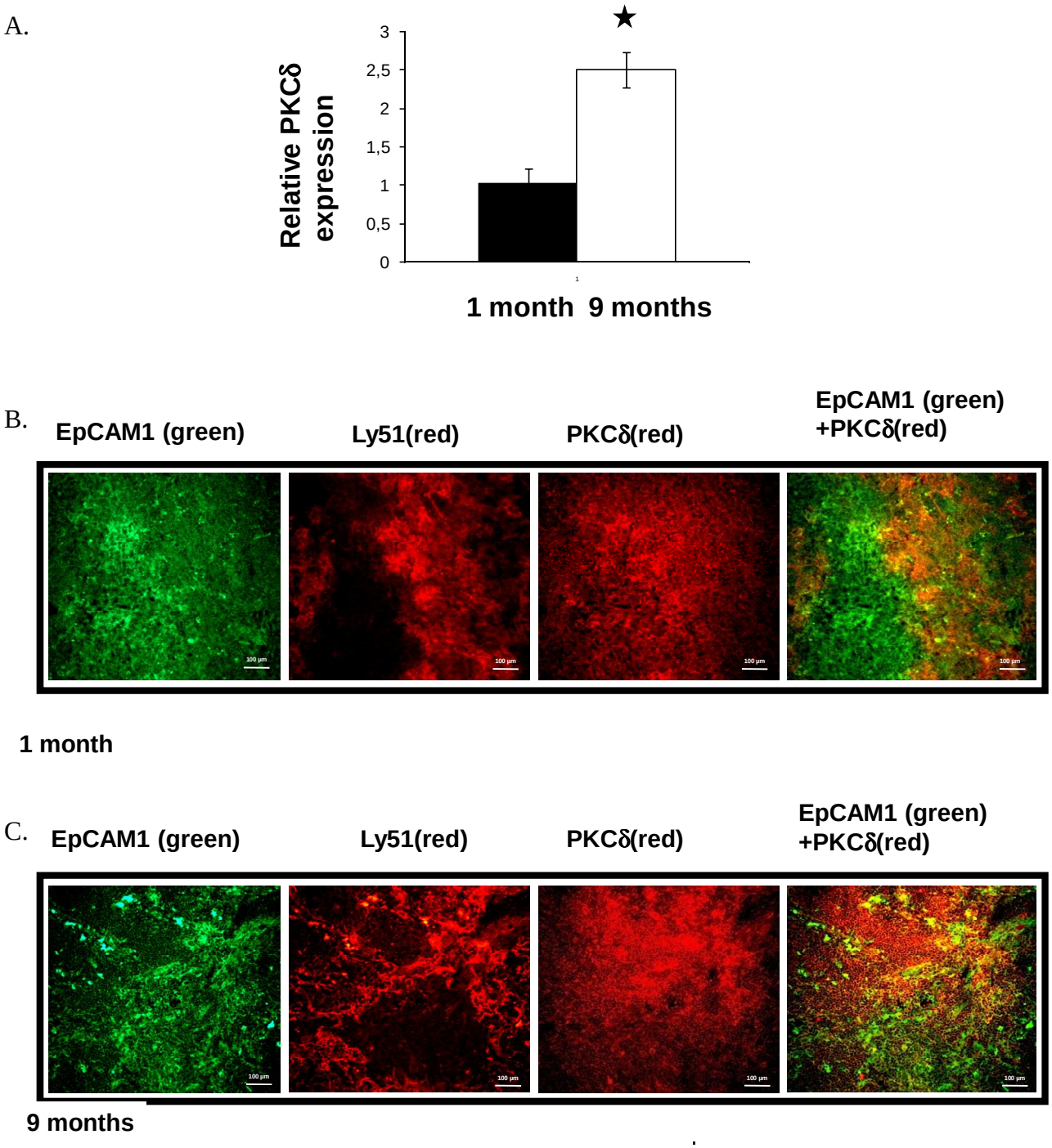


Figure 14. PKCδ expression pattern changes during ageing

(A) Gene expression of PKCδ changes with age by qRT-PCR. TECs were purified from young (1 month) and ageing (9 months) mice. Data were normalized to β-actin housekeeping gene. Statistically significant differences are marked by asterisks. (B, C) Age associated changes in PKCδ expression by histology. Cryostate sections of 1 month and 9 months old mouse thymi were stained with anti- PKCδ– NL663, anti-EpCAM1-FITC and anti-Ly51-PE. Characteristic stainings are shown from a minimum of five repeats (bar=100μm).

6.1.7. Identification of Wnt4 target genes in TECs using microarray analysis

To be able to identify molecular interactions within the Wnt4 specific signal transduction process, modification of specific signalling molecules became necessary. However, to detect Wnt4 specific effects, identification of reliable read-out markers became essential.

To investigate Wnt4 ligand specific gene activity, TEP1 cells were exposed to Wnt4 enriched medium for 1hr after a thorough wash with protein free medium. Microarray analysis of gene expression changes identified several genes that were affected by Wnt4 treatment (Table 2).

TABLE 2. A selected list of gene expressions modulated by Wnt4

Gene	Fold change upon Wnt4 exposure
CTGF	**4,31±0,20**
c-Jun	**2,15±0,11**
IER2	**1,87±0,14**
IER3	**2,52±0,17**
ANKRD	**2,11±0,10**
LNFR	**0,51±0,05**
PHLDA1	**4,58±0,22**
SEMA-Domain	**0,84±0,05**

Wnt4 specific effect on gene expression modification was tested by depletion of Wnt4 from the medium using a Wnt4 specific antibody (Abcam). The Wnt4 depletion assay demonstrated abrogation of increased gene transcription indicating that gene expression changes were triggered by Wnt4 (ANKRD: from 2.1 times induction to 1.5 times, c-jun: from 2.5 times induction to 1.2 times etc.).

Since connecting tissue growth factor (CTGF) showed significant and reliable regulatory pattern in the experimental system, it was chosen as a read-out gene in Wnt4 signalling studies. To investigate, whether CTGF is only an immediate early gene following Wnt4 exposure or its expression is prolonged in a Wnt4 rich environment, gene expression profile of the Wnt4 over-expressing cell line was studied (Fig. 15). As CTGF transcript was detectable above control levels, CTGF was deemed suitable as a read-out gene for signalling studies during ageing.

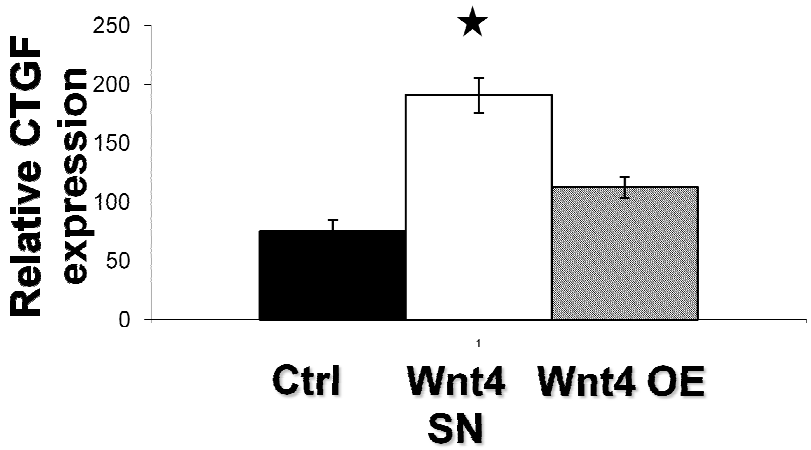


Figure 15. Analysis of Wnt4 target gene (CTGF) expression

QRT-PCR analysis of CTGF expression in control, Wnt 4-treated, Wnt4 over-expressing TEP1 cells. Statistically significant differences are marked by asterisks.

6.1.8. PKCδ in Wnt4 signalling

To investigate PKCδ involvement in Wnt4 signalling, PKCδ activity was modified by over-expression of wild type PKCδ (Fig. 16A-C) or by silencing PKCδ translation using commercially available siPKCδ (Santa Cruz) (Fig. 16D). CTGF was used as a read-out gene in the experiments. TEP1 cells with increased or decreased PKCδ levels were exposed to control and Wnt4 rich SNs for 1 hr, and then CTGF expression was analysed (Fig. 17A-B).

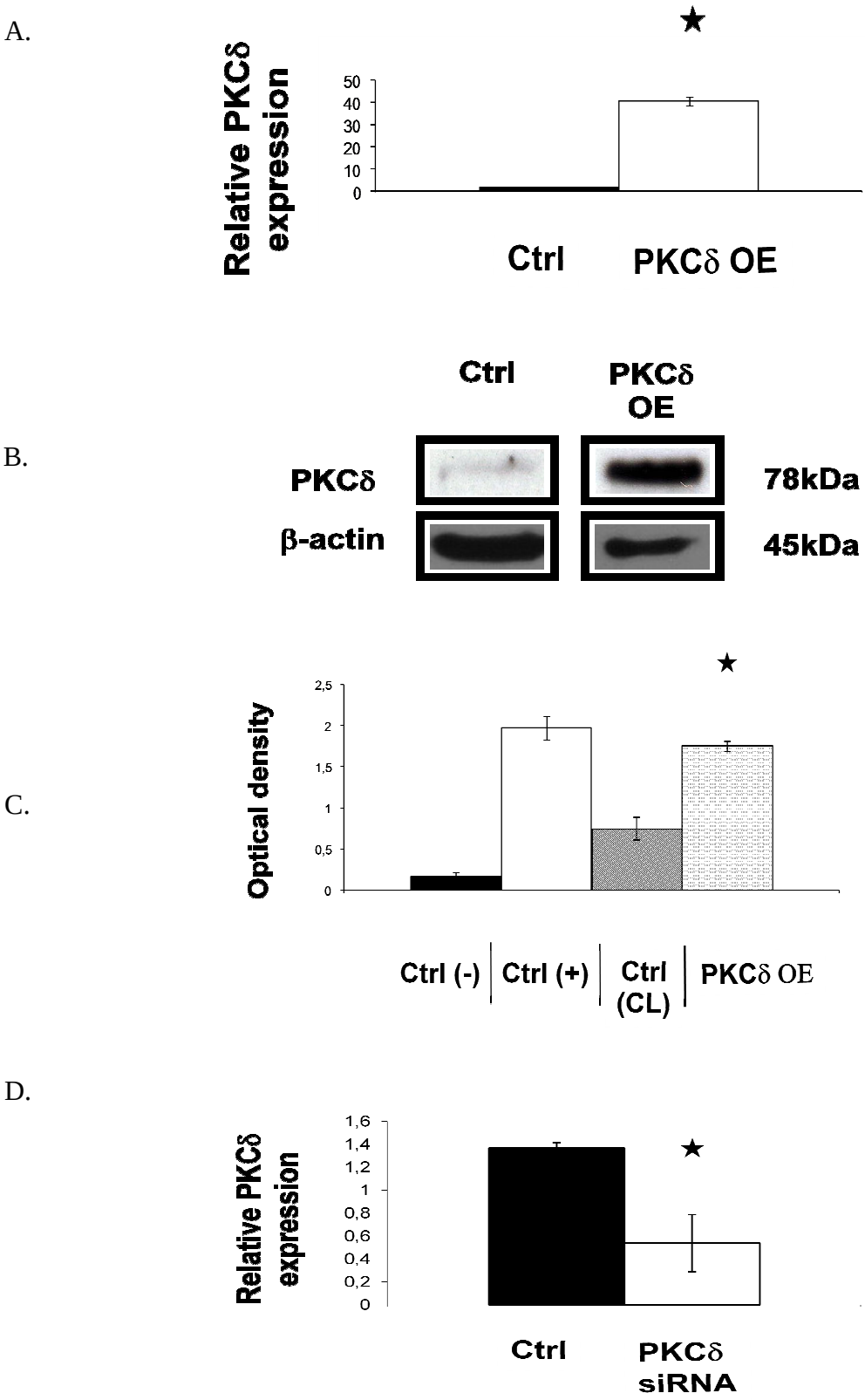
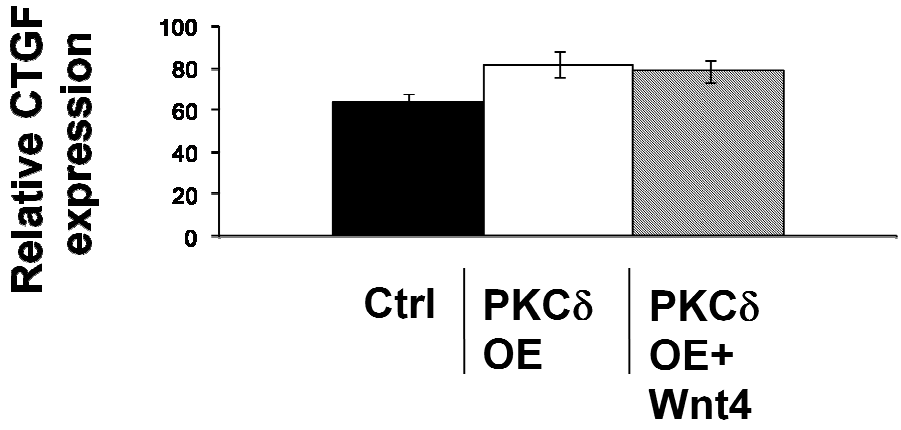


Figure 16. Gene-expression levels of PKCδ in over-expressing and silenced TEP1 cell line

(A) PKCδ expression by qRT-PCR in control and PKCδ over-expressing TEP1 cells. (B) PKCδ expression by Western blot using control and PKCδ overexpressing TEP1 cells. (C) PKCδ activity was also measured by colorimetric ELISA assay, relative absorbance values of controls, GFP only and PKCδ over-expressing cells are shown. (D) PKCδ specific RNAi silencing measured by qRT-PCR in mock and PKCδ specific siRNA-transfected TEP1 cells. Statistically significant differences are marked by asterisks.

A.



B.

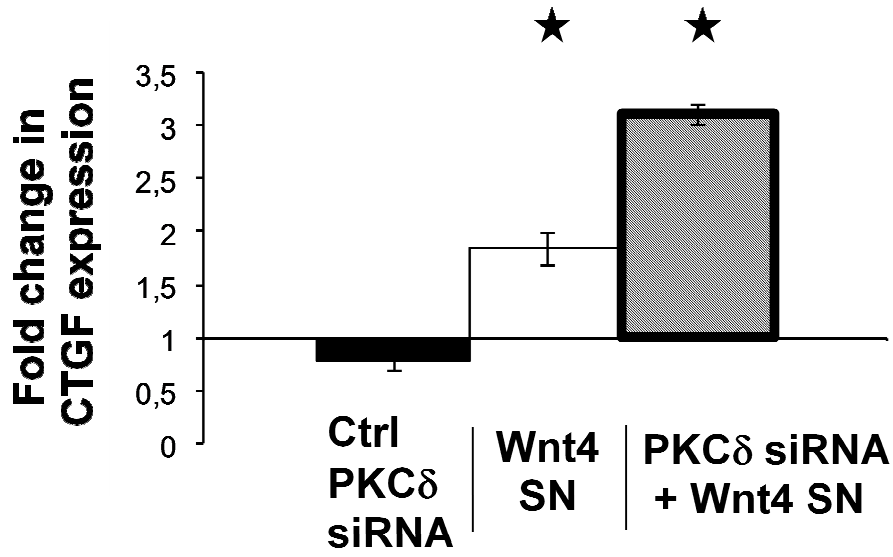


Figure 17. CTGF expression changes in Wnt4 signalling in TEP1 cell lines

(A) qRT-PCR analysis of CTGF expression in control and Wnt4 treated PKCδ over-expressing TEP1 cell line. (B) qRT-PCR analysis of CTGF expression in control and Wnt4 treated in TEP1 cell lines pre-treated with siPKCδ. Data were normalized to β-actin. Statistically significant differences are marked by asterisks.

Surprisingly, although over-expression of PKCδ had no radical effect on Wnt4 target gene transcription (Fig. 17A), even moderate down-regulation of PKCδ was able to significantly increase CTGF expression in the presence of Wnt4 (Fig. 17B), indicating that PKCδ might be involved in a negative regulatory loop.

6.1.9. Co-immuno-precipitation of PKCδ with Dvl, Fz-4 and Fz-6

As Fz-6 has been implicated in previous studies as a negative regulator of β-catenin dependent signalling, it was important to determine whether PKCδ is preferentially associated with either Wnt4 receptors. Co-immuno-precipitation patterns of PKCδ with Fz-4 and Fz-6 were analysed in control and Wnt4 SN treated cells revealing association of PKCδ and its active cleavage products with both Fz-4 (1.2 fold, 2 fold and 1.4 fold, respectively) and Fz-6 (1.4 fold, 1.5 fold and strongly detectable over non-detectable, respectively) upon Wnt4 treatment (Fig. 18A and B).

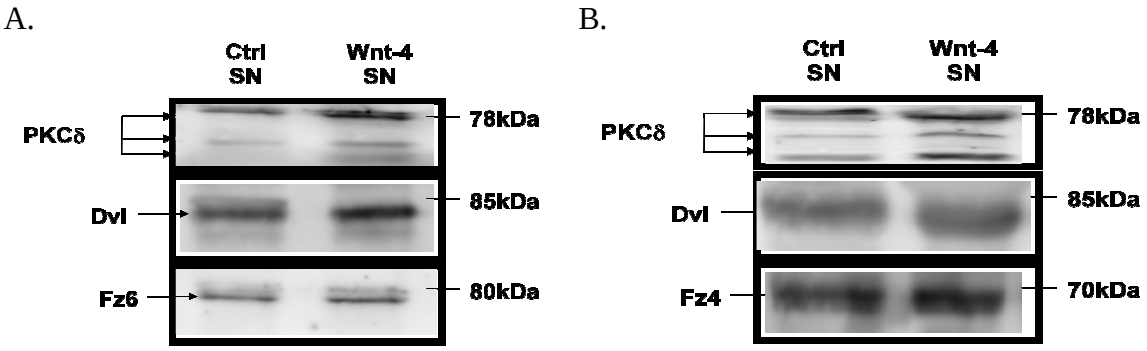
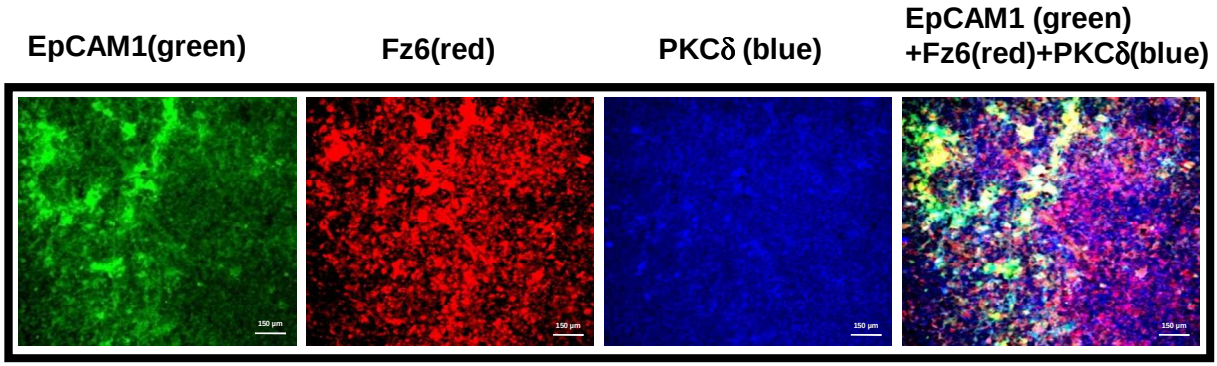


Figure 18. Analysis of Fz-4, Fz-6, Dvl and PKCδ co-localisation

(A, B) Western blot analysis following immuno-precipitation using a-Fz-6-antibody (A) or a-Fz-4-antibody (B) shows increased association of Fz-6 with PKCδ and Dvl upon Wnt4 treatment. Representative blots are shown from two repeats.

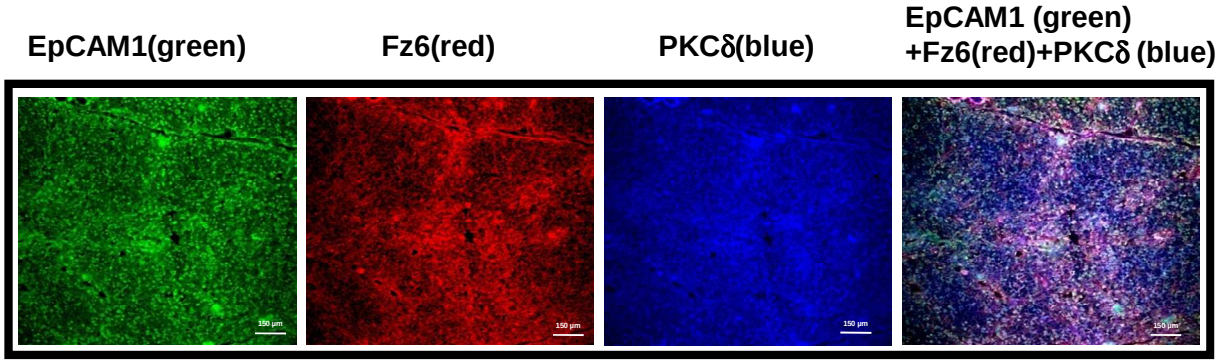
To find out whether PKCδ co-localises with Fz-6 in primary thymic tissue, immuno-histochemistry was performed. Experiments demonstrated age dependent increase of both Fz-6 and PKCδ as well as co-localisation of Fz-6 and PKCδ staining (Fig. 19A and B). While in the young thymus Fz-6 and PKCδ co-localisation is more pronounced in the thymic cortex (Fig. 19A), in the ageing thymus it is the medulla that exhibits stronger staining for both proteins (Fig. 19B).

A.



1 month

B.



9 months

Figure 19. Immuno-histochemical analysis of Fz-6 and PKCδ co-localisation

(A, B) Histology of 1 month and 9 months old mouse thymi using anti-PKCδ– NL663, anti-Fz-6-NL557 and anti-EpCAM1-FITC antibodies. Size markers are shown in the corner of all histology figures. Characteristic stainings are shown from a minimum of five repeats (bar=150μm).

6.1.10. Increased expression of CTGF and Fz-8

While increased expression and activity of the Fz-6 receptor, a suppressor of the canonical Wnt signalling pathway explains some aspects of uneven target gene transcription following manipulation of PKCδ activity, parallel changes like up-regulation of Fz-4 also occur during ageing that might add to the complexity of the signalling process. Increase in Fz-4 levels in ageing mice correlated with increased CTGF gene expression (Fig. 20A).

If Fz-6 that also increases during senescence is truly a suppressor of β-catenin signalling then CTGF expression should have decreased or remained unchanged as Fz-4 transmitted signals would have been quenched by Fz-6 signalling. To test the above hypothesis, we have considered the following: CTGF has recently been reported to negatively regulate canonical Wnt signalling by blocking β-catenin stabilisation *via* GSK3β activation leading to phosphorylation and consequent degradation of β-catenin (Luo, Kang et al. 2004), indicating that CTGF might be part of a negative feed-back loop. As CTGF is a secreted protein, expression of Fz-8 (Mercurio, Latinkic et al. 2004) a recently reported receptor for CTGF was analysed in purified TECs of 1 and 9 months old thymi using qRT-PCR. As parallel with CTGF, Fz-8 mRNA levels increased (Fig. 20B) in ageing mice, while the direct target of β-catenin dependent Wnt4 signalling (Balciunaite et al., 2002) FoxN1became undetectable (data not shown) (Kvell et al., 2010), indicating the existence of an additional negative feed-back loop.

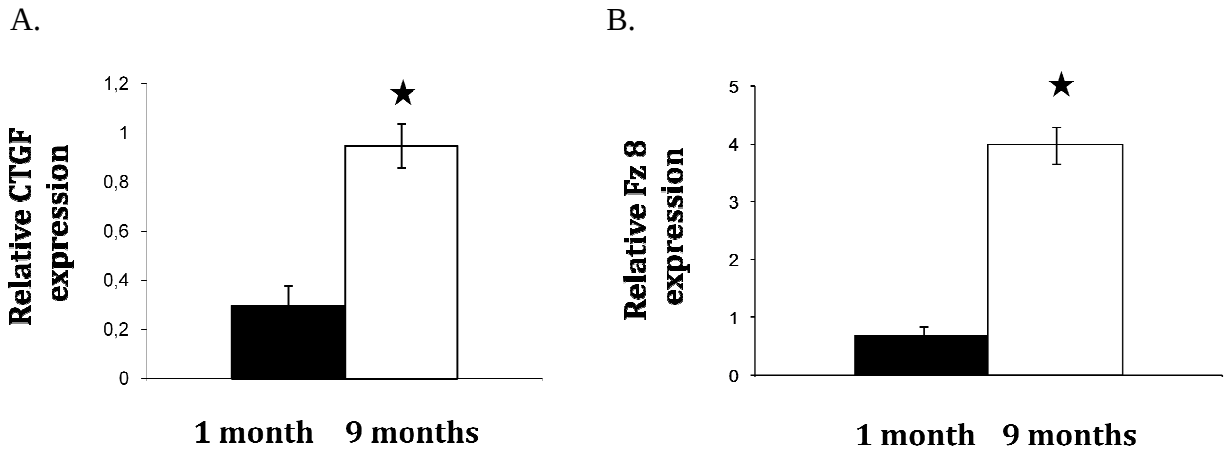


Figure 20. CTGF and Fz8 expression during thymic senescence

(A, B) qRT-PCR analysis of CTGF and Fz-8 expression in young (1 month) and ageing (9 months) mouse thymic epithelium. Data was normalised to β-actin. Statistically significant differences are marked by asterisks.

6.2. Steroid induced thymic senescence

A commonly held view is that the thymus involutes at puberty, and this model is based primarily on studies showing that growth hormone (GH) and sex steroids can affect T-cell production in the thymus and that their concentrations change with age (Min, Montecino-Rodriguez et al. 2006), GH and insulin-like growth factor-I (IGF-I) as well have thymopoietic effects (Burgess, Liu et al. 1999) and their production declines with age. However, it is the increased production of sex steroids at puberty that is most often associated with thymic involution. The previous part of this thesis describes mechanisms associated physiological thymic senescence. As steroids are frequently applied medications, investigations were extended to identify similarities in induced and physiological senescence and potential mechanisms that might be able to reduce adipoid involution of the thymus.

6.2.1. Effects of single-dose GC administration

Similarly to physiological senescence, the level of both FoxN1 the transcription factor essential for thymic organogenesis and maintenance and its regulator Wnt4 decreased in EpCAM1^{+} TECs within 24 hrs following a single dose DX injection and remained at low levels over 1 week (Fig. 21).

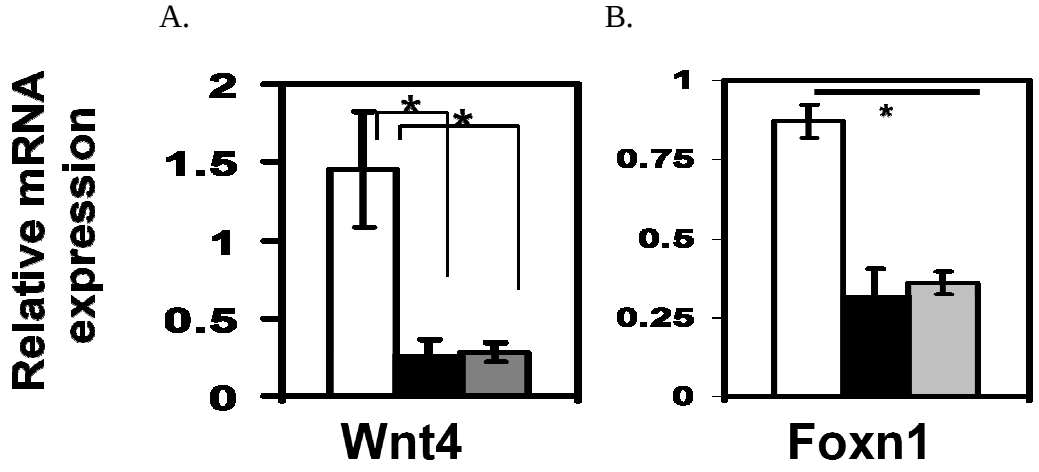


Figure 21. Dexamethasone (DX)-induced effects on Wnt4 and FoxN1 expressions

Wnt4 (A) and FoxN1 (B) expressions in DX-treated thymic epithelial cells (TEC) control (white) and treated (black). Gray bars represent gene expressions 168 h after DX treatment and asterisks indicate significant differences.

Down-regulation of FoxN1 and Wnt4 correlated with up-regulation of pre-adipocyte markers LAP2α and PPARγ (Fig. 22), indicating further molecular similarities with physiological senescence.

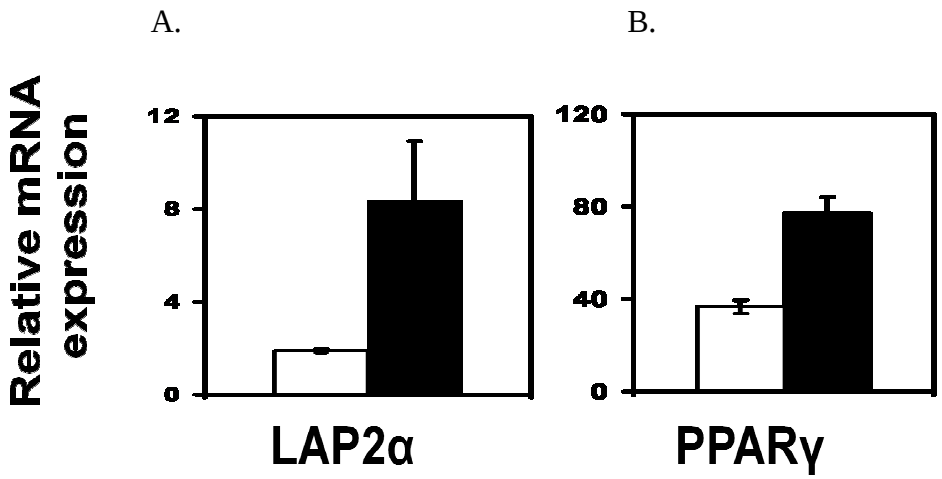


Figure 22. Dexamethasone (DX)-induced effects on expressions of LAP2α and PPARγ

Expression of LAP2α (A) and PPARγ (B) were tested in TECs 168 h after a single DX injection and were found to be elevated in DX-treated samples (black) compared to control (white). Gene expression was normalized to 18S rRNA. Results are representative of three independent experiments.

6.2.2. Effects of sustained GC administration

In clinical treatments GC analogues are widely used for extended periods of time. To mimic this pattern of clinical application, mice were injected with DX repeatedly for a time course of 1 month. Both Wnt4 and FoxN1 levels were drastically down-regulated measured by qRT-PCR (Fig. 23), while the adipocyte differentiation factor ADRP, down-stream target of LAP2α and PPARγ was significantly increased by this advanced time point (Fig. 23). The results indicated that adipocyte-type trans-differentiation is completed over a time period where the early trigger signal is an increase in LAP2α expression that is followed by PPARγ, then ADRP up-regulation.

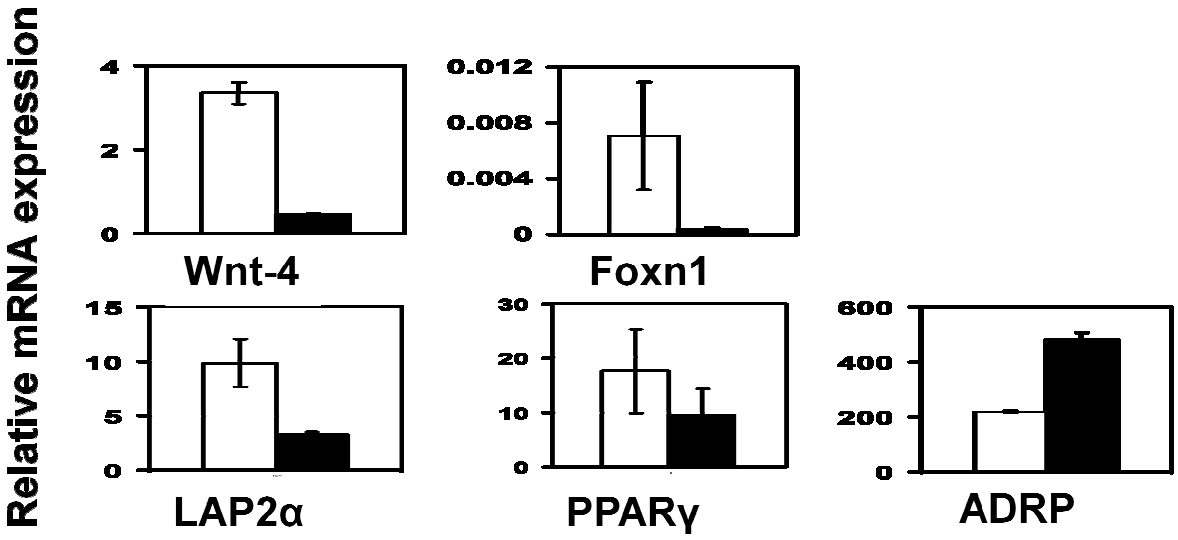


Figure 23. Effect of sustained DX administration (1 month) on TEC gene expressions

Wnt4, FoxN1, LAP2α, PPARγ, and ADRP expression of control and DX-treated TECs. The levels of Wnt4, FoxN1, and LAP2α were found to be reduced after repeated *in vivo* DX injections, whereas PPARγ was unaltered. Additionally, ADRP was found to be elevated in DX-treated samples. Note the scale differences for gene expression.

6.2.3. Wnt4-mediated inhibition of steroid-induced adipose trans-differentiation

To test whether Wnt4 can prevent adipocyte-type trans-differentiation, Wnt4 overexpressing TEP1 cell line was exposed to solvent or DX for a week then gene expressions were analysed compared with control TEP1 cell line. While in the control cell line DX exposure induced up-regulation of adipoid trans-differentiation markers, within the Wnt4 over-expressing cell line, none of the adipoid trans-differentiation markers were up-regulated (Fig. 24) indicating that Wnt4 can protect TECs against adipoid trans-differentiation.

As the TEP1 cell line does not express FoxN1 (unpublished observation), it has become evident that the absence of Wnt4 alone is sufficient to allow pre-adipocyte-type trans-differentiation, and apparently FoxN1 has no significant role in the process.

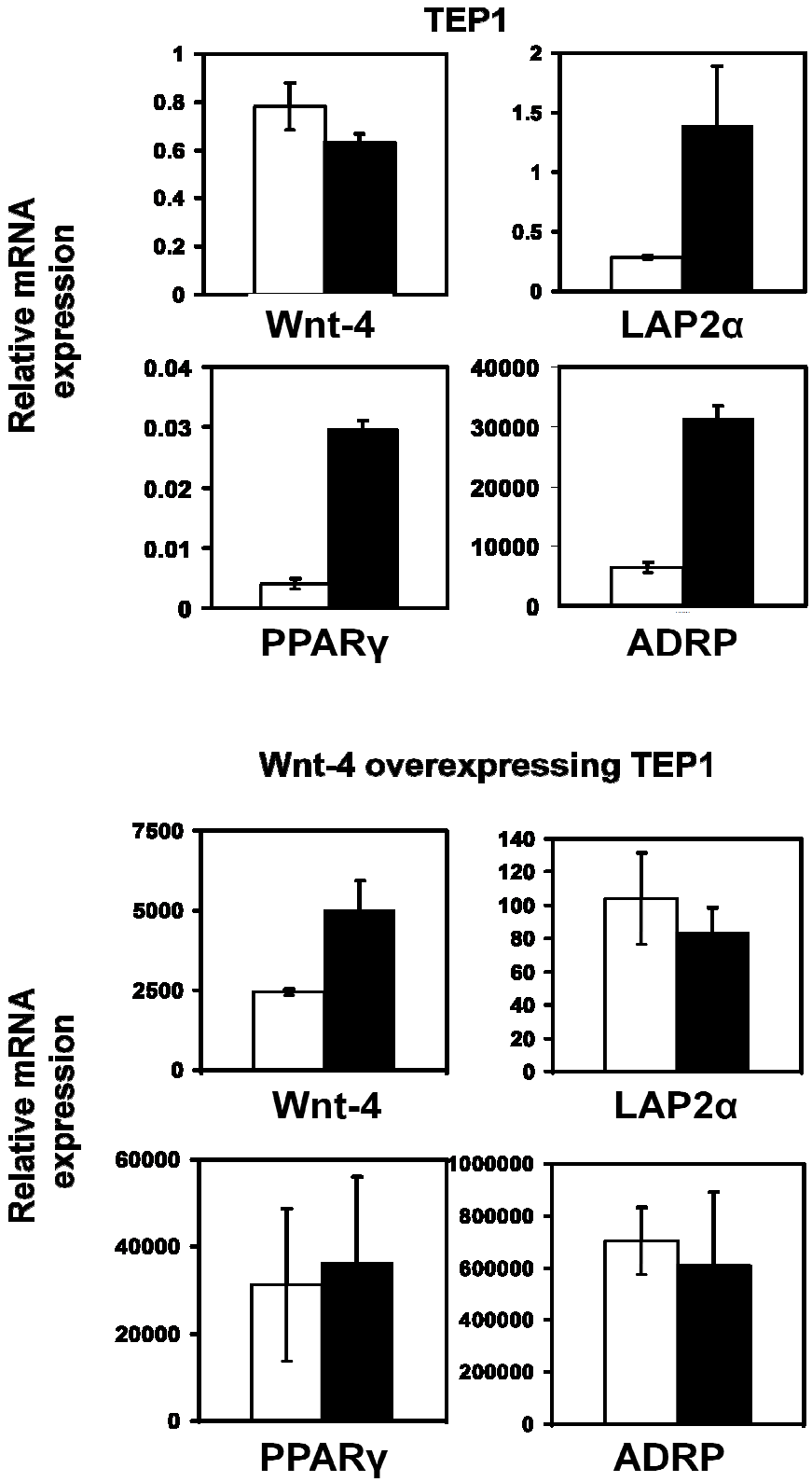


Figure 24. Wnt4 overexpression rescues the DX-induced adipocyte-related gene expressions in TEP1 cells

Gene expression changes in Wnt4 over-expressing TEP1 - cell line following 168 h of DX-treatment. LAP2α, PPARγ, and ADRP expressions of solvent and DX-treated control and Wnt4 over-expressing TEP1 cell lines. White bars indicate solvent-treated control and black bars show the values of DX-treated samples. Results are representative of three independent experiments. Note the different scales for gene expression.

7. Discussion

Wnts are involved in many cellular mechanisms in various organs including the thymus. Wnt4 is expressed abundantly in the thymus and regulates cell-cell interactions, migration, proliferation, and activates different target gene expressions during thymic organogenesis and physiological function of the developed thymus. The sequence of Wnt4 is highly conserved in mammals and shows multiple roles in organogenesis and homeostasis as well as differentiation. While Wnt4 can elicit its effects *via* the non-canonical signalling network, within the thymus Wnt4 has been regarded as the canonical - β-catenin signalling – pathway activator. As Wnts in general and Wnt4 in particular are regulators of both thymocyte development and maintenance of TEC identity, the question has risen naturally, what role Wnts have in regulating thymic senescence?

The model system was chosen carefully, as rodent tissues do not necessarily mimic age associated alterations in human beings. Based on our immuno-histochemical analysis of thymic tissues in ageing mice, our experiments provided evidence that in mice the thymic structure becomes just as disorganized with age as it does in the human tissue. The border of medullary and cortical area becomes less defined and the medullary region occupies less space. Using GFP transgenic animals and adipose tissue staining, the mechanism was proved to be highly similar to adipose involution that is normally detected in ageing human thymi.

Molecular analysis of ageing thymic tissue revealed that morphological changes were associated with down-regulation of Wnt4 and up-regulation of molecules (LAP2α, PPARγ and ADRP) responsible for adipoid trans-differentiation. To be able to determine whether TECs can directly change into adipocytes or they turn into fibroblasts first, a simple immuno-fluorescent staining experiment was performed, where co-localization of the epithelial marker EpCAM1 and the fibroblast marker ER-TR-7 was detected indicating gradual changes during the trans-differentiation process. Our experiments have provided evidence that adipocytes do not migrate into the thymus from perithymic area during ageing, instead they are produced locally *via* multiple cellular trans-differentiation steps.

In summary, dedifferentiation of TECs triggers EMT first, then the resulting fibroblasts undergo the conventional route of differentiation program towards adipocyte-lineage commitment regulated by the continually changing ratio of Wnt4 and LAP2α. Our current understanding of the adipoid involution process is summarized in figure 25.

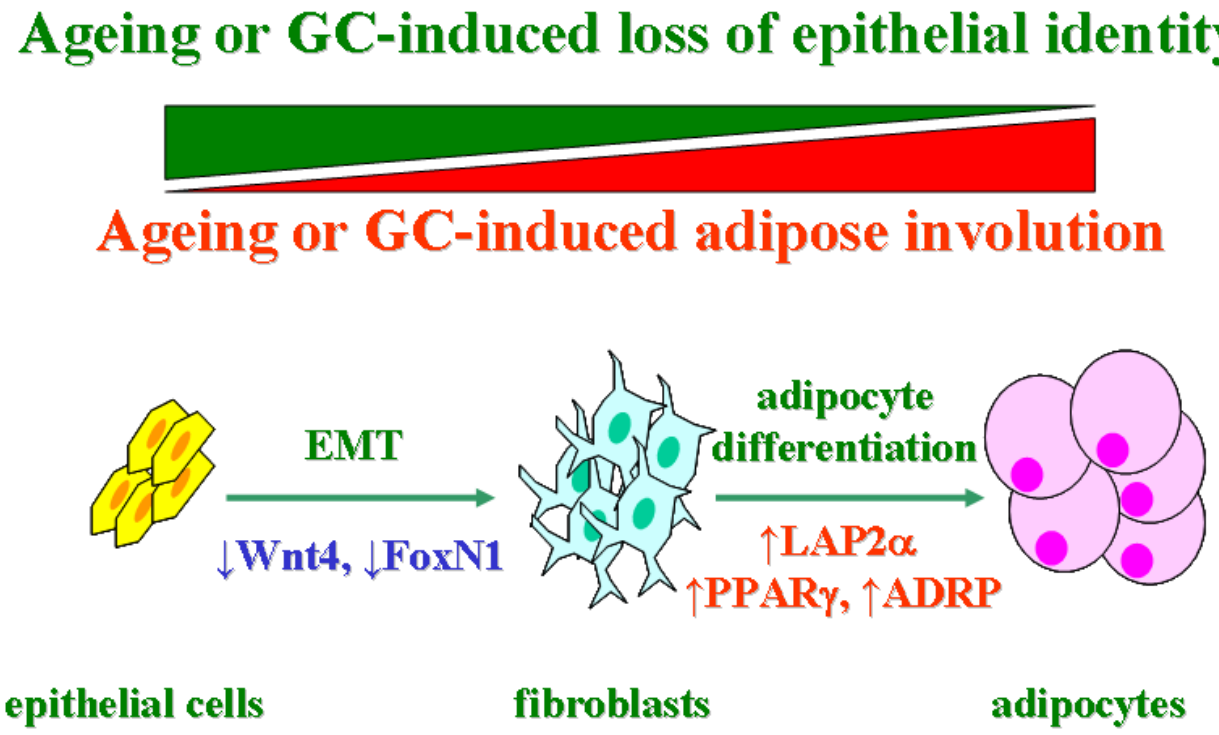


Figure 25. Model of adipose involution in thymic ageing

Interestingly, GCs induce similar molecular changes observed during physiological senescence and also lead to adipose involution. Wnt4 plays a central role in the involution process, as loss of TEC identity and adipose trans-differentiation are tightly associated with decreasing Wnt4 and FoxN1 levels. As the involution process can be reversed and TEC identity restored by the up-regulation or added Wnt4, these results highlighted the regulatory role of Wnt signalling in thymic involution.
While the physiological model is important, the question remains: what signals trigger down-regulation of the β-catenin dependent Wnt pathway in such a forceful way that allows the initiation of thymic involution?

Signalling studies using cloned Wnt4 have ensued with determination of Wnt4 specific target genes to provide a reliable read-out system. Modification of signalling molecules associated with Wnt4 signals transmitted from Fz-4 and Fz-6 receptors became the target of the investigation. It has become evident that PKCδ is involved in signal transduction from both receptors and that PKCδ preferentially associates with Fz-6, a negative regulator of β-catenin dependent signalling. Additionally, our studies have revealed that down-regulation of β-catenin dependent Wnt signalling is progressively down-regulated during ageing *via* activation of multiple negative feed-back loops in the following steps: During the ageing process, Wnt4 levels decrease, while receptor expression

increase with proportionally higher expression of Fz-6. The β-catenin dependent Fz-4 signals lead to increased expression of CTGF, a β-catenin dependent target gene that is also part of a negative-feedback loop. CTGF receptor Fz-8 is also up-regulated leading to enhanced activation of GSK3β that phosphorylates β-catenin accelerating proteosomal β-catenin degradation in the cytosol. All these signalling events lead to FoxN1 down-regulation, loss of TEC characteristics and provide an opening for molecular events leading up to adipocyte type trans-differentiation. The molecular events associated with thymic ageing are summarized in figure 26.

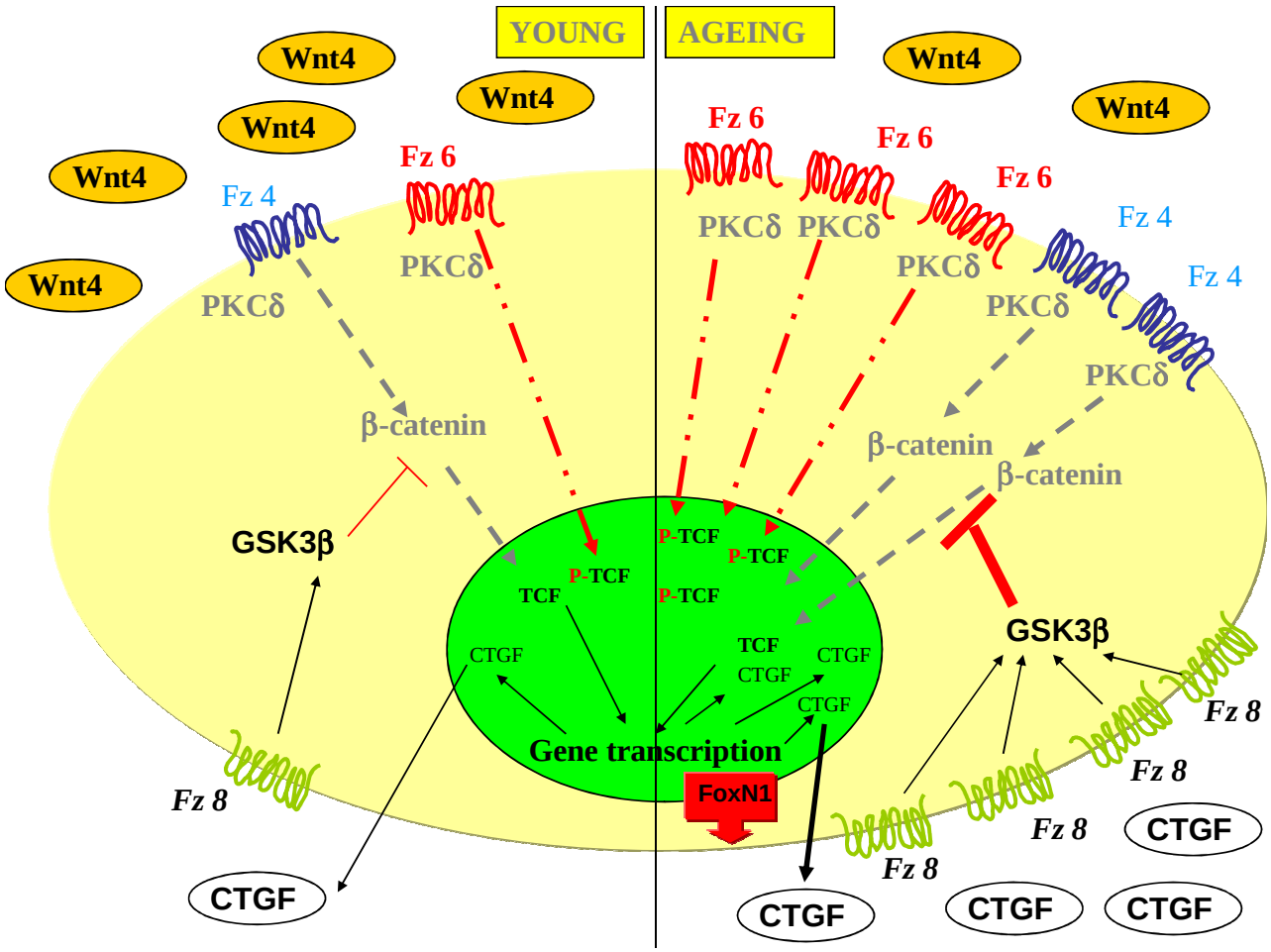


Figure 26. Model of molecular mechanisms in thymic ageing

It has been demonstrated in the present work that Wnt4 and LAP2α are key regulators in physiological and induced ageing. The tightly regulated balance of these two factors determines the rate of thymic senescence. Details of the signal transduction of the ageing process raises the possibility to find molecular targets for restoration of the physiological T-cell out-put or to find therapeutic targets in laminopathies, where lamins including LAP2α – a member of lamin protein family – play an important role.

8. Conclusions

Main conclusions of the thesis

1. In mice, similarly to human thymic tissue, the highly organized structure of the thymic epithelium becomes disorganized followed by adipose involution making the mouse thymus a suitable model to study the molecular background of thymic senescence.

2. Both physiological and glucocorticoid induced senescence are regulated by decreased Wnt4 and increased LAP2α signalling.

3. Up-regulation of Wnt4 can protect against adipoid trans-differentiation and thymic involution.

4. Down-regulation of Wnt4 levels initiate EMT (Fig. 25).

5. Up-regulation of LAP2α initiates adipocyte type trans-differentiation (Fig. 25) in the fibroblast like cells results of the EMT process.

6. PKCδ is involved in Wnt4 signalling from both Wnt4 receptors, Fz-4 and Fz-6. Nevertheless, PKCδ preferentially associates with Fz-6 that receptor transmits negative, β-catenin inhibitory signals that leads to suppression of β-catenin dependent gene transcription.

7. The β-catenin target gene, CTGF, and one of its receptors, Fz-8 are up-regulated during thymic senescence. As CTGF-Fz-8 signals are involved in a negative feedback loop inhibiting β-catenin dependent signalling, this signalling pathway contributes to down-regulation of TEC identity.

8. Thymic ageing is a continuous, multi-component process that is regulated by complex molecular interactions leading to suppression of the canonical Wnt pathway allowing adipocyte-type trans-differentiation (Fig. 26).

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10. List of Publications

10.1. Publications related to the thesis:

10.1.1. Papers:

Varecza, Z., Kvell, K., Talaber, G., Miskei, G., Parnell, S.M., Anderson, G., Jenkinson, E.J. Pongrácz, J.E.: Multiple suppression pathways of canonical Wnt signalling control thymic epithelial senescence.
2011. Mech Ageing Dev. (in press, available online 27. April 2011) (IF: 4.2)

Talabér, G., Kvell, K., **Varecza, Z.**, Anderson, G.,Jenkinson, J.E., Boldizsar, F., Berki, T., Pongrácz, J.E.:Wnt4 protects thymic epithelial cells against Dexamethasone-induced senescence.
2011. Rejuv.Res., 14(3) Epub (IF: 4.2)

Kvell, K., **Varecza, Z.**, Bartis, D., Hesse, S., Parnell, S., Anderson, G., Jenkinson, E.J., Pongracz J.E.: Wnt4 and LAP2alpha as pacemakers of thymic epithelial senescence.
2010. PLoS One, 5(5):e10701 (IF: 4.35)

Impact factor: 12.75

Total impact factor: 20.75
Total citations: 36

10.1.2. Poster presentations related to the thesis:

Varecza, Z, Kvell, K, Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Wnt Modulates Notch Pathway Associated Gene Expressions in Primary Thymic Epithelium of Balb/c Mice
2009 Wnt meeting, LOMBARDI COMPREHENSIVE CANCER CENTER, GEORGETOWN UNIVERSITY, Washington DC, USA, 11-14 June 2009.

Varecza, Z, Kvell, K Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Novel and atypical PKCs are involved in non-canonical Wnt signaling
Wnt Signaling in Development and Disease, Max Delbrück Communications Center, Berlin-Buch, 12 – 15 September 2007.

Varecza, Z, Kvell, K Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Novel PKCs are involved in Wnt signaling
IV. International Conference on Molecular Recognition (Pecs), Aug. 15-18, 2007.

Varecza, Z, Kvell, K Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Novel and atypical PKCs are differentially involved in non-canonical Wnt signalling in thymic epithelial development in mice
The traditional Wnt meeting, UCSD, La Jolla (California), June 21-23, 2007.

Kvell, K, **Varecza, Z**, Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Wnt glycoprotein-triggered changes of gene expression in murine thymic epithelial cells The traditional Wnt meeting, UCSD, La Jolla (California), June 21-23, 2007.

Varecza, Z, Kvell, K, Miskei, G. Anderson, G, Jenkinson E.J. ' Pongrácz, J.E.
PKCs differentially regulate Wnt signalling in thymic epithelium in mice
Annual meeting of the Hungarian Society for Physiology, Pecs, June 5-8, 2007.

Varecza, Z, Kvell, K, Miskei, G, Anderson, G, Jenkinson E.J, Pongrácz, J.E.
Novel and atypical PKCs are involved in Wnt signalling in thymic epithelium in mice
Annual meeting of the Hungarian Society for Membrane Transport, Sümeg, May 22-25, 2007.

Varecza, Z, Kvell, K Miskei, G., Parnell, S.M., Anderson, G. Jenkinson E.J. and Pongracz, J.E.
Protein kinase C dependent expression of AIRE transcription factor regulates autoimmunity
Annual meeting of PhD students, Semmelveis University, Budapest, Apr. 12-13, 2007.

10.2. Further publications

Papers:

Varecza Z, Elekes K, László T, Perkecz A, Pintér E, Sándor Z, Szolcsányi J, Keszthelyi D, Szabó A, Sándor K, Molnár TF, Szántó Z, Pongrácz JE, Helyes Z.:
Expression of the Somatostatin Receptor Subtype 4 in Intact and Inflamed Pulmonary Tissues.
2009. J Histochem Cytochem., 57(12):1127-1137. (IF:2.5)

Jakab F., Horvát G., Ferenczic E., Sebők J., **Varecza Z**.,Szűcs G.,
Detection of Dobrava hantaviruses in Apodemus agrarius mice in the Transdanubian region of Hungary
Virus Res. 2007 Sep;128(1-2):149-52. Epub 2007 May 23 (IF:2.56)

Pócsi I, Molnár Z, Pusztahelyi T, **Varecza Z**, Emri T.
Yeast-like cell formation and glutathione metabolism in autolysing cultures of Penicillium chrysogenum.
Acta Biol Hung. 2007 Dec;58(4):431-40 (IF:0.55)

Varecza Z., Emri T., Pusztahelyi T., and Pócsi I.
A novel aspect of NADPH production in ageing Penicillium chrysogenum.
Acta Biol Hung. 2006 Mar;57(1):115-21. (IF:0.55)

Emri T., Molnár Z., Pusztahelyi T., **Varecza Z.,** and Pócsi I.
The FluG-BrlA pathway is involved in the regulation of autolsys in *Aspergillus nidulans*
Mycol Res. 2005 Jul;109(Pt 7):757-63 (IF:2.92)

Sámi, L., Pusztahelyi, T., Emri, T., **Varecza, Z**., Grallert, Á., Karányi, Zs., Kiss, L. and Pócsi, I.
Autolysis and ageing of *Penicillium chrysogenum* cultures under carbon starvation: Chitinase production and antifungal effect of allosamidin.
(2001) J. Gen. Appl. Microbiol. , 47, pp. 201-211 (IF:0.95)

Book chapters:

Pócsi I., Emri T., **Varecza Z.**, Sámi L. and Pusztahelyi T.
Allosamidin inhibits the fragmentation and autolysis of *Penicillium chrysogenum*.
(2000) In: Advances in Chitin Science, Vol. 4. Eds. Peter, M.G., Domard, A. and Muzzarelli, R.A.A. pp. 558-564.

Further poster presentations:

Bovari J., Miskei G., Kvell K., Dorn A., Demeter A., **Varecza Z.**, Thickett D. and Pongracz J.E.
Salbutamol differentially regulates the expression of Wnts and their target genes in A549 cells
Wnt Signaling in Development and Disease, Max Delbrück Communications Center, Berlin-Buch, 12 – 15 September 2007.

Pongrácz J.E., Miskei G., Bartis D., Molnár T., László T., Kvell K., **Varecza Z.**, Stockley R.A., Tickett D.
Inflammatory mediator production is mediated by Wnts in primary human lung cell tissue.
Wnt signaling in Development and Disease, Berlin, Germany, 2007.

Miskei G., Bartis D., Kvell K., Molnár T., **Varecza Z.**, Balasa T., Kovács B., László T., Pongrácz J.E.:
Retinoic acid affects Wnt signalling both in the alveolar epithelial cell line and in primary human lung tissue.
Wnt signaling in Development and Disease, Berlin, Germany, 2007.

Varecza Z., Emri T., Molnár Z., Pusztahelyi T. and Pócsi I.
Yeast like cell formation and gluthatione metabolism in *Penicillium chrysogenum* and *Aspergillus nidulans*
X. Fermentation Kollokvium, Keszthely (2004)

Varecza Z., Emri T. and Pócsi I.
A new aspect of NADPH production in ageing *Penicillium chrysogenum*
15th International Congress of the Hungarian Society for Microbiology, Keszthely (2004)

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The protein FluG is indispensable in the initialization of autolysis in *Aspergillus nidulans*

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Cloning, expression and purification of the mysterious presenilin 1 in yeast expression system

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Co-infection of Sf9 cells with baculovirus harbouring Ku70/80 and TRF1

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Varecza Z., Yannone S. M., Jap B. K. and Chen D. J.

Optimization of the expression of Ku 70/80 protein in baculovirus expression system

Department of Energy Collaboration Meeting, Lawrence Berkeley National Laboratory, (2001)

Pócsi I., Emri T., Sámi L., **Varecza Z**., Pusztahelyi T., Grallert Á., and Kiss L.

Effects of allosamidin on the fragmentation, autolysis and cryptic growth of ageing cultures of *Penicillium chrysogenum*

15. Conference of the Hungarian Chemotherapic Society, Hajdúszoboszló (2000).

Sámi, L., Emri, T., **Varecza, Z**., Pusztahelyi, T., Pócsi, I.,

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Allosamidin inhibits the fragmentation and autolysis of hyphae in ageing cultures of *Penicillium chrysogenum*

First Hungarian Conference of Mycology, Budapest (1999)

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