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Central Immune Senescence, Reversal Potentials

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1. Introduction

1.1 Ageing in focus

Ageing is a complex process that affects all living organisms. Senescence is not only conceivable in multicellular organisms, but also in unicellulars. Unlike certain diseases that have specific morbidity rates, ageing is a physiological process that affects all individuals that live long enough (unaffected by i.e. predation or famine) to experience senescence.

A pioneer of ageing research, August Weismann has established two rather opposing concepts for aging and even today both gather numerous followers. One is the adaptive concept, according to which ageing has evolved to cleanse the population from old, non-reproductive consumers. The other, non-adaptive concept suggests that ageing is due to greater weight on early survival / reproduction rather than vigour at later ages. This latter has been reshaped by the theory of antagonistic pleiotropy (Ljubuncic et al. 2009).

Due to advances in biomedical research and care, currently an average 55-aged person is expected to live up to 85 years of age at death on average in the Western societies. This number is expected to increase if biomedical research continues to develop at the current rate and by the year 2030 an average 55-aged person is expected to live up to 115 years of age at death (according to SENS plans) (de Grey 2007). If such forecasts prove to be true, it is of extraordinary significance and will likely trigger immense social and economical conflicts.

1.1.1 Ageing and society

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

At present life-span is still significantly increasing in the Western civilisations, however, this increase is not accompanied by proportional increase in life spent in overall good health referred to as 'health-span'. There are current efforts to prolong health-span within expanding life-span. This would not only extend life spent in appropriate quality of life, but

also has the potential to alleviate pressure on current public health systems. This chapter focuses on central immune senescence and therefore will enumerate potential mechanisms of extending human central immune fitness in the elderly.

1.1.2 Ageing of the immune system

Impaired immunological responsiveness in the elderly poses a major difficulty. 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. 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 lymph-node 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 immune senescence accompanied by 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 an involution process that results in a decline of *de novo* T-cell production.

1.1.3 Significance of thymic involution studies

In contrast to the extensive studies addressing haemopoietic cells, the in-depth analysis of determinants for stromal competence during immunological ageing is far less detailed, despite its clear significance related to immunological responsiveness in the elderly. There is literature describing quantitative changes that occur during immunological senescence in peripheral immunologically competent tissues like the spleen or lymph nodes. Probably the best characterised, significant example is that of FDCs. Compared to young counterparts the aged follicular dendritic cells express significantly less CD21 ligand and FcγRII. As a consequence aged FDCs lose their ability to trap immune complexes and present antigens to B cells. This in turn leads to impaired germinal centre reactions and antibody production (Aydar et al. 2004). However, even these well characterised quantitative changes of the peripheral lymphoid tissues are less dramatic than the adipose involution of the thymus.

The manipulation of thymic immune senescence and the restoration of de-novo T-cell production should provide direct benefits for both adult and elderly patients. Such interventions shall increase health-span within life-span significantly reducing the healthcare costs alleviating the burden on healthcare systems.

1.2 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 et al. 2006) $CD4^+CD8^+$ (double positive or DP) thymocytes undergo positive (functional TCR) and negative (non self-reactive TCR) selection in the cortex and medulla, to finally leave the thymus for the periphery as $CD4^-CD8^+$ (cytotoxic) or $CD4^+CD8^-$ (helper) single positive (SP), mature, naïve T-cells.

1.3 Thymic microenvironment in *de novo* T-cell production

Successful T-cell development requires the interaction of thymocytes with the thymic stroma, creating the special thymic microenvironment for T-cell differentiation and selection. A large proportion of the thymic stroma consists 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 et al. 2009) – that is essential for $Mts24^+$ epithelial progenitors (Bennett et al. 2002; Gill et al. 2002) to develop into various epithelial subsets (Dooley 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 et al. 2002) protein. The mature thymic epithelium consists 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 establishing the special thymic microenvironment. The thymic epithelial network regulates homing, intrathymic migration, and differentiation of developing T-lymphocytes through release of cytokines (e.g. interleukin-7 (Alves et al. 2009)), secretion of extracellular matrix components, and establishment of intercellular connections (Crisa L et al. 1996) (Schluns et al. 1997). Thymocytes bearing diverse TCR repertoire are selected by MHC (major-histocompatibility-complex) molecules and MHC bound-antigens presented by the thymic stroma, including epithelial cells. During T-cell development, characterised by progression through phenotypically distinct stages (Lind et al. 2001), thymocytes reside in spatially restricted domains of the mature thymus. T-cell precursors enter the thymus at the cortico-medullary junction (Blackburn et al. 2004), then migrate to the subcapsular zone of the outer cortex, back through the cortex, then to the medulla, where they finally leave to the periphery (Blackburn et al. 2004). Functional studies have shown, that the cortex is important in producing chemokines, which attract pro-thymocytes (Bleul et al. 2000) and are also essential for mediating positive selection (Anderson et al. 1994). Meanwhile the medullary epithelium has been implicated in driving the final stages of thymocyte maturation (Ge et al. 2000) and has a crucial role in tolerance induction (Farr et al. 1998; Derbinski 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 et al. 2005), Notch (Valsecchi 1997), and Wnt (Pongracz et al. 2003) family members.

1.4 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 into 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 functional thymic epithelial tissue mass, the thymus can no longer support the same output of naïve T-cell production (Ribeiro et al. 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 et al. 2007; Gui et al. 2007; Grubeck-Loebenstein 2009). Since the thymic epithelium has also a key role in deleting auto-reactive T-cell clones, functional impairment increases the chances of developing auto-immune disease (Hsu et al. 2003). The transcription factor FoxN1, characteristic in thymus development is also affected by age. FoxN1 (Mandinova et al. 2009) is not only essential for progenitor epithelial cells of the thymic rudiment to develop into various epithelial subsets (Dooley et al. 2005) but also to maintain TEC identity in the differentiated, adult thymus. Decreased level of FoxN1 expression in adult TECs results in accelerated thymic involution (Chen et al. 2009; Cheng et al. 2010).

1.5 Thymic involution: developmental programme or senescence?

It has long been known that the thymus begins adipose involution and senescence rather early, but how early is that exactly? Recent studies have analysed the kinetics of thymic function and thymic mass versus age. It has been confirmed in both mouse and human thymic samples that the functional peek of thymic activity significantly precedes the peek of thymic mass and the first signs of adipose involution. In the mouse thymic activity is largely decreased by the age of one month compared to the newborn age (thymocyte precursor immigration at 6% and mature T cell emigration at 7% where 100% is measured at newborn age), yet the thymus reaches its largest size at one month of age (Shiraishi et al. 2003). Similar tendency has been described in humans where thymic function reaches its peak around the age of one year followed by the first signs of adipose infiltration by the age of approx. five years (Shiraishi et al. 2003). However, most studies describe significant thymic adipose involution starting around puberty / young adulthood. Therefore there is apparently significant detachment of thymic activity peak and thymic mass peak, and surprisingly activity peak significantly precedes mass peak.

The above described phenomenon raises the issue whether the early appearance of thymic involution belongs to senescence or developmental programme and how strictly these two may be separated? Similar questions are raised by the detection of miniature atherosclerotic lesions detected already at foetal age, a currently fashionable topic (Leduc et al. 2010).

1.6 Trans-differentiation of fibroblasts into adipocytes

The nuclear lamina consists of a matrix of proteins located next to the inner nuclear membrane. The lamina family of proteins makes up the matrix and that is highly conserved in evolution. The family of lamina associated polypeptides (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 LAP2α protein family (Dorner 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 et al. 1996; Hutchison et al. 2001). LAP2α is synthesized in the

cytoplasm and is then transported into the nucleus by a PKC-dependent mechanism (Dreger et al. 1999). The mere over-expression of LAP2α in fibroblasts is known to directly up-regulate PPARγ expression, an acknowledged marker and key transcription factor of pre-adipocyte differentiation (Dorner 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 programme in the absence of additional stimuli (Dorner et al. 2006).

1.7 Wnt signalling

1.7.1 Wnt signalling

The Wnt family of 19 secreted glycoproteins controls 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 splits into the polar cell polarity (PCP) or c-Jun-N-Terminal Kinase (JNK) / Activating Protein (AP1) dependent and the Ca^{2+} 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 et al. 1996) and non-canonical pathway activators (Wnt5a, Wnt4, Wnt11) (Torres et al. 1996), although promiscuity is a feature of both ligands and receptors.

1.7.2 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 et al. 2001; Staal 2001; Xu et al. 2003) as well as in the thymic epithelium (Balciunate et al. 2001, Pongracz et al. 2003). Generally, in the absence of canonical Wnt-s, 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 phosporylated 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 Wnt-s, 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 consequent inhibition of GSK-3β. Inhibition of GSK-3β results in stabilisation and finally cytosolic accumulation of β-catenin, which then translocates to the nucleus where is required to form active transcription complexes with members of the T-Cell Factor (LEF1, TCF1, TCF3, TCF4) transcription factor family (Staal et al. 2003) and transcription initiator p300 (Labalette et al. 2004). Successful assembly of the transcription complex leads to the activation of various target genes including cyclin-D1 (Shtutman et al. 1999; Tetsu et al. 1999), c-myc (He et al. 1998), c-jun (Mann et al. 1999), Fra-1 (Mann et al. 1999), VEGFR (Zhang et al. 2001).

1.7.3 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 (Yamanaka et al. 2002) and the PKC/CAMKII/NFAT dependent Ca^{2+} pathway (Wang et al. 2003), become activated following the formation of Wnt-Fz-LRP6 complex just like the canonical Wnt pathway. Although the non-canonical pathways differ from the canonical pathway in their dependency on the type of G-proteins (Malbon et al. 2001), activation of Dvl, downstream of Frizzled, is critical for further signal transduction in both (Boutros et al. 1998; Sheldahl 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 involve JNK or PKC and CaMKII, and trigger the transcription of different target genes. It has been proposed for non-canonical Wnt-signalling 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 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 et al. 2003) and matrix metalloproteinases (Nateri et al. 2005). Certainly, canonical Wnt signals can be rechanneled into the JNK pathway through naturally occurring, intracellular molecular switches, like the Dvl inhibitors, Naked-s (Nkd-1, Nkd-2) (Yan et al. 2001) leading to AP1 rather than TCF activation. AP1 is not a single protein, but a complex of various smaller proteins (cJun, JunB, JunD, cFos, FosB, Fra1, Fra2, ATF2, and CREB), which can form homo- and heterodimers. The composition of the AP1 complex is a decisive factor in the activation of target genes, therefore the regulation of AP1 composition is important. Two prominent members of the AP1 complex cJun and Fra1 are both targets of the canonical Wnt pathway (Mann et al. 1999), indicating strong cross-regulation between the canonical and the non-canonical JNK dependent Wnt signalling cascades (Nateri et al. 2005).

While there are shared ligands (Rosso et al. 2005; Wang et al. 2005) and target genes (Shtutman et al. 1999; Schwabe et al. 2003) in the canonical and JNK dependent Wnt pathways, Ca^{2+}/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 et al. 2001). Generally, Ca^{2+} and PKC-dependent signals are frequently linked to AP1, NFkB and NFAT activation.

1.7.4 Inhibitory Wnt pathway

Besides the canonical and non-canonical Wnt pathways, inhibitory Fz pathways have also been described. Fz1 and Fz6 are, for example, able to transmit inhibitory Wnt signals. While Fz1 inhibits Wnt signal transduction via a G-protein dependent manner (Roman-Roman et al. 2004) (Zilberberg et al. 2004), Fz6 (Golan 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 et al. 2003; Smit et al. 2004) via a Ca^{++} dependent signalling cascade. NLK phosphorylates TCF that consequently

cannot bind to β-catenin, and the formation of active transcription complex becomes inhibited (Smit et al. 2004) .

1.7.5 Wnt-s in ageing

As Wnt-s are important regulators of stem cell survival and differentiation, recent studies have started to investigate the role of Wnt family members in ageing. Most studies confirmed that drastically reduced Wnt levels can trigger ageing as tissue specific stem cells fail to replenish mesenchymal tissues 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 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 (Brack et al. 2007).

1.7.6 Wnt-s 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 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 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 Wnt-s 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. Recent data (Pongracz et al. 2003) revealed differential expression of Wnt ligands and receptors in thymic cell types raising 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 et al. 2001; Staal 2001; Pongracz et al. 2003; Xu 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 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 Wnt-s. So far, signalling studies have revealed, that Wnt4 can activate both the canonical (Lyons et al. 2004) and the non-canonical (Torres et al. 1996) (Chang et al. 2007; Kim et al. 2009) Wnt-pathways.

1.8 Steroids and ageing

Physiological steroids are implicated in the regulation of thymic ageing. For example both surgical and chemical castration have been demonstrated to decrease the progression of thymic ageing (Qiao et al. 2008) indicating that high steroid levels would accelerate the

ageing process of the thymus. Still, steroids used in therapy have not been fully investigated for their effects on immune senescence. Autoimmune diseases and haematological malignancies are often treated by steroids, as they effectively promote apoptosis of leukaemia cells and trigger complex anti-inflammatory actions (Stahn et al. 2007). Apart from triggering decreased expression of cytokines and MHC class II (MHC II) molecules, glucocorticoid (GC) analogues like dexamethasone (DX) also induce apoptotic death of peripheral (Wust et al. 2008) and developing T-cells. In mouse models, GCs cause massive thymocyte depletion, especially in the $CD4^{+}CD8^{+}$ (DP) thymocyte population, (Wiegers et al. 2001; Berki et al. 2002; Jondal et al. 2004) blocking *de novo* T-cell production. Experiments have also demonstrated that high-dose GCs induce a dramatic (Blomgren et al. 1970) and apoptosis-associated (Boersma et al. 1979) involution of the thymus, and not only thymocytes but also TECs are seriously affected (Dardenne et al. 1986). Recent reports (Fletcher et al. 2009) have highlighted that TEC depletion appears reversible, and thymic epithelial stem cells play an important role in this process.

2. Thymic senescence – Current opinion

2.1 Physiological thymic senescence

2.1.1 Disintegration of epithelial network, adipose involution

Senescence exhibits characteristic histological changes in both the human and mouse thymus (Oksanen 1971; Marinova 2005). In young adult mice (at 1 month of age), histology reveals strict segregation of epithelial cell compartments by staining for medullary ($EpCAM1^{++}$, $Ly51^{-}$) and cortical ($EpCAM1^{+}$, $Ly51^{++}$) epithelial cellular subsets (Kvell et al. 2010). Thymic morphology shows high level of integrity just preceding puberty/early adulthood. However, the highly organized structure disintegrates and becomes chaotic by the age of 1 year. By this age the strict cortico-medullary delineation becomes disintegrated, degenerative vacuoles appear surrounded by areas showing strong co-staining with both epithelial markers. Also significant cellular areas appear that lack staining with either epithelial markers, a pattern completely absent at the young adult age. Staining for extracellular matrix components of fibroblast origin ($ER\text{-}TR7^{++}$) identifies mesenchymal elements. The staining pattern with ER-TR7 and EpCAM1 is strikingly different at the two ages examined. In young adult thymic tissue sections, a-EpCAM1 and a-ER-TR7- show little tendency for co-localization. In stark contrast, already by the age of 9 months a-EpCAM1 and ER-TR7-staining show significant overlap within the thymic medulla. The disorganization of thymic epithelial network is followed by the emergence of adipocytes. If thymic sections of senescent mice are co-stained with neutral lipid deposit-specific stains then histology shows the presence of relatively large, inflated cells in which the cytoplasm is pushed to the periphery by red-staining neutral lipid deposits, a pattern characteristic of adipose cells (Kvell et al. 2010).

2.1.2 Gene expression changes in the thymic epithelium during ageing

To investigate the underlying molecular events of thymic epithelial senescence, the gene expression changes may be investigated in TECs purified from 1 month and 1 year old mice (Kvell et al. 2010). 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 et al. 2002). At the same time, mRNA levels of pre-adipocyte differentiation markers PPARγ and ADRP rise with age. 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. 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 et al. 2006) and the same is suggested by our reports performed, however, with epithelial cells (Kvell et al. 2010).

According to literature, EMT is associated with differential expression of E- (decrease) and N-cadherin (increase) (Seike 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 and trans-differentiation.

2.1.3 Studies of LAP2α and Wnt4 effects on TEC

The hypothesis that both LAP2α and Wnt4 play important though opposite roles in thymic senescence may be addressed using LAP2α over-expressing or Wnt4-secreting transgenic TEP1 (mouse primary-derived thymic epithelial) cell lines. 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 et al. 1983). Using such cells quantitative RT-PCR analysis revealed that LAP2α over-expression triggers an immense surge of PPARγ expression. 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 also becomes up-regulated although to a lesser extent. On the other hand in Wnt4-secreting cells the mRNA level of both PPARγ and ADRP decreased (Kvell et al. 2010).

2.1.4 Fz-4 and Fz-6 expression and distribution 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 the Wnt4 receptors, Fz-4 and Fz-6 were analysed in thymi of young adult and mature adult (1 month and 9 months old) mice (Varecza et al. 2011). Q-RT-PCR analysis of TECs showed increased expression of both Fz-4 and Fz-6 mRNA with age. Immune-histochemistry using Fz-4 and Fz-6 specific antibodies confirmed elevated levels of both receptor proteins. 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 preferentially stained for

Fz4 and Fz6, the cortex (EpCAM1$^+$/Ly51$^+$) only faintly stained for these receptors. In contrast to the young tissue, the 9 month old thymus shows a different pattern as the whole section including the cortex has become increasingly positive for both receptors (Varecza et al. 2011).

2.1.5 PKCδ translocation and its relation with Wnt4 signalling

Since Wnt4 levels as well as its receptors are modulated during the ageing process, further studies were performed to investigate active receptor signalling that is invariably associated with modified level of phosphorylation of receptor associated signalling molecules. Since Fz-s associate with Dvls that are phosphorylated by the δ isoform of PKCs, PKCδ activity was in focus. To test the involvement of PKCδ in Wnt4 signal transduction, increased Wnt4 levels were achieved using the supernatant of Wnt4-transgenic cell line (Varecza et al. 2011). Wild type TEP1 cells were exposed to SNs of control and Wnt4-secreting cells for 1 hour, then cytosolic and membrane fractions were isolated from cell lysates. Similar to previous studies with Wnt-5a (Giorgione et al. 2003), Western blot analysis revealed that within one hour of Wnt4 exposure PKCδ translocated into the membrane fraction where the cleavage products (Kanthasamy et al, 2006) characteristic of PKCδ activation were detectable. Additionally, increased membrane localisation of PKCδ was also detected in the Wnt4-overexpressing cell line. As 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, up-regulation of PKCδ was also detected in the ageing thymi. To investigate the role of PKCδ involvement in Wnt4 signalling, PKCδ activity level was modified by either over-expressing wild type PKCδ or by silencing PKCδ translation using siRNA technology. CTGF was used as a read-out gene based on data of previous experiments (Varecza et al. 2011). Surprisingly, although over-expression of PKCδ had no radical effect on Wnt4 target gene transcription, even moderate down-regulation of PKCδ was able to significantly increase CTGF expression in the presence of Wnt4, indicating that PKCδ might be involved in a negative regulatory loop.

2.1.6 Negative regulatory loops of signalling during senescence

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. Experiments demonstrated age dependent increase of both Fz-6 and PKCδ as well as co-localisation of Fz-6 and PKCδ (Varecza et al. 2011). While in the young thymus Fz-6 and PKCδ co-localisation is more pronounced in the thymic cortex, in the ageing thymus it is the medulla that exhibits stronger staining for both proteins. 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.

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 et al. 2004), indicating that CTGF might be part of a negative feed-back loop. The expression of Fz-8 (Mercurio et al. 2004) a recently reported receptor for CTGF increased in ageing mice, while FoxN1 the direct target of β-catenin dependent Wnt4 signalling (Balciunaite et al. 2002) became undetectable (Kvell et al. 2010).

2.2 Thymic senescence model

2.2.1 Steroid induced accelerated 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 cell production in the thymus and that their concentrations decrease with age (Min et al. 2006). 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 adipose involution of the thymus.

Similar to physiological senescence, the level of FoxN1 transcription factor and its regulator Wnt4 decreased in TECs within 24 hours following a single dose DX injection and remained low for over 1 week (Talaber et al. 2011).

However, in clinical treatments GC analogues are widely used for extended periods of time, rather than single shots. 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 measured drastically down-regulated, while the adipocyte differentiation factor ADRP, down-stream target of PPARγ was significantly increased. The results indicate that adipocyte-type trans-differentiation is completed at the molecular level over a much shorter time period following exogenous steroid-induced senescence compared to physiological rate senescence (Talaber et al. 2011).

2.2.2 Wnt4 inhibits steroid-induced adipose trans-differentiation

To test whether Wnt4 can prevent adipocyte type trans-differentiation, Wnt4 over-expressing TEP1 cell line was exposed to DX for a week. While in the control cell line DX exposure induced up-regulation of adipose trans-differentiation markers, within the Wnt4 over-expressing cell line, none of the adipose trans-differentiation markers were up-regulated indicating that Wnt4 alone can efficiently protect TECs against exogenous steroid-induced adipose trans-differentiation (Talaber et al. 2011).

3. Conclusions

3.1 Physiological thymic epithelial senescence

There are characteristic changes in the gene expression profile of purified thymic epithelial cells during thymic epithelial senescence (Kvell et al. 2010). Of note, Wnt4 level decreases, while LAP2α level increases. Also, the expression of the transcription factor FoxN1 required for maintaining thymic epithelial identity diminishes with age. On the other hand, adipose

differentiation is confirmed at the molecular level by the increased expression of PPARγ and ADRP. This process is accompanied by shift from E-cadherin to N-cadherin, typical for EMT (epithelial to mesenchymal transition). These pioneer experiments confirm in both a model cell line and purified primary cells rendered transgenic for either Wnt4 or LAP2α that their opposing effects antagonistically influence adipose trans-differentiation of thymic epithelial cells via EMT. This has lead to the establishment of a novel, confirmed theory for the source of adipose cells replacing functional thymic epithelial network during senescence (see **Figure 1**). Apparently, these cells do not differentiate from invading or resident mesenchymal cells, but rather trans-differentiate (via EMT) from thymic epithelial cells (Kvell et al. 2010).

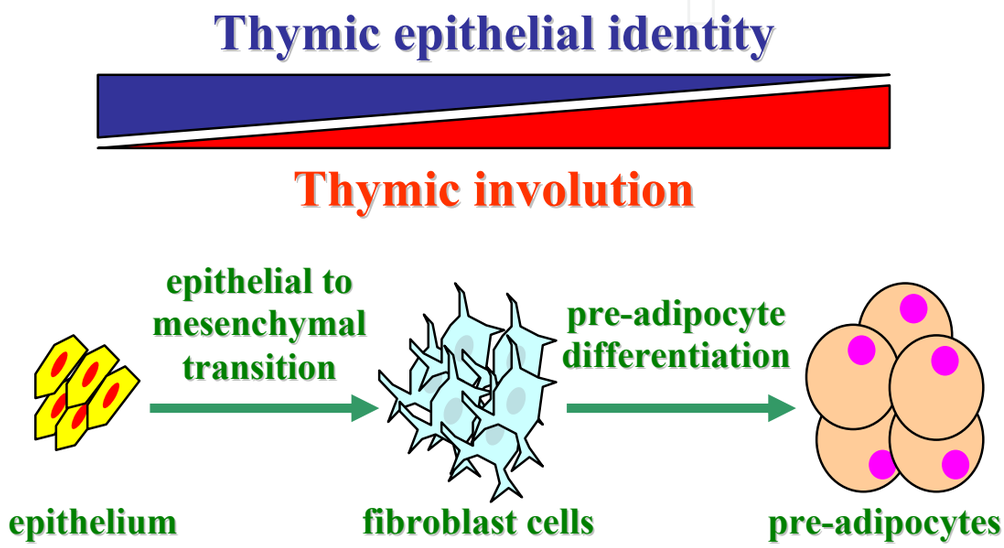


Fig. 1. Model of thymic involution process
Dedifferentiation of thymic epithelial cells triggers EMT (epithelial to mesenchymal transition) first, and then the resulting fibroblast cells undergo the conventional route of differentiation program towards adipocyte-lineage.

3.2 Signal transduction mechanisms involved in thymic epithelial senescence

While individual molecules, such as Wnt4 or LAP2α can serve as therapeutic targets to modify the ageing process, identification of complex interactions amongst signalling networks can provide further details. Investigation of Wnt signal transduction in the thymic epithelium has revealed that signalling pathways are activated or inhibited in an orderly fashion (Varecza et al. 2011). Initially, both Wnt4 receptors, Fz-4 and Fz-6 are up-regulated at young adult age. However, signals from Fz-4 and Fz-6 are different. While signals from Fz-4 initiate β-catenin dependent gene transcription, Fz-6 signals lead to suppression of β-catenin dependent signalling via increased activities of TGFβ-Activated Kinase (TAK) and Nemo-Like-Kinase (NLK). Fz-associated signals also require PKCδ to transmit Wnt signals. PKCδ associates with Fz-6 aiding suppression of β-catenin dependent signalling. Additional to Fz-6 signalling, connective tissue growth factor (CTGF, a β-catenin target gene) can also

feedback on β-catenin dependent signal transduction. CTGF can interact with Fz-8 as well as LRP6, an important co-receptor of Wnt signalling and can trigger activation of GSK3β. This latter leads to accelerated proteasomal degradation of β-catenin and hence suppression of Wnt signals. Multiple signalling mechanisms that lead to suppression of Wnt signalling is summarized in **Figure 2** (Varecza et al. 2011).

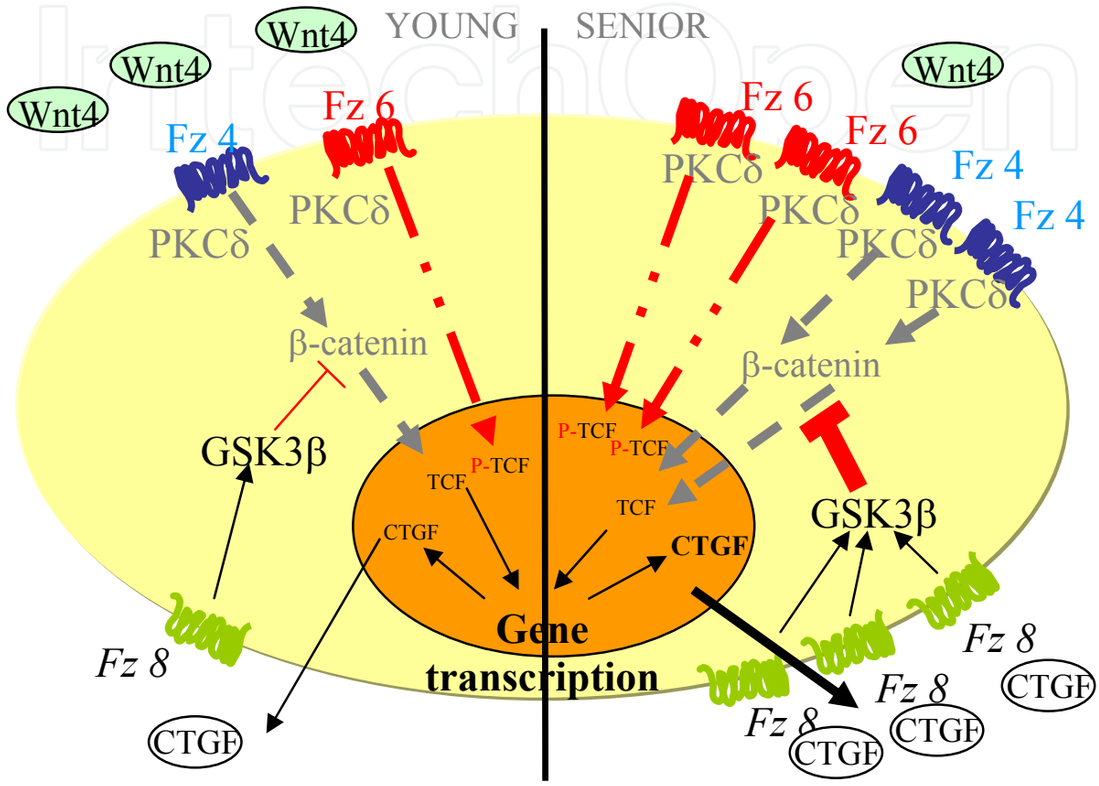


Fig. 2. Model of molecular mechanisms in thymic aging
At young age, Wnt4 levels are high and Wnt4 molecules compete for a moderate number of Fz receptors. While Fz-4 activates canonical Wnt signalling, signals from Fz-6 inhibit β-catenin dependent gene transcription keeping Wnt4 dependent signalling in balance. During the ageing process, Wnt4 levels decrease, while receptor expression increases with proportionally higher Fz-6. The β-catenin dependent Fz-4 signals lead to increased expression of CTGF. The CTGF receptor Fz8 is also up-regulated leading to enhanced activation of GSK3β. All these signalling events lead to loss of thymic epithelial cell characteristics and provide an opening for molecular events leading up to adipocyte type trans-differentiation.

3.3 Accelerated-rate, induced model of thymic epithelial senescence

Glucocorticoids are immunosuppressive drugs often used for treatment of autoimmune diseases and haematological malignancies. Although glucocorticoids can induce apoptotic cell death directly in developing thymocytes, how exogenous glucocorticoids affect the

thymic epithelial network that provides the microenvironment for T cell development has been poorly characterised. The effect of DX (dexamethasone) on thymic epithelial cells has been tested both *in vitro* (model cell line) and *in vivo* (mouse model) (Talaber et al. 2011). *In vivo*, following single treatment with pharmacologically relevant dose of DX reversible changes in gene expression profile identical to physiological thymic epithelial senescence have been recorded, but occurring at a highly accelerated pace (see **Figure 1**). Specifically, the expression of Wnt4 and FoxN1 decreased, while LAP2α and PPARγ levels increased. Moreover, sustained DX treatment has induced the elevation of ADRP expression as well. The same changes of gene expression profile have been observed using the model TEP1 (thymic epithelial) cell line, however, *in vitro* studies have shown the molecular level rescue of thymic epithelial cells from adipose trans-differentiation due to the over-expression of Wnt4. These studies reveal the currently neglected effect of steroid therapy on thymic epithelial cells in patients receiving sustained or even single dose treatment and highlights novel potential side-effects appearing in the form of accelerated thymic senescence (Talaber et al. 2011).

4. Perspectives

4.1 Intervention possibilities of thymic rejuvenation

This chapter summarised current knowledge on thymic senescence, a central immune tissue that suffers significant morphological changes and functional impairment during ageing. The epithelial network is in focus that provides the niche for developing thymocytes until adipose involution begins. We have discussed physiological thymic epithelial senescence in detail with respect to the signalling pathways involved in the process (Kvell et al. 2010). It has also been shown that steroid induced accelerated rate thymic epithelial senescence quite resembles physiological rate senescence (except for its speed) at the molecular level (Talaber et al. 2011). The data presented confirm that Wnt4 can efficiently rescue thymic epithelial cells from steroid-induced adipose involution at the molecular level (Talaber et al. 2011). Since physiological and steroid-induced thymic epithelial senescence are identical at the molecular level, it is anticipated that sustained Wnt4 presence in the thymic context can efficiently prolong FoxN1 expression, maintain thymic epithelial identity and prevent trans-differentiation towards adipocyte lineage. The same works identify LAP2α as a pro-ageing molecular factor promoting the trans-differentiation of thymic epithelial cells into pre-adipocytes via EMT. The thymus selective decrease of LAP2α activity through small molecule compounds could theoretically shift the delicate molecular balance towards the same direction as increased Wnt4 presence.

However, there are also other methods that can efficiently support major functions of the thymus: T cell maturation and selection. An example is the thymus-specific enrichment of transgenic IL7 proteins using IL7-CCR9 fusion proteins that selectively home and accumulate in the thymic context to reinforce thymocyte development and maturation (Henson et al. 2005). This method has been characterised in detail and is currently being geared up towards potential human application in the form of inhalation products selectively delivering IL7 to the thymus (Aspinall et al. 2008).

The thymus-specific ablation of sex steroids also offers a target point for such interventions. Major involution in thymus mass occurs in parallel with the advance of puberty and

correlation has been drawn with sex steroid levels. The use of thymus selective 11β-HSD1 inhibitor compounds could also theoretically decrease thymocyte sensitivity to steroid-induced apoptosis and steroid-induced epithelial molecular senescence, providing synergistic mechanism of action. Such artificial compounds (like the PF-00915275) have already been tested in healthy volunteers and were approved for safety (Courtney et al. 2008). However, these compounds do not specifically accumulate in the thymus and have not been tested in the thymic context.

Alternative methods for thymic rejuvenation include those targeting KGF, ghrelin and GH signal transduction pathways (Aspinall et al 2008). The ideal future thymus rejuvenation system that works selectively in the thymus at high efficiency and low side-effect ratio would likely constitute a combination of the above outlined methods and would efficiently aid restoration of immune competence.

4.2 Social and economic impact

By targeting and specifically inhibiting the molecular pathways that drive thymic adipose involution / immune senescence, it is possible to extend immune health-span within life-span, and improve health and quality of life, and also significantly decrease healthcare costs. This effect is expected to be very significant as – opposed to certain diseases – the physiological process of immune senescence affects all individuals, including currently healthy people.

It is to be evaluated whether an immune-fitness extending treatment would be predominantly useful as preventive treatment applied in younger individuals or would rather be useful as a reversal treatment in elderly individuals with various stages of thymic adipose degeneration. Both scenarios would affect vast segments of the population and would yield similarly significant economic and social benefit.

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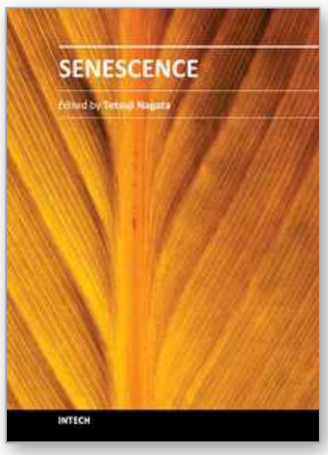
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