Final report of OTKA PD grant No. 78310

Title: The identification of pro-aging molecular mechanisms in the thymus

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1. Physiological thymic senescence

1.1. Disintegration of epithelial network, adipose involution (Figure 1)

Senescence exhibits characteristic histological changes in both the human and mouse thymus. 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. 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-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 contrast, by 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 large, inflated cells in which the cytoplasm is pushed to the periphery by red-staining neutral lipid deposits, a pattern characteristic of adipose cells.

1.2. Gene expression changes in the thymic epithelium during ageing (Figure 2)

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. 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. 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 and the same is suggested by our reports performed, however, with epithelial cells.
According to literature, EMT is associated with differential expression of E- (decrease) and N-cadherin (increase). 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.

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. 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.

2. Steroid-induced thymic senescence

2.1. Steroid-induced accelerated thymic senescence model (Figure 3)

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. 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.
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 drastically down-regulated measured, while the adipocyte differentiation factor ADRP, down-stream target of PPARγ and LAP2α 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.

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.

3. Signal transduction

3.1. Signal transduction mechanisms involved in thymic epithelial senescence (Figure 4)

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. 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 together lead to the suppression of Wnt signalling.

4. Conclusion (Figure 5)

4.1. Physiological thymic epithelial senescence

There are characteristic changes in the gene expression profile of purified thymic epithelial cells during thymic epithelial senescence. 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 confirmed in both model cell line and purified primary cells rendered transgenic for either Wnt4 or LAP2α show their opposing effects on 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. Apparently these cells do not differentiate from invading or resident mesenchymal cells, but rather trans-differentiate (via EMT) from thymic epithelial cells.

4.2. 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). *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. 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.

5. Future plans

5.1. Identification of small molecule inhibitors of LAP2α

We plan to utilize a cellular micro-environment array system (provided by collaborative partner Karl Willert PhD) to test a small-molecule library. The library (offered by service-based Vichem Ltd, http://www.vichem.hu/) contains 17,000 compounds divided into 300 groups. These 300 compound mixtures will be tested for the changes in PPARγ target gene expression in the cellular micro-environment array system. The 17,000 compounds have been grouped with overlaps in a manner so that a single run of 300 groups allows for the identification of individual candidate molecules. The runs will be performed in triplicates to allow for statistical analysis. Once individual molecules have been identified (with the help of Vichem Ltd that holds the key for compound allocation pattern among the 300 groups) Vichem will synthesize sufficient amounts of the select candidate molecules for their individual tests. This will be performed by re-running the cellular micro-environment arrays using the selected individual molecules. We expect to have a handful of novel, patentable small compounds capable to halt thymic epithelial trans-differentiation by preserving epithelial identity. Our research group has submitted an OTKA K proposal (No. 104500) to finance the small molecule inhibitor tests.

6. Acknowledgements

I wish to thank the continuous support of my previous supervisor Judit PONGRACZ PhD in aiding the first years of my career as a principal investigator. I am also truly grateful for OTKA for providing me the chance to show my capabilities as a principal investigator. I hope the scientometric figures (3 open-access publications in international, peer-review, high-impact journals and a bookchapter) and the current summary together prove that the project was worth the credits. I assume that the recently submitted OTKA K grant proposal (No. 104500) will gain further support and so the project can advance towards potential therapeutic applications.

7. Figures

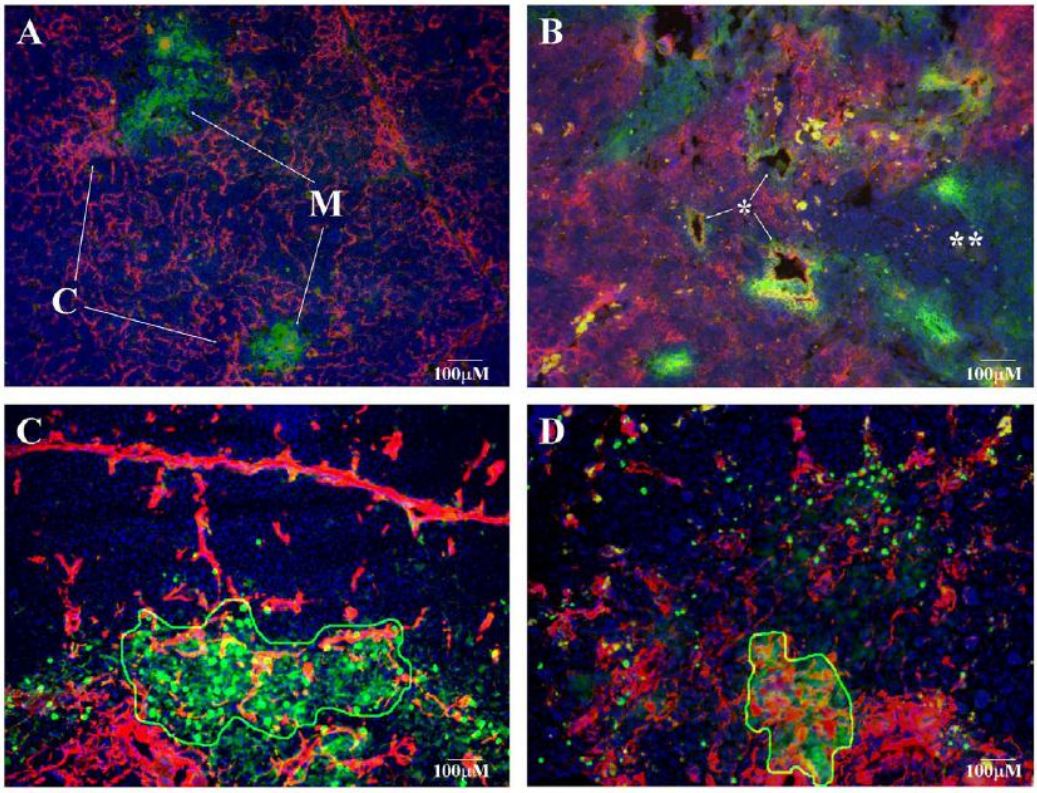
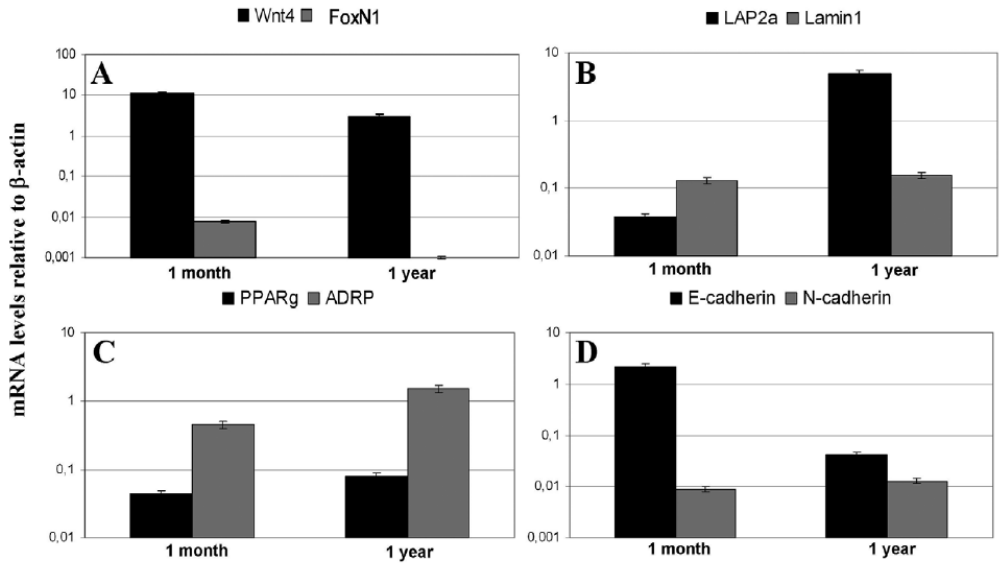


Figure 1A section of 1 month, figure 1B section of 1 year old BALB/c mouse thymus. Staining pattern: anti-EpCAM1-FITC (green), anti-Ly51-PE (red), DAPI (blue). 'M': medullary, 'C': cortical epithelial compartments. *: degenerative vacuoles, **: loss of epithelial staining. Figure 1C section of 2 month, 1D section of 9 month old thymus. Staining pattern: anti-EpCAM1-FITC, ER-TR7-PE, DAPI (blue). EpCAM1++ thymic medulla is outlined.



Figures 2A–D demonstrate gene expression changes of MACS purified thymic epithelial cells measured by Q-PCR. Please note that the Y-axis scale is logarithmic. Error bars show ±1 SD.

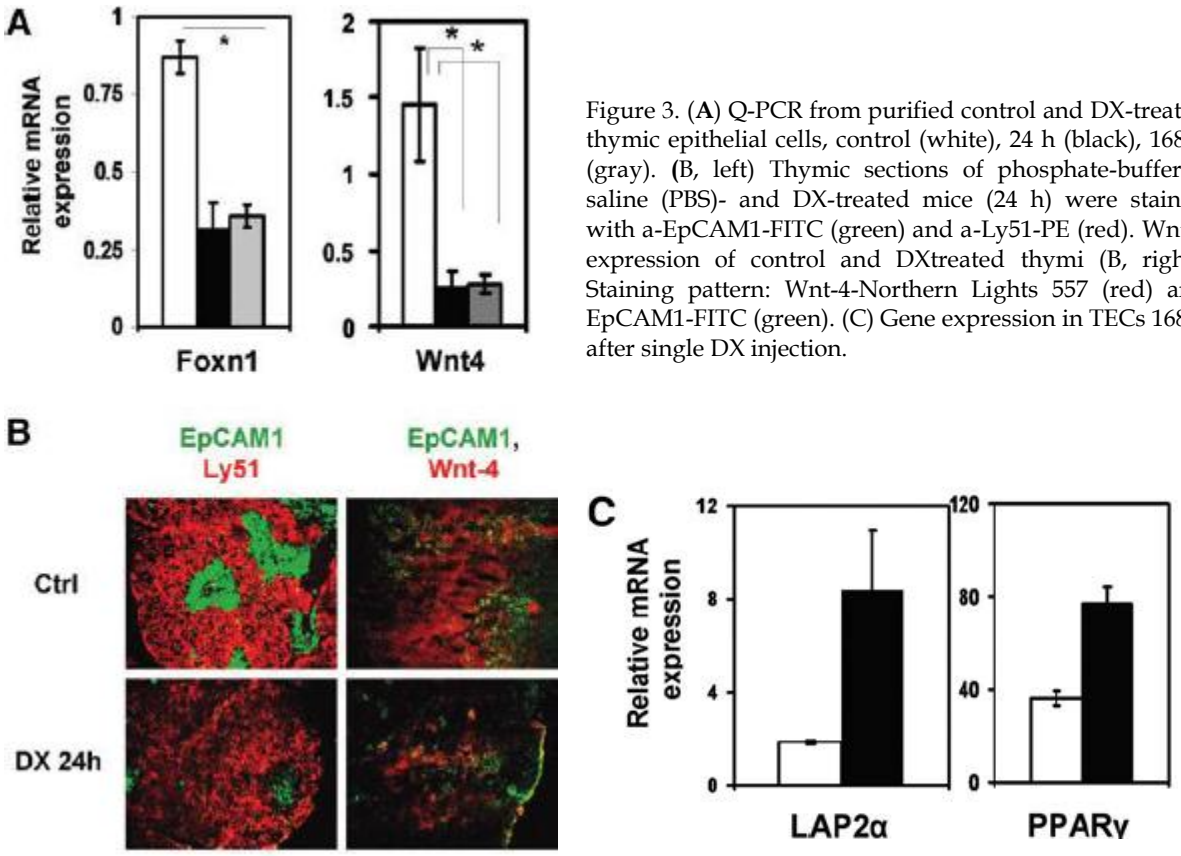


Figure 3. (**A**) Q-PCR from purified control and DX-treated thymic epithelial cells, control (white), 24 h (black), 168 h (gray). **(**B, left) Thymic sections of phosphate-buffered saline (PBS)- and DX-treated mice (24 h) were stained with a-EpCAM1-FITC (green) and a-Ly51-PE (red). Wnt-4 expression of control and DXtreated thymi (B, right). Staining pattern: Wnt-4-Northern Lights 557 (red) and EpCAM1-FITC (green). (C) Gene expression in TECs 168 h after single DX injection.

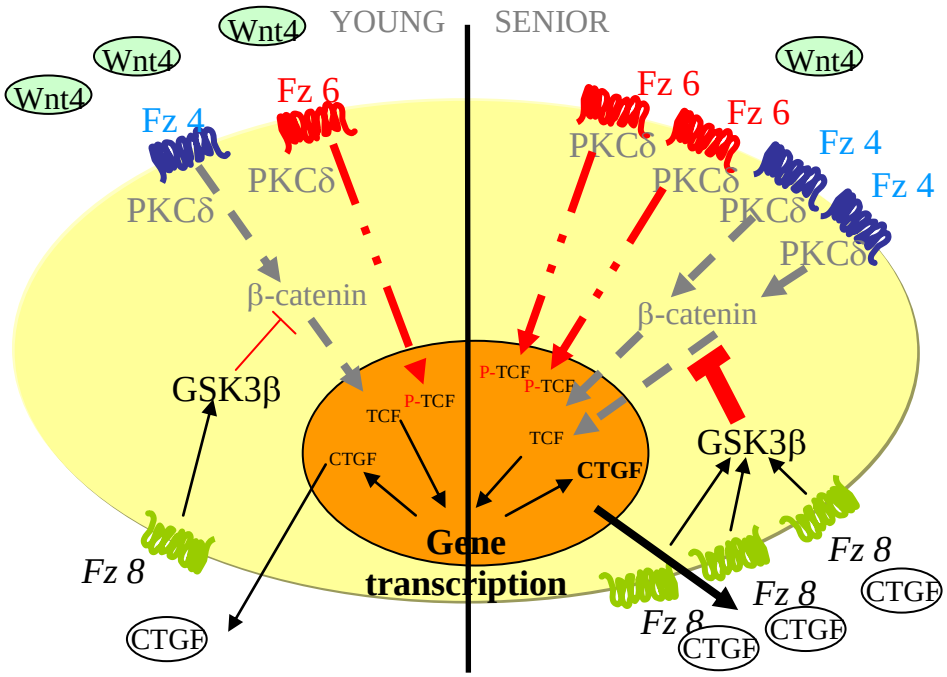


Figure 4. At young age, Wnt4 levels are high. During the ageing process, Wnt4 levels decrease, while receptor expression increases with proportionally higher Fz-6. The □-catenin dependent Fz-4 signals lead to increased expression of CTGF. The CTGF receptor Fz8 is also up-regulated leading to enhanced activation of GSK3β.

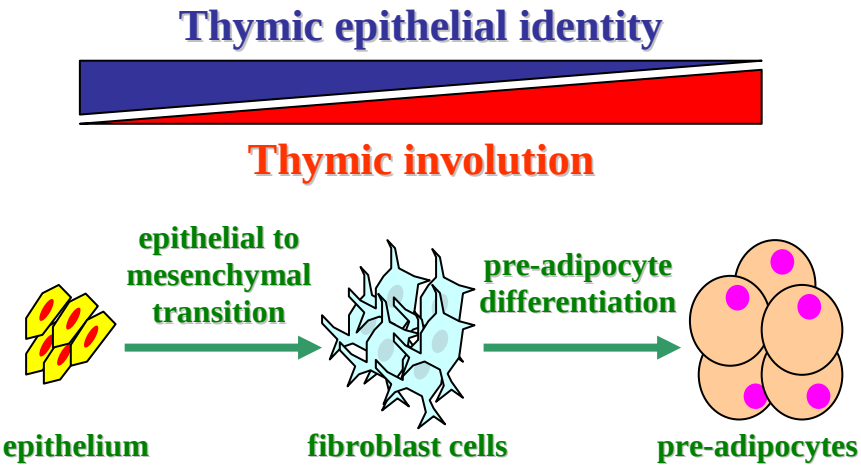


Figure 5. Dedifferentiation of thymic epithelial cells triggers EMT (epithelial to mesenchymal transition) then the resulting fibroblast cells undergo the conventional route of differentiation program towards adipocyte-lineage. The process occurs during both physiological and steroid—induced thymic adipose involution.

8. References

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