Ph.D. thesis

The role of Wnt signalling in thymic senescence

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1. Introduction

Ageing in focus

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.

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. 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.

Significance of thymic involution studies

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.

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. As the thymic epithelium is replaced by adipose tissue, the whole process is called adipose involution. Due to decrease in thymic epithelial tissue mass, the thymus can no longer support the same output of T-cell production. 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. One of the transcription factors, FoxN1 that is characteristic in thymus development is also affected by age. FoxN1 is not only essential for progenitor epithelial cells of the thymic rudiment to develop into various epithelial subsets 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.

Wnt-s in ageing

As Wnt-s 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. Based on the experiments on KLOTHO mice it has been proposed that increased Wnt signalling leads to continuous stem cell proliferation which finally can also result in depletion of the stem cell pool causing accelerated ageing.

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. Initial experiments, by manipulating the level of some Wnt-s and soluble Fz-s, have shown perturbation of T-cell development 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. 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 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 and the non-canonical Wnt-pathways.

Inhibitory Wnt pathway

Besides the canonical and non-canonical Wnt pathways, inhibitory Fz pathways have also been described. Fz-1 and Fz-6 are, for example, able to transduce inhibitory Wnt signals. While Fz1 inhibits Wnt signal transduction *via* a G-protein dependent manner, Fz-6 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) *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.

PKC-s in the thymus

Members of the PKC family regulate a wide variety of cellular processes including proliferation differentiation and apoptotic death. Catalytically active PKC-s usually relocate from the cytosol to cellular or nuclear membranes. Although PKC-s have been described as having a non-redundant role in signal transduction of various immune cell types including mature T-cells and developing thymocytes, the expression of PKC family members and their function in the thymic epithelium remains obscure.

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 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. Experiments have also demonstrated that high-dose GCs induce a dramatic and apoptosis-associated involution of the thymus, and not only thymocytes but also TECs are seriously affected.

2. Materials and Methods

Antibodies

Western blot analysis

For western blot analysis we used rabbit polyclonal and goat polyclonal and rat monoclonal primary antibodies and donkey HRP-conjugated secondary antibodies.

Animals

BALB/c 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.

Cell cultures.

Cell lines

TEP1 (thymic epithelial) 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.

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. 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.

Dexamethasone treatment of cells and animals

Cell lines were treated with DX with a final concentration of 1 µM for 1 week. 4 week-old BALB/c mice were used for the experiments. Animals received a single dose (20 mg/kg) Dexamethasone injection intraperitoneally, then were sacrificed 24 and 168 hours after injection or received PBS or DX for 3 months. 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.

Manipulation of gene expression

Retroviral Constructs

Wnt4: The Wnt4 sequence was purchased and subcloned from a 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.

Wnt4 and PKCδ sequences were amplified and cloned into the MIGRI retroviral vector. Retrovirus was produced by transfecting the plasmid DNA into the Phoenix packageing cell using Lipofectamine 2000.

Transient transfection of siRNA PKCδ

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.

Detection of gene transcription

cDNA generation

cDNA was generated both from cell lines and primary cells by isolating total RNA either by TRI-reagent or by using an RNA isolating kit. Reverse transcription of 0.5 µg of total RNA was performed in 50 µl total volume using random hexamer primers.

Microarray analysis

Three independent replicate microarray experiments were performed.

Microarray and microarray data analysis was performed by the Center for Genomics, University of Debrecen.

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 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.

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. Sorted cells were collected for mRNA and protein extraction, microarray, qRT-PCR and Western-blot analysis.

PKCδ activation assay

The kinase assay was performed using the HTScan Kinase –assay Kit using a biotinylated substrate peptide in the presence of PKCδ diluted in kinase buffer. 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 (Soft Flow Hungary Kft. Pecs).

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 and standard centrifugation protocol was applied for collecting membrane proteins. Proteins of cytosolic and membrane fractions were separated on 10% SDS PAGE and PKCδ protein was detected using Western blotting.

Immuno-precipitation

TEP1 cells were lysed in RIPA-buffer supplemented with protease, and phosphatase inhibitors 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.

Western blotting

TEP1 cells were collected, whole cell lysates were resolved in 10% SDS–PAGE. Gels were blotted onto nitrocellulose membranes. Proteins were visualised by enhanced chemiluminescence as described in the manufacturer's instructions and analysed by Fuji LAS4000 image station using different exposure times from 0.5 – 6 min for best quality.

Immuno-histochemistry and immuno-fluorescency

Frozen thymic sections (7-10 μm thick) were fixed in cold acetone or in 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. 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.

3. 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.

4. Results

Summary of Results

Physiological thymic senescence

1. The mouse thymus undergoes morphological changes during senescence, similarly to humans
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 transdifferentiation
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

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 transdifferentiation

5. 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 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 immune-hystochemical 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, 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 immune-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 1.

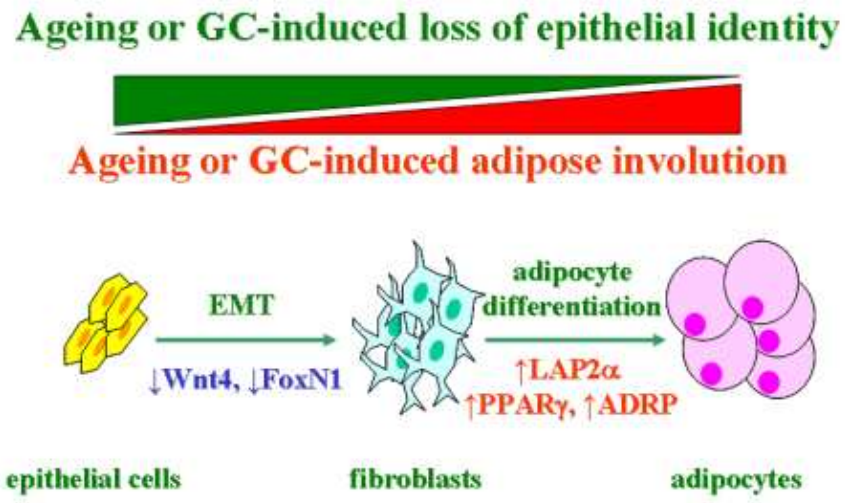

Figure 1. Model of adipose involution in thymic ageing

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 Fz4 and Fz6 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 Fz6, 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 Fz8 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 2.

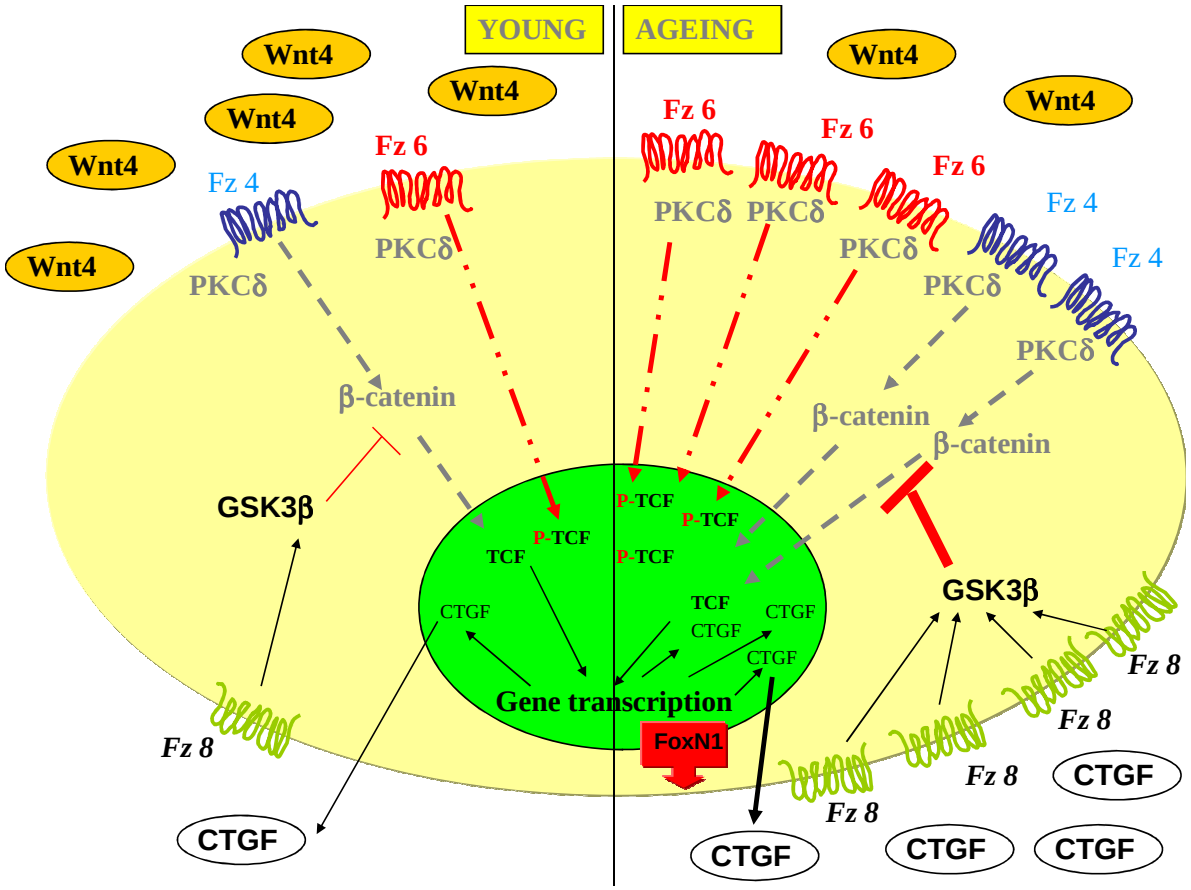

Figure 2. Model of molecular mechanisms in thymic ageing

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.

6. 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

5. Up-regulation of LAP2α initiates adipocyte type trans-differentiation (Fig. 1.) 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, Fz8 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. 2.).

7. List of Publications

Publications related to the thesis:

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.
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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.
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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

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.

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PKCs differentially regulate Wnt signalling in thymic epithelium in mice

Annual meeting of the Hungarian Society for Physiology, Pecs, june 5-8,2007

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Novel and atypical PKCs are involved in Wnt signalling in thymic epithelium in mice

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Further publications

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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.

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