THE ROLE OF EXTRACELLULAR VESICLES IN INHIBITING THYMIC AGING

PhD thesis

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Introduction

The immune system is a complex network, where circulating blood cells in lymphoid tissues act together against external pathogens. The lymphoid system consists of primary and secondary lymphatic tissues, where the bone marrow and the thymus belong to the group of primary lymphatic tissues. Among other, the main effector cells of the immune system are lymphocytes. Immunodeficiency can occur if components of the immune system become defective. This condition can be inherited, mainly presenting during infancy or at young ages. Recurrent infections can also highlight immunodeficiencies. Acquired immunodeficiency may develop at senior ages, and may be caused by medication, radiation, measles or HIV infection. In addition, various environmental factors can also influence the development of autoimmune diseases. The goal of this study was to emphasize the regenerative capacity of the thymus, which offers potential for therapeutic treatment. In the future transgenic Wnt4-exosomes of the thymus may be applied for immunodeficiencies, autoimmune- and malignant diseases.

Literature review

Structure of the young thymus

The thymus is a primary lymphoid tissue responsible for the development of T-cells. The epithelial cells of the thymus are in close interaction with the developing T-cells. The thymus regulates, when the mature T-cells can enter to the circulation. It also eliminates non-functional and auto-reactive T-cells generated during the maturation process. Also, the developing- and mature T-cells can stay hidden from external pathogens until they become ready to enter the periphery. The two separated lobes of the thymus are further divided into cortical and medullary compartments and are segregated by the cortico-medullary junction. In the cortical region developing thymocytes are controlled via thymic nurse cells (epithelial cells), and macrophages that eliminate defective and autoreactive T-cells. Along the cortico-medullary junction dendritic cells present self-antigens to T-cells in order to filter out unwanted T-cells. Hassal's corpuscles are typical for the medullary part of the thymus in human. Differentiation of thymic epithelial cells is indispensable for the selection of T-cells, regulated by different growth factors and regulatory molecules including FoxN1 and Wnt4. Cortical epithelial cells express cytokeratin 8, EpCAM1, Ly51, CD205, MHCI and MHCII, while medullary epithelial cells contain cytokeratin 5, EpCAM1, MHCII and CD80 cell surface markers.

T-cell maturation: from the bone marrow to the thymus

Upon hormonal induction the hematopoetic stem cells migrate from the red bone marrow to the thymus. These T-cell progenitors go through a complex developmental process, where their T-cell receptor (TcR) genes undergo rearrangement. Arriving to the thymus, thymocytes are $TcR^-/CD3^-CD4^-CD8^-$ double negative (DN). According to the expression of CD44 and CD25 cell adhesion molecules, the initial part of the DN phase can be further divided into four subgroups. During the DN1 phase thymocytes are CD44 positive, but CD25 negative, while entering the DN2 phase thymocytes mature into $CD44^{low}$ and $CD25^+$ cells. Thymocytes in the DN3 phase first lose their CD44 positivity, then their CD25 expression in the DN4 phase and finally turn into DP (or $CD8^+CD4^+$) cells. IL-7 has an important role during early T-cell development, without its expression the T-cell maturation process halts. Finally DP thymocytes go through a negative selection procedure, where they show affinity to either MHCI molecules (and become $CD8^+$ T-cells) or to MHCII molecules (and become $CD4^+$ T-cells). Non- functional DP cells are eliminated via apoptosis. At the end of the maturation process $CD4^+$ or $CD8^+$ (SP) T-cell migrate further to the medullary compartment of the thymus binding to the ligands of medullary thymic epithelial cells via CCR7 chemokine receptors. In summary, T-cell development is a complex process, only those functional SP $CD4^+$ or $CD8^+$ T-cells reach the circulation that show tolerance towards self-antigens.

Aging of the thymus

During aging the thymus goes through a process, where thymic epithelial cells differentiate via epithelial-to-mesenchymal transition (EMT) and finally become adipocytes. In human this degenerative process already starts in childhood and accelerates with puberty due to hormonal induction. The process shows identical kinetics in mouse. It is estimated that by the age of 50 years in human (approx. 15 months in mouse), the thymus loses approximately 90% of its function: naive T cell production. The consequences of impaired thymus function cause elevated incidence of infections, cancer- and autoimmune disorders as observed at senior ages.

Transcription factor FoxN1 is the mastermind of thymus organogenesis and identity, and is also an acknowledged direct molecular target of the glycolipoprotein Wnt4. As a consequence, Wnt4 plays a key role during embryonic thymus development and the maintenance of its identity in adulthood. Over time thymic epithelial cells secrete less Wnt4, while their Frizzled receptors (Fz4 and Fz6) become up-regulated indicating a potential

compensatory mechanism and possibly enhanced Wnt4-binding. This results in the suppression of the Wnt pathway, allowing PPARgamma to take over and initiate adipocyte differentiation.

We have previously shown that the addition of exogenous Wnt4 reinforces thymic epithelial identity and confers resistance in a steroid-induced model of senescence through suppressing PPARgamma. Previous records show that Wnt4 loses its activity when purified as a sole compound, but remains active as supernatant fraction. Recent publications of various tissue contexts have suggested that Wnt molecules (including Wnt4) travel in conjunction with extracellular vesicles (EVs), more specifically exosomes. It has also been reported that a significant portion of the Wnts (including Wnt4) may actually be displayed on exosomal surfaces. Along with Wnt4, the Wnt pathway activator miR-27b has also been shown to inhibit PPARgamma activity. Similar to the Wnt molecules, miRNA species can be also found and are transported in EVs, especially in exosomes.

Molecular background of adipose tissue formation in the aged thymus

Next to PPARgamma, transcription factor TBX-1 also plays an important role in thymic adipose involution. TBX-1 is a key molecular player in thymus organogenesis during embryonic development. Impaired thymus organogenesis leads to deficient thymocyte development, naive T-cell production, and immune functions. This function was investigated in the embryonic setting, but the potential role of persistent TBX-1 expression during adulthood has not been addressed yet. TBX-1 has another pivotal role in the development and function of a recently described subtype of adipose tissue: beige adipose tissue. Beige adipose tissue also responds to adrenergic stimuli via thermogenesis, similar to brown adipose tissue. TBX-1 is considered as a beige-specific marker, but other beige-indicative markers have also been described. Mitochondrial uncoupling protein-1 (UCP-1) has been reported to be expressed by brown/ beige adipose tissue. EAR2 was reported to efficiently promote adipose tissue development, while CD137 is an acknowledged beige adipocyte surface marker. The adult thymus expresses TBX-1 and UCP-1 in the stromal compartment, both known to promote beige adipose tissue development. Yet to date thymic adipose tissue that develops with age has not been accurately positioned on this white-beige-brown continuum of adipose tissue subtypes. Our work shows that TBX-1 expression is present in adulthood, with a transient decrease in its expression (in human at approx. 23 years, in mouse at approx. 12 months of age). This might reflect the functional switch of TBX-1 from embryonic thymus morphogenic to adult adipose morphogenic. We have also investigated other beige adipose

tissue markers, such as PPARgamma, UCP-1, CD137, EAR-2 and Lipid TOX, which all showed increased expression after steroid treatment of mouse (TEP1) and human (1889c) TEC cells serving as cellular senescence model. Furthermore, immune-fluorescent staining and TEM showed the presence of adipose tissue traits in the form of intracellular multilocular neutral lipid drops, which also suggest that thymic adipose tissue has beige/brown characteristics.

General characterization and types of extracellular vesicles

Extracellular vesicles (EVs) are 30-1000 nm membrane-delimited structures secreted into the extracellular space from any type of eukaryotic or prokaryotic cell. They are present in high abundance in all types of natural fluids (e.g. blood, saliva, urine or breast milk, but also fresh- and saltwater). Cell cultures *in vitro* also secrete vesicles into the cell culture medium. The vesicles differ from each other in composition, size and biogenesis, thus differentiating apoptotic bodies, microvesicles and exosomes. Microvesicles are 100-1000 nm vesicles, while apoptotic bodies range between 1000-5000 nm. The smallest sized particles are exosomes varying between 30-100 nm in diameter and are the most examined and investigated vesicle population.

Exosomes

Exosomes are the smallest (30-100 nm) vesicle population, their membrane contains many lipids, proteins and lipid rafts (microdomains). Similar to cells, their membranes also contain a phospholipid (PL) bilayer, but the outer layer also contains phosphatidylserine (next to phosphatildylcholine), losing the PL-asymmetry. Exosomes are generated via invagination of the plasma membrane and packaged into multi-vesicular bodies (MVB). Finally they fuse with the plasma membrane of the cell and are released via exocytosis into the extracellular space. This process is regulated by several proteins including TSG-101 and the Alix proteins. Exosomes can also contain molecules from endosomes, or cell surface receptors. Moreover, signaling molecules, integrins and other receptors may also be found in vesicles. Additionally, exosomes contain heat shock proteins, which play an important role during antigen presentation. Many proteins can be found either membrane-bound or lipid-bound on their surfaces including tetraspanin proteins, annexin and flotillin. Proteins can also comprise their cargo including enzymes, cytoskeletal proteins, cytosolic proteins and other signal transduction molecules. Beyond lipids and proteins, exosomes also contain nucleic acids like

nuclear or mitochondrial DNA fragments, mRNA, miRNA or other non-coding RNA-s. These exosomal RNAs may be taken up by adjacent cells or circulate.

Isolation and detection techniques of extracellular vesicles

According to literature data there are numerous methods to isolate extracellular vesicles from various biological fluids. The most commonly used method is ultracentrifugation, but several other methods may also be used for vesicle isolation such as precipitation, immune-affinity, size-exclusion chromatography or ultrafiltration. If isolating EVs from cell culture supernatant it is mandatory to avoid FBS, since vesicles in the serum have the same size and density as vesicles of interest. Several methods are used for the detection of EVs: transmission electron microscopy, flow cytometry, ELISA, western-blot and mass spectrometry.

Biological function, diagnostic and therapeutic application of extracellular vesicles

EVs have various biological functions, they take part in cell-and tissue regeneration processes, blood coagulation, regulation of the immune response and their role during pregnancy has also been published. The surface molecules of vesicles match with the molecules of the plasma membrane of the original cell, allowing for tracing experiments. Under pathological conditions the vesicular composition and quantity of the secreting cells may change, which gives the chance to isolate and identify these EVs from various body fluids. Previous literature data confirms that EVs are present in various diseases, they trigger immune suppression in malignant diseases, the development of metastatic niches as well as the process of angiogenesis. With the analysis of miRNA content of EVs secreted by cells into the periphery, predictive diagnostic markers may be identified. There is also a possibility for horizontal gene transfer, where the function of the recipient cell is modified via inducing protein expression with the help of delivering miRNA or mRNA molecules. Furthermore miRNAs are able to target viral genomes to have antiviral effect.

Role of extracellular vesicles in the context of the thymus and T-cell development

In the past few years several studies have shown that the thymic epithelium is a rich source of immunologically relevant exosomes. Thymus originating exosomes have been analysed using proteomic and miRNA detection methods to contain annexins and Rab

proteins. Cytokeratins have been also identified, which confirms the thymic epithelial origin of thymus exosomes.

Our research group has also performed experiments related to the effect of Wnt4 exosomes on $CD34^+$ hematopoetic stem cells. Peripheral blood-bourne $CD34^+$ cells have been added to a 3D human thymic epithelial (1889c) cell culture model, where we monitored the effect of Wnt4 exosomes on T-cell development after incubation for several weeks. We found that the addition of Wnt4 exosomes accelerated T-cell development, where tymocytes have reached a developmental stage close to SP condition. This proved that Wnt4 together with FoxN1 can induce the thymic epithelium to render T-cell development more efficient. Knowing this, the regenerative and T-cell development inducing capacity of Wnt4 exosomes could be used for therapeutic applications.

The impact of thymic aging on the efficiency of the immune system

Immune-senescence is a process, where the immune system starts to decline functionally over time. Due to physiological aging the aged population becomes more susceptible to infections while vaccination becomes less efficient. Life expectancy of the global population has increased in the past few decades, which mandate for solutions to prevent the development of age-related infections, malignant- and autoimmune diseases. Senior citizens are severely affected by type A influenza virus (H1N1), West-Nile virus (WNV) or the new Coronavirus (SARS-CoV-2 causing COVID-19). Recently it has been shown, that exosomal miRNAs play an important role during viral infection. These data also confirm that the aged population shows a need for improved vaccination and novel therapeutic solutions which aim the regeneration of the immune system.

During aging a chronic inflammation status called „inflammaging" develops, in which autoreactive T-cells are released from the dysfunctional thymus into the circulation causing tissue inflammation. Latent viral infections, pro-inflammatory cytokines produced by senescence-associated cells are also responsible in the development of inflammaging. Lately it has been shown that EVs isolated from young mice are able to counteract thymus aging. These exosomes contain miRNAs, which can act as post-translational modifiers and temporarily restore the microstructure and weight of the thymus. These EVs can decrease the number of autoreactive T-cells and chronic inflammation.

Yet to date literature is lacking on the effect of thymic epithelial exosomes on thymus tissue regeneration. Thymus senescence may be accelerated by specific toxins, viruses or heavy metals, where it is important to reinforce tymic epithelial identity.

Aims of the thesis

Our goal was to characterize transgenic exosomes from Wnt4 over-expressing thymic epithelial cell line, to determine their Wnt4 and miR-27b content, test their thymus tissue regenerative capacity and examine their distribution *in histo* and *in vivo*.

The following objectives were in focus during the experiments:

1. Establish and apply efficient and reproducible transgenic thymic epithelial exosome isolation protocol.

2. Qualitative and quantitative characterization of transgenic thymic epithelial exosomes with diverse methods.

3. Investigate the biological effect of transgenic thymic epithelial exosomes, test and prove their anti-aging effect in the thymus.

4. Label transgenic thymic epithelial exosomes in order to carry out *in histo* and *in vivo* binding and tracking experiments.

5. Monitor the tissue binding activity of labeled exosomes during *in histo* experiments, evaluate their binding pattern on thymus sections of different ages.

6. Track the route of labeled transgenic thymic epithelial exosomes during *in vivo* experiments, evaluate their topological distribution in various organs.

7. Examine the tissue regenerative capacity of exosomes in mice and investigate their potential in human applications.

8. Determine the miRNA profile of steroid-induced thymic aging, provide insight into the exosomal miRNA regulation processes during aging.

Materials and methods

Cell cultures

In vitro experiments were performed using the TEP1 primary derived (BALB/c) thymic epithelial cell line. A549 human lung epithelial cell line served as a control in some experiments. The Wnt4 over-expressing version of TEP1 and the Wnt5a over-expressing version of A549 were generated via lentiviral transfection linked to green fluorescent protein (GFP) expression. Cells were maintained in DMEM supplemented with 10% FBS, penicillin-streptomycin (P/S), L-glutamine, Hepes buffer, non-essential amino acids, and ß-mercapto-ethanol. In order to differentiate thymic epithelial cells (TECs) into adipocytes, steroid treatment was used. Dexamethasone (DX) was diluted to a final concentration of 1μM and added for a week. To reveal the miRNA profile of the aging model we treated human thymic epithelial cells (1889c) with DX. Cells were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamin, P/S and Hepes buffer. All cells were incubated at 37°C and 5% CO_2.

Flow-cytometry

Both control and Wnt4 over-expressing TEP1 cell lines were verified for presence of Wnt4 over-expression via analyzing GFP expression. In both cases 150,000 cells were washed with 1x PBS then fixed using paraformaldehyde containing PBS solution. BD FACSCanto II flow-cytometer was used for data acquisition at a medium flow rate and stopped at 10,000 events. Measurements were performed and analyzed with BD FACSDiva Software version 6.1.3.

Exosome collection, isolation and labeling

Control mouse TECs, Wnt4 overexpressing mouse TECs and Wnt5a over-expressing human A549 cells were cultured in T-Cell Expansion Medium and serum-free DMEM until they reached 80– 90% of confluence. Equal volume (10-10 ml) of FBS-free cell culture media were collected from T75 tissue culture flasks and centrifuged at 2,000 g for 30 min to remove cell debris and apoptotic bodies. Supernatants were filtered through a 0.45μm filter and incubated overnight at 4◦C using Total Exosome Isolation (TEI) Reagent. Following an hour long centrifugation at 10,000 g, pellets were collected and re-suspended in sterile PBS for further use. Exosomes were fluorescent-labeled using DiI lipid stain. DiI lipid-stain was added to the cell culture medium the day before collecting cell supernatant. DiI lipid-stain 50 mg/mL stock solution was diluted 10,000-fold.

Transmission electron microscopy (TEM)

Pelleted exosomes were added in 50 µl of PBS onto mesh grids and dried overnight without the use of fixative. Counter-staining was performed using uranyl-acetate and lead-citrate. Exosomes were examined using a Jeol Jem-1200Ex electron microscope. Images were acquired using an integrated MegaView III digital camera.

Immune-fluorescent staining

Immune-fluorescent staining was performed on 2-month old and 21-month-old mouse thymus cryosections. Tissue section of 7µm were mounted onto glass slides and dried overnight. Tissue samples were fixed with cold acetone then unspecific protein-protein interactions were blocked with 5% BSA in PBS solution before applying fluorochrome-conjugated primary antibodies. FITC-conjugated a-mouse CD326 (EpCAM) was used at 1:100 dilution and DAPI (1:1,000) was added as nuclear counterstain. Slides were also incubated overnight with DiI-stained Wnt4 exosomes. Following washing steps with 1x PBS, samples were imaged using Nikon Eclipse Ti-U microscope equipped with a CCD camera (Andor 4Zyla 5.5) and images were captured using NIS-Elements Software. Images were analyzed using ImageJ Software. Thymus lobes of iv-injected mice were sectioned to 5µm thickness and the same staining procedure was used as described above.

RNA isolation, cDNA preparation, qRT-PCR, TaqMan array

Total RNA was isolated and cDNA was prepared to perform qPCR analysis using PikoReal Real-Time PCR System. Gene expression was normalized to mouse ß-actin and HPRT1 housekeeping genes. To detect miR-27b levels in exosomes, Total Exosome RNA & Protein Isolation Kit was used for miRNA isolation. Reverse transcription was carried out using specific primers for U6B as endogenous control and miR-27b as target gene. Quantification of miR-27b was performed using specific gene targeted TaqMan MicroRNA Assay. The miRNA profiling of control and steroid-treated 1889c samples were analyzed with Quantstudio 12K Flex Real-Time PCR System platform using Taqman Array Human miRNA A and B panels, where 7 housekeeping genes (RNU44, RNU48, ath-miR159a and 4 U6 snRNS) and 377 human miRNAs were detected.

Nanostring assay

Total RNA (100ng) was used to detect up 880 miRNA targets with the hybridization-based nCounter SPRINT Profiler using nCounter Human v3 miRNA Expression Assay and

nCounter CodeSet following sample preparation. Quantified data were analyzed using nSolver Analysis Software version 4.0. Threshold count was determined using negative control as background noise. Gene expression changes were visualized on heat map using GraphGad Prism version 7.04.

ELISA

Human Wnt4 ELISA Kit was used to measure the Wnt4 protein levels of isolated exosomes. Exosomes isolated from TEP1 cell culture media were used as control. We aimed to quantify surface and total exosome protein levels separately. To detect surface proteins, pelleted exosomes were resuspended in PBS (for intact exosomes), while for total protein concentration, exosomes were diluted in Exosome Resuspension Buffer (for disintegrated exosomes). Plates were measured at 450 nm using EnSpire Multimode Plate Reader.

Ultracentrifugation

In order to compare TEI efficiency with standard ultracentrifugation 1ml serum-free medium containing exosomes was centrifuged at 100,000 g for 3 h at 4°C using Sorvall MTX 150 Micro-Ultracentrifuge. Pelleted exosomes were resuspended in PBS and used for further experiments.

***In vivo* Exosome Homing**

Eight week-old BALB/c mice were used for intravenous introduction of DiI lipid-stained exosomes in a pilot study. For control purposes non-stained TEP1 exosomes and DiI lipid-stained Wnt5a exosomes were used. Another mouse was injected with the same dosage of DiI lipid-stained Wnt4 exosomes as treatment. After 24 h, mice were sacrificed and their organs were analyzed for fluorescence intensity with the help of IVIS Lumina III in vivo Imaging System. Imaging of the thymus, lungs, liver, and spleen was performed at 520 nm using Living Image Software. Thymus lobes were embedded into cryomold and sectioned to confirm tissue homing. Mice were housed under minimal disease conditions and kept in the Laboratory Animal Core Facility of the University of Pécs. Experimental procedures were carried out according to the "1988/XXVIII act of the Hungarian Parliament on Animal Protection (243/1988)". All animal experiments were performed with the consent of the Ethics Committee on Animal Research of the University (ref. no.: #BA02/2000-46/2016).

Statistical analysis

Statistical analyses were performed using SPSS version 22. Descriptive statistics (mean ± SD) were calculated for all data. Normality was assessed using the Shapiro-Wilk test ($n < 50$). Comparisons were performed using one sample t-test and independent samples t-test. Statistical significance level was marked as follows: ns: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$.

Results

Characterization of transgenic exosomes

We focused on the characterization of exosomes secreted by the Wnt4 over-expressing BALB/c TEC (thymic epithelial cell) transgenic cell line. Purity and transgenic status of cell lines was confirmed by GFP expression and flow-cytometric analysis. Using a commercially available PEG-based kit we isolated transgenic exosomes (TEI) from TEC line supernatant. The enriched exosomes were confirmed by TEM to contain of uniform-sized exosomes of approx. 40 nm in diameter. Along with Wnt4, miR-27b has also been reported to suppress PPARgamma. For this reason, we have measured both Wnt4 protein and miR-27b RNA quantities in transgenic exosomes relative to their control counterparts, using ELISA and TaqMan qPCR methods. As expected, both Wnt4 and miR-27b showed statistically significant elevated levels in exosomes of transgenic TECs compared to controls. Furthermore, through the lysis of exosomes we certified that a significant portion of Wnt4 is surface displayed, in harmony with literature data. Of note, UC has provided poor exosomal Wnt4 protein and miR-27b miRNA yield as compared to TEI.

In vitro biological effect of transgenic exosomes

We have previously reported that steroid-treatment triggers adipose transformation of TECs via epithelial-to-mesenchymal transition. We have used enriched Wnt4-transgenic exosomes, which efficiently blocked the aging process via preserving TEC identity, blocking EMT and adipose transformation from developing.

In histo binding and distribution of transgenic exosomes

Previously we have reported that Wnt4- binding Frizzled receptors (Fz4 and Fz6) are up-regulated in aged TECs. Next, we have tested whether thymic histological sections can bind transgenic exosomes using standard immune-fluorescent staining protocol. We were also

interested to see if histological binding shows a particular pattern and if this pattern changes with the age of the epithelium. For this purpose, DiI lipid-stained transgenic exosomes have been applied on thymic sections of young (2-month-old) and aged (21-month-old) mice for immune-histology staining. As our results show, both young and old mouse thymic sections efficiently bind transgenic exosomes. However, medullary regions showed preferential binding at young age, while a more profound cortical binding pattern was observed at old age. Not only does the binding pattern change with age, but binding frequency also increases with age. This statistically significant change is shown by DiI/fluorescent integrated pixel density at old age compared to young age.

***In vivo* binding and distribution of transgenic exosomes**

We were interested to see if *in vivo* binding of transgenic exosomes also occurs in the thymic epithelium. For this reason, DiI-stained Wnt5a-transgenic (control) human (A549), and Wn4- transgenic (sample) BALB/c (TEP1) exosomes have been injected into tail veins of young adult BALB/c mice to check their *in vivo* topological distribution after 24 h. It has been reported that certain organs (e.g. liver, lungs, and spleen) capture a significant portion of exosomes rapidly and non-specifically following systemic administration. Using IVIS Lumina III imaging and performing topological reconstruction of murine organs over standard mouse contour, we were able to record homing of transgenic exosomes to the thymus despite significant non-specific capture by other organs. Fluorescent signals of DiI lipid-stained sample Wnt4-transgenic TEC (thymic epithelial cell) exosomes exceeded control values of control Wnt5a-transgenic exosomes. Fluorescent staining of *in vivo* Wnt4-transgenic exosome-infiltrated thymus sections revealed the histological level homing pattern that allows for the quantitative evaluation of transgenic exosome homing.

miRNA profile of epithelial cells of *in vitro* aging model

Our goal was to determine miRNome profile of steroid-induced human aging model (1889c cells) using two distinct platforms (Quantstudio and Nanostring). The amplification-based QS measures 768 miRNAs, while the amplification-free NS is able to determine 880 miRNAs. Several of the identified miRNAs have relevance to thymus senescence with special focus on adipose tissue development, epithelial-to-mesenchymal transition, cell proliferation and senescence.

Discussion

As shown above, our results confirm that the thymic epithelium sheds high amounts of exosomes. The Wnt4 over-expressing transgenic TEC line proves to be a reliable source of transgenic exosomes that are easy to visualize, enrich to high purity, characterize, and apply in experiments. As expected, the transgenic exosomes contained elevated levels of Wnt4 protein, as well as miR-27b, which potentially synergize to counteract PPARgamma.

Based on our results obtained with the cellular model system of thymus aging, the applied transgenic exosomes efficiently prevent steroid-triggered adipose transformation due to reinforced epithelial identity (increased FoxN1 and CIITA expression), lack of EMT (sustained E-cadherin and low N-cadherin expression) and resistance to adipose differentiation (low PPARgamma expression).

During analysis of *in histo* transgenic exosome binding we experienced slight medullary preference at young age (2-month), while at old age (21-month), transgenic exosomes show a moderate cortical preference in thymic epithelium binding. Furthermore, transgenic exosomes show significantly higher overall binding frequency at an old age, suggesting that the senescent thymic epithelium readily adsorbs Wnt4-transgenic exosomes. This is in harmony with the fact that a significant portion of exosomal Wnt4 is surface-displayed and Wnt4-binding Frizzled receptors (Fz4 and Fz6) are up-regulated in senescent TECs. These results suggest that as Wnt4 secretion decreases with age consequent medullary involution may precede cortical involution due to the medullary lack of Wnt4-effect. Our *in vivo* transgenic exosome homing assay shows homing to the thymus using mouse TEC-derived Wnt4-transgenic exosomes. Histological analysis of Wnt4-transgenic exosome-infiltrated thymus sections also confirmed their presence.

miRNA profile analysis identified a number of context-relevant copy number alterations. Of note, miR-27a and miR-106b are beige adipose tissue regulators and miR-155 is an inhibitor of brown/beige adipose tissue formation. Furthermore, miR-128a-3p, miR-1825, miR-301a-5p, miR-30d, miR-425-5p, miR-550a, and miR92b-3p also influence adipose tissue formation. In addition, epithelial-to mesenchymal transition (EMT) related miRNAs showed changes in copy number in steroid-induced TECs like miR-105-5p, miR-200a-3p, miR-597-5p, miR-888, and miR-99b. Finally, from a senescent perspective miR-125a-3p, miR-125a-5p, miR-15b-5p, miR-181a-5p, miR-323-3p, and miR-331-3p affect cellular / tissue level senescence with focus on the thymus also showed significant changes. In summary, steroid-treatment in TECs affects the same miRNA species that were reported in

connection with senescence-related thymus adipose involution that yields beige adipose tissue.

As mentioned before miRNAs play an important role in the defense mechanisms against different infections. Certain miRNAs can decrease the accumulation of viral particles, such as miR-323 and miR-485. In our aging model studies we have detected the reduction of these miRNAs, which confirms that these small non-coding RNAs could have an antiviral effect supporting the immune system. Furthermore, miR-15b-5p, miR-30e and miR-548d also show decreasing tendency during aging and these are associated with chronic diseases and have antiviral effect on SARS-CoV-2 (causing COVID-19). It is important to mention, that COVID-19 mainly affects the aged population, especially when associated with chronic diseases such as cardiovascular-, chronic lung-, liver and kidney diseases, diabetes mellitus or cancer. These all verify that because of the increasing life expectancy the aged population requires new therapeutic solutions that support the reinforcement of the thymus. In this regard the tissue regenerative, anti-aging and T-cell development supporting effect of Wnt4 transgenic exosomes has a high therapeutic potential.

Summary of new results

1. Our results prove that the supernatant of Wnt4 over-expressing thymic epithelial cell line (TEP1) is a reliable source to produce and isolate large amounts of exosomes. We also showed that PEG-based exosome isolation yields higher amount of proteins (Wnt4) and miRNA (miR-27b) than ultracentrifugation.

2. We successfully managed to characterize Wnt4 transgenic exosomes in size, protein- and miRNA content. We also showed that a large portionWnt4 proteins are surface exposed.

3. Our *in vitro* thymic aging model showed that Wnt4 transgenic exosomes isolated from Wnt4 over-expressing TEP1 cell line supernatant can decrease PPARgamma expression, thereby counteract steroid-induced aging process.

4. We also showed *in histo* that the senescent thymic epithelium readily adsorbs Wnt4-transgenic exosomes as a significant portion of exosomal Wnt4 is surface-displayed and Wnt4-binding Frizzled receptors (Fz4 and Fz6) are up-regulated in senescent TECs.

5. *In vivo* experiments showed that DiI-lipid stained transgenic Wnt4 exosomes efficiently bind to the thymus and also enrich in other tissues.

6. We have successfully detected miRNA changes supporting that thymus senescence yields bona fide beige adipose tissue. We also detected miRNA species participating in the general aging processes.

7. We have proved that Wnt4 transgenic exosomes can potentially be used for regenerative purposes of the immune system in mice. This raises the possibility of human application, as exosomes are applicable in transferring therapeutic molecules and these molecular processes are similar in human and mouse.

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