PhD thesis

Alteration in the Wnt microenvironment directly regulates molecular events leading to pulmonary senescence

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Introduction

Aging-an overview

The elderly population is increasing with an unprecedented rate within this century putting an enormous pressure on healthcare, labor market and pension system alike.

The EU27 population is projected to become older with the median age expected to rise from 40.4 years in 2008 to 47.9 years by 2060. The proportion of people aged 65 years or over in the total population is estimated to increase from 17.1% (84.6 million in 2008) to 30.0% (151.5 million) in 2060.

Aging in the lung

In the aging lung the total tissue mass decreases along with the number of capillaries. Formation of new alveoli is also limited. As senescence progresses, lung tissue becomes prone to inflammation, fibrosis and tumors. Studies suggest that the senile lung is characterized by airspace enlargement similar to acquired emphysema that is also detected in non-smokers above 50 years of age.

While molecular processes of other organs are studied widely, there is relatively limited background knowledge concerning pulmonary senescence. Most studies have been performed in mice and although similarly to humans, aging of the mouse lung is associated with homogeneous airspace enlargement, mice are still not the best model organism to study molecular and micro-environmental changes during pulmonary senescence as not all the physiological processes or responses are identical to humans. Partly, as in mice the aging process initiates earlier and the whole process is faster.

Normal lung function

At the gas-exchange region of the alveoli there are two types of epithelial cells, Alveolar type I (ATI) and alveolar type II (ATII) cells. The role of ATI cells are quite simple, the gas exchange is going through them. But the roles of ATII cells are much more complicated. Apart from self-renewal, ATII cells are capable of trans-differentiation into ATI cells. ATII cells are also important in producing surfactant proteins, which are responsible for lowering surface tension in the alveoli aiding gas exchange and stabilizing alveolar structure. At the alveolar region of the lung a well-maintained, surfactant producing ATII cells population is essential.

ATII cells are unable to take up triglycerides directly from the blood stream. Triglycerides are taken up and transported to ATII cells by lipofibroblasts. Lipofibroblasts or lipid laden fibroblasts are fibroblasts adjacent to ATII cells at the alveolar region. The lipid accumulation process is regulated by a peroxisome proliferator-activated receptor gamma (PPARγ) and adipose differentiation-related protein (ADRP) dependent mechanism. In the absence of PPARγ, lipofibroblasts trans-differentiate into myofibroblasts which cannot provide lipids and therefore support normal lung function. A decrease in lipofibroblasts therefore can lead to increased sensitivity to develop lung diseases including chronic obstructive pulmonary disease (COPD) or lung fibrosis.

PPARγ

Peroxisome proliferator- activated receptors (PPARs) are members of the nuclear hormone receptor superfamily, which function at the transcriptional level to regulate a wide range of physiological activities. PPARγ is one of the PPAR receptors, is expressed in a broad range of tissues.

PPARγ is most highly expressed in white adipose tissue (WAT) and brown adipose tissue (BAT), where it is a master regulator of adipogenesis as well as a potent modulator of lipid metabolism and insulin sensitivity of the entire body.

Within the lung, PPARγ expression has been reported in the epithelium, as well as in fibroblasts. While PPARγ is a very important molecule in the maintenance of the lipofibroblast phenotype (see above), epithelial PPARγ is necessary for the establishment and maintenance of normal lung structure, through regulation of epithelial cell differentiation and control of lung inflammation. PPARγ is also important for postnatal lung maturation. Targeted deletion of PPARγ in epithelial cells changed the epithelial structure of the lung but not the maturity and the SPC production of alveolar epithelial cells.

Wnts and β catenin pathway

The Wnt family of 19 secreted glycoproteins controls a variety of developmental processes. There are two main signaling pathways involved in the signal transduction process from the Wnt receptors called Frizzleds: the canonical or β-catenin dependent, and the non-canonical pathways.

Generally, in the absence of canonical Wnts, glycogen synthase kinase-3β (GSK-3β) in complex with adenomatous polyposis coli (APC) and axin is active and phosphorylates β-

catenin. The phosphorylated β-catenin is targeted for ubiquitination and proteasome-mediated degradation. In the presence of Wnt-s, activation of Dvl leads to phosphorylation and consequently inhibition of GSK-3β-APC-axin complex. Inhibition of GSK-3β-APC-axin complex results in the stabilization and finally cytosolic accumulation of β-catenin, which then translocate to the nucleus, where is required to form active transcription complexes.

Experimental procedures

Ethical Statement

Lung tissue samples were collected during lung resections at the Department of Surgery, University of Pécs, Hungary. The project was approved by the Ethical Committee of the University of Pécs. Patients had given written consent to provide samples for research purposes. All collected samples were treated anonymously.

Animals

For the experiments Balb/C inbred, albino mice were used from both genders. The mice were kept under standardized conditions, where tap water and food was provided ad libitum. They were let to age till 1, 3, 6, 12, 18, 24 months before sacrifice.

Sky scan microCT

Mice anesthetized intraperitoneal with sodium pentobarbital (eutazol), were placed in the SkyScan 1176 microCT (Bruker, Kontich, Belgium) machine equipped with a large format 11 megapixel camera.

Lung cell isolation

Mice were anesthetized with sodium pentobarbital through intraperitoneal injection. The mice were perfused through right ventricle with phosphate buffer saline (PBS) to reduce lung blood content. Lungs were removed from the chest and cleaned from connective tissue. Pulmonary lobes were dissected into smaller pieces and digested in collagenase-dispase with continuous stirring. Digested lung cells were filtered with 70µm cell-strainer single cell suspension was created.

Cell sorting

Single cell suspension isolated from mouse lungs were labeled with anti CD45-FITC produced at the University of Pécs, Department of Immunology and Biotechnology and anti EpCAM1 (G8.8 clone (ATCC) anti-rat-PE). Cell sorting was performed by FACSAria III (BectonDickinson) cell sorter.

Cell lines

For in vitro experiments TEP1 and Wnt4 over-expressing TEP1 cell lines supernatants were used. The cells were cultured in DMEM (Dulbecco's Modified Eagle's medium Lonza) supplemented with 10% FCS, penicillin, streptomycin and β-mercapto-ethanol. They were kept at 37°C in a humidified termostate where the CO_2 concentration was 5%.

Three dimensional (3D) human lung tissue cultures

Three dimensional (3D) human lung tissue cultures were set up using the protocol patented by Judit E. Pongracz Primary Small Airway Epithelial cells (SAEC) and Normal Human Lung Fibroblast (NHLF) cells were purchased from Lonza. Small Airway Epithelial Growth medium (SAGM) or fibroblast growth medium (FGM) medium were used for the initial expansion of SAEC or NHLF respectively, as recommended by the manufacturer (Lonza) For the creation of 3D lung tissue the two cell types were mixed in 1:1 ratio. Then 3D lung tissues were kept in 24 well plate (Sarstedt) in mixed SAGM:FGM (1:1 ratio) medium.

Recombinant Adeno (rAd) and Lenti (L) viral constructs and rAd and L-viral infecton of pulmonary epithelium (SAEC) and fibroblasts (NHLF)

ICAT sequence was amplified by PCR reaction using forward (5') 5'-ATGAACCGCGAGGAGCA-3' and reverse (3') 5'-CTACTGCCTCCGGTCTTCC-3' primer sequences and cloned into the bi-cistronic GFP (green fluorescence protein) Adeno Shuttle and Lenti pWPTS vectors. The Shuttle vector was cloned by homologous recombination into the adenoviral vector. Adenovirus was produced by transfecting the linearized plasmid DNA into the 293 packaging cell line (American Type Culture Collection, Rockville, MD) using Lipofectamine 2000 (Invitrogen). The resulting plaques were amplified; the adenovirus purified and concentrated using the adenoviral purification kit (BD Biosciences).

Late second generation lentiviral vectors were prepared by co-transfection of three plasmid constructs (envelope construct pMD.G, packaging construct R8.91 and transfer construct pWPTS) into 293T cells using the calcium-phosphate method as described previously. Biological titration was performed with HeLa cells. Viral particles were concentrated 1000-fold in volume; biological titers reached 10^8 TU/ml. The HIV-1 derived lentiviral system was kindly provided by Prof. Didier Trono (CMU, Geneva, Switzerland).

For ICAT delivery to epithelial cells, complete SAEC-NHLF spheroids were incubated in the rAd virus containing media for 1 hour, then the SAEC-(ICAT-GFP)-NHLF and SAEC-(GFP)-NHLF shpereoids were washed and incubated for 7 days before RNA isolation. For ICAT delivery to fibroblasts, NHLF cells were exposed to L-virus containing media for 1

hour, then cells were washed and incubated for 2 days in 2D monocultures. NHLF cells were then harvested and spheroids were produced as described above. SAEC-NHLF-(ICAT-GFP) and SAEC-NHLF-(GFP) shperoids were cultered for an additional 5 days before RNA isolation.

Western blot analysis

Lung tissues of young (1 months) and old (24 months) lysed in lysis buffer (20mM HEPES pH 7.4, 1mM $MgCl_2$, 1mM $CaCl_2$ 137mM NaCl, 50mM β-glycerophosphate, 2mM EGTA, 1% Triton X100 supplemented with 1mM DTT, 2mM PMSF, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatine) on ice for 20 min, then snap frozen in liquid nitrogen and stored at −70 °C until used. Just before loaded on 10% SDS-PAGE, the samples were boiled in 2× SDS sample buffer. Protein concentrations of lung extracts were measured with bicinchoninic-acid kit (Sigma), then the same amount of proteins were loaded to polyacrylamide gels then transferred to nitrocellulose membrane. The membrane was blocked with TBS buffer containing 1% of BSA and 0.05% of Tween and incubated with primary antibodies (anti-β-catenin, anti-Wnt4 both purchased from Santa Cruz, both at 1:1000 dilution) overnight. HRP conjugated anti-Mouse (Sigma) and anti-Goat (Sigma) were used as secondary antibodies (both at 1:1000 dilution). Blots were visualised using the chemiluminescent Supersignal kit (Pierce) and densitometrically scanned for quantification (LAS 4000 GE Healthcare Life Sciences, Little Chalfont, UK).

RNA isolation

Cell samples were homogenized in RA1 reagent, and RNA was isolated using the NucleospinII RNA isolation kit (Macherey-Nagel, Dueren, Germany). DNA digestion was performed on column with RNase free DNase. The concentration of RNA samples was measured using Nanodrop (Thermo Scientific, Waltham, MA, USA).

Real-Time quantitative PCR

cDNA was synthesized with high capacity RNA to cDNA kit (Life technologies Inc., Carlsbad, CA, USA) using 1μg of total RNA according to manufacturer's recommendation. Reverse transcription was performed in 20μl total volume using random hexamer primers. RT-PCR was used for gene expression analysis. Gene expression levels were determined by gene specific RT-PCR using ABsolute QPCR SYBR Green Low ROX master mix (ABGene, Thermo Scientific) and 100 nM primers on the Applied Biosystems 7500 thermal cycler system. For normalization β-actin was used as housekeeping gene. The primer sequences are

shown in the table below. PCR conditions were set as follows: one cycle 95°C for 15 minutes, 40 cycles 95°C for 15 seconds, annealing temperature was 58°C and 72°C for 1 minute for elongation. Specification of the PCR reaction was determined by using a dissociation stage. The calculation of the RT-PCR results was performed as follows: The mean Ct values are determined by calculating the average of the parallel samples. ΔCt is calculated by subtracting the mean Ct of the housekeeping gene from the mean Ct of the gene of interest. ΔΔCt is constituted by the difference between the old sample Ct and the young sample as a control Ct values Finally, the relative quantity (RQ), which is presented in the diagrams, can be calculated by applying the formula: $RQ=2^{-\Delta\Delta Ct}$

Primer sequences

Primers	Forward	Reverse
mouse primers		
mouse β-Actin	TGGCGCTTTTGACTCAGGA	GGGAGGGTGAGGGACTTCC
mouse PTHrPR	GGCGAGGTACAAGCTGAGAT	ACACTTGTGTGGGACACCAT
mouse Wnt4	CTCAAAGGCCTGATCCAGAG	TCACAGCCACACTTCTCCAG
mouse Wnt5a	AAGCAGGCCGTAGGACAGTA	CGCCGCGCTATCATACTTCT
mouse Wnt11	GCTCCATCCGCACCTGTT	CGCTCCACCACTCTGTCC
mouse PPARγ	CCCAATGGTTGCTGATTACAAA	AATAATAAGGTGGAGATGCAGG TTCT
mouse ADRP	CGCCATCGGACACTTCCTTA	GTGATGGCAGGCGACATCT
mouse Sirt1	GACGATGACAGAACGTCACA	GATCGGTGCCAATCATGAGA
mouse Sirt7	TGAGACAGAAGAGGCTGTCG	TGGATCCTGCCACCTATGTC
human primers		
human β-Actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTCC
human PPARγ	GGTGGCCATCCGCATCT	GCTTTTGGCATACTCTGTGATCTC
human S100A4	TGGAGAAGGCCCTGGATGT	CCCTCTTTGCCCGAGTACTTG
human IL1β	TCAGCCAATCTTCATTGCTCAA	TGGCGAGCTCAGGTACTTCTG

Immunofluorescence

Mice were anaesthetized with sodium pentobarbital intraperitoneally and then they were perfused through the right ventricle with PBS solution as described above. Then lungs were filled up with 1:1 ratio of PBS:cryostate embedding media (TissueTek Alphen an den Rijn, Netherland), and freeze down at -80 °C. The sections were made by Leica cryostate at -30°C. The sections were 7-9 um.

The human samples were kept in PBS containing 1% of FCS at room temperature till processing. The filling, freezing and sectioning steps were performed as described above.

At the endpoint of the treatment the 3D microtissues were carefully removed from the 24 well plates and embedded into TissueTek embedding media and immediately frozen down at -80°C.

For histological observations, cryostat sections (7-9 μm) were fixed in cold acetone for 10 minutes.

Antibodies, Fluorescent staining

Following rehydration and blocking step (for 20 minutes in 5% BSA in PBS) immunofluorescent staining was performed. Anti-Wnt5a (Santa Cruz, Santa Cruz, CA, USA) antibodies were used as primary antibodies, and anti EpCAM1-FITC (clone G8.8, American Type Culture Collection (ATCC) directly labeled antibody was used as a control staining for 1 hour. For human samples anti pro-SPC antibody (Millipore Billerica, MA, USA) was used. For the spheroids anti-KRT7 (DAKO, Agilent, Santa Clara, CA, USA) antibodies and anti E-cadherin (AbCam, Cambridge, UK) antibodies were applied.

The secondary antibodies were northern light anti-mouse NL-493 and northern light anti-rabbit NL-557 (R&D systems, Minneapolis, MO, USA). The nuclei were counterstained with DAPI (Serva, Heidelberg, Germany). Pictures were captured using Olympus IX81 fluorescence microscope equipped with CCD camera and analysis software. Images were processed and analyzed with ImageJ.

Fluorescent images were analyzed, and the mean intensity was calculated with StrataQuest software (Biotech-Europe, Prague, Czech Republic).

Hematoxylin-eosin staining

Following sectioning (see sectioning above), samples were immediately stained with hematoxylin and eosin. Pictures were scanned with Pannoramic Desk machine (3D Histech, Budapest, Hungary) then analyzed by Pannoramic viewer software (3D Histech).

Neutral lipid staining

Mouse lung sections were fixed in cold acetone fixed stained with anti-EpCAM1 antibody directly conjugated with FITC (ATCC clone G8.8), then the LipidTox (Life Technologies Inc.) staining was performed. Fluorescent images were captured and analyzed as described above.

Statistical analysis

If applicable, data are presented as mean ± standard deviation (SD), and the effects between various experimental groups were compared with the Student t-test. $p<0.05$ was considered as significant. The normal distribution was tested with Kolmogorov-Smirnoff test.

Results

Morphological changes in the aging lung

During aging, pulmonary function degenerate due to pulmonary inflammation and structural changes described as senile emphysema. Micro computed tomography and hematoxilyn-eosin staining of lung sections confirm such changes in Balb/c mice and human samples, respectively. Both techniques highlighted enlarged airspace both in old mice and in aging human lungs.

To confirm degeneration of the epithelial surface layer during aging, single cell suspensions were generated from the pulmonary tissues of mice and cells were sorted based on EpCAM-1 and CD45 cell surface antigens. Analysis of density plots revealed a significant increase in CD45+ leukocyte level showing a marked increase in both macrophage and B-cell populations, whereas the number of epithelial cells significantly decreased in the senescent lung.

Both PPARγ expression and lipid levels decrease with age

While senescence-associated low level chronic inflammation could explain tissue destruction, the reasons for ineffective regeneration are not so easily explained. As lipofibroblasts are essential for maintenance of ATII-s molecular studies were designed to identify molecules involved in lipid production to define the presence and activity of lipofibroblasts during pulmonary senescence. PPARγ mRNA as well as its down-stream target, adipose differentiation-related protein (ADRP) were measured in purified EpCAM-1^+ epithelial and EpCAM-1^- non-epithelial cells using qRT-PCR. Compared to 1 month old Balb/c mice, both PPARγ and ADRP levels decreased at 24 months of age in both cell populations indicating reduced ability for surfactant synthesis and triglyceride uptake in the aging lung. To confirm the qRT-PCR data, lipid levels were assessed in pulmonary tissues using neutral lipid staining of 1 month and in 24 months old mouse lung sections. While lipid staining co-localized with nuclear staining in young (1 month old) mice, lungs of old (24 months) mice contained enlarged lipid droplets not associated with nuclear staining. Western blots were performed using protein extracts of 1 month and 24 months mouse lungs. The results show an age associated reduction in PPARγ protein levels indicating a loss of lipofibroblasts.

According to Torday et al. parathyroid hormone-related protein (PTHrP) expression is necessary for differentiation of mesenchymal lipofibroblasts, which induce ATII cell differentiation making PTHrP receptor transcript levels indicative of alterations in

lipofibroblast differentiation. Quantitative RT-PCR analysis revealed a drastic reduction in PTHrP receptor mRNA levels within the EpCAM1$^-$/CD45$^-$ cell population due to loss of lipofibroblasts in the aging lung.

Alteration of the Wnt microenvironment during pulmonary senescence

Recent studies have highlighted the importance of Wnt signaling in the regulation of PPARγ activity. Takada reviewed that canonical Wnts antagonize the effect of PPARγ in osteoblast-adipocyte differentiation. Moreover Talabér et al have shown that overexpression of Wnt4 in TEP1 thymic epithelial cell line results in reduced PPARγ expression and consequent inhibition of thymic adipose involution indicating a strong involvement of Wnts in pulmonary senescence also.

When measured, several Wnts, including Wnt4 as well as the inflammatory mediator, Wnt5a were expressed in both epithelial and non-epithelial cells of mouse lungs. While Wnt4 mRNA levels increased during aging in both epithelial (EpCAM-1$^+$) and non-epithelial (EpCAM-1$^-$) cells, both Wnt5a and Wnt11 expression decreased in epithelial (EpCAM-1$^+$) and increased in non-epithelial (EpCAM-1$^-$) cells with age. Western blot analysis of protein extracts of 1 month and 24 months mouse lungs supported the age associated increase in Wnt4 levels. Protein studies performed on human lung samples using Wnt5a staining of 73 years old and 21 years old supported similarities between the mouse and human pulmonary senescence program. Corresponding to mouse qRT-PCR data, Wnt5a staining intensity increased with age in the human lung and Wnt5a was detected in the non-epithelial, cytokeratin-7 negative cell population.

β-catenin dependent regulation of PPARγ expression

Our previous data as well as Takada's results designated the canonical Wnt signaling pathway as regulator of PPARγ expression. As Wnt4 but not Wnt5a or Wnt11 can act via the canonical Wnt signaling pathway, our attention was focused on Wnt4. To be able to investigate molecular changes triggered by Wnt molecules in human lung tissue *in vitro*, 3D lung spheroids were exposed to control and Wnt4 supernatants of TEP1 cells for 7 days. Using qRT-PCR analysis, reduced mRNA expression levels of PPARγ were detected. Thereafter canonical Wnt pathway activity was modified using chemical activators and inhibitors of the β-catenin pathway. LiCl was used as an activator of the β-catenin pathway that inhibits the activity of GSK3-β, and therefore protects β-catenin from phosphorylation and consequent

proteosomal degradation. IWR is an inhibitor of the β-catenin pathway that acts via stabilization of Axin protein complexes aiding β-catenin destruction.

To investigate our theory, initially primary human lung fibroblasts (NHLF) were used and treated with LiCl at 10 mM, IWR at 1 μM concentration and a Wnt4 enriched supernatant for 7 days, then PPARγ mRNA levels were measured using qRT-PCR. The expression of PPARγ was drastically reduced after LiCl and Wnt4 treatment, while inhibition of β-catenin signaling increased PPARγ levels. β-catenin levels were also assessed by Western blot analysis after treatment of NHLF cells with LiCl, Wnt4 and IWR. While LiCl and Wnt4 treatment increased, IWR treatment reduced β-catenin protein levels indicating that amplified Wnt4 production during aging is likely to initiate reduction of lipofibroblast differentiation via a β-catenin dependent mechanism. To examine the possibility that Wnt4 induced reduction of PPARγ levels affect SPC production, the 3D in vitro tissue culture was applied, as 3D culture conditions stimulate surfactant production of SAEC. The lung tissue model was treated with Wnt4-enriched supernatants and rhWnt5a, respectively. Following 7 days of incubation, Wnt4 reduced pro-SPC expression demonstrating that Wnt4 can regulate pro-SPC levels.

Reduced β-catenin activity is necessary in pulmonary epithelial cells to produce pro-SPC

While the above data support that PPARγ levels in fibroblasts are necessary for lipofibroblast-like differentiation and maintenance of surfactant production in ATII-type cells, it is still not clear whether PPARγ activity is needed within the ATII cell population for surfactant synthesis. It is an important question as age associated decline of PPARγ mRNA affected not only fibroblasts but epithelial cells also. As the aging process in the human lungs was associated with reduced pro-SPC levels the presence and activity of PPARγ in pulmonary epithelium might be equally important to that in lipofibroblasts.

β-catenin activity was modulated therefore using the physiological inhibitor of the β-catenin dependent Wnt signaling pathway, ICAT. ICAT was introduced into epithelial as well as fibroblast cells using recombinant viral gene delivery methods in the 3D human lung model. To specifically target epithelial cells (SAEC), recombinant Adeno viruses (rAd-GFP and rAd-ICAT-GFP) were used, while fibroblasts (NHLF) were transfected using lentiviruses (rL-GFP and rL-ICAT-GFP). For lentiviral gene delivery, NHLF cells were infected before the generation of 3D lung tissue spheroids to avoid transfection of epithelial components. Following 7 days of exposure to ICAT, PPARγ expression was measured. Inhibition of β-catenin activity either in epithelium or in fibroblasts drastically increased PPARγ expression

indicating that inhibition of β-catenin signaling modulates lipid metabolism of both cell types. Pro-SPC staining increased drastically in rAd-ICAT-GFP infected tissues identifying a β-catenin regulated and PPARγ dependent mechanism as an important element of pro-SPC production in ATII cells.

Wnt signaling in myofibroblast-like differentiation

According to a previous study transdifferentiation of lipofibroblasts to myofibroblasts is characterized by loss of PTHrP receptor expression and triglyceride content. Our results support previous findings as PTHrP mRNA levels decreased with age in the non-epithelial cell population. To investigate if changes in the Wnt microenvironment are responsible for increased myofibroblast differentiation, the myofibroblast marker S100A4 mRNA was measured after exposure of NHLF cells and the 3D lung tissue spheroids to Wnt4 and Wnt5a, respectively. Interestingly, both Wnt4 and Wnt5a were able to increase S100A4 transcript levels indicating involvement in the regulation of myofibroblast- differentiation.

Epigenetic regulation of the pulmonary aging mechanism

As it is accepted that environmental factors affect aging, it was predicted that epigenetic mechanisms might be able to modulate pulmonary senescence. Sirtuins are often connected with aging and different senescent mechanisms. In order to unveil their function in pulmonary senescence, an initial screening of Sirtuin expression was performed. mRNA level was measured in both $EpCAM^+$ (epithelial) and $EpCAM^-$ (fibroblast like, non-epithelial) pulmonary cells of young and old mouse lung samples. Interestingly, both Sirt1 and Sirt7 message levels were increased with age, although Sirt7 showed a higher, nearly two fold increases in both cell types. At protein level the difference between the young and old lungs was more pronounced. While Sirt1 protein expression increased significantly in old human lungs supporting mRNA data, Sirt7 protein remained undetectable both in young and old lungs. Unfortunately, based on the general view of the anti-aging function of Sirt1, it was difficult to explain our findings. Recent studies, however, have revealed that Sirt1 acts against adipogenesis and inhibits PPARγ protein function. To investigate whether Sirt1 can inhibit PPARγ activity in lung tissues, experiments were set up using 10 nM concentration of resveratrol, a small molecule that mimics Sirt1 function, and Wnt4 that was able to activate the β-catenin pathway and inhibit PPARγ in previous experiments. As both resveratrol and Wnt4 act as inhibitors of PPARγ expression, and their effects were additive in co-treatment.

Conclusion

The alveolar region of the lung changes during aging and the useful lung surface decreases.

The Wnt microenvironment changes with age and while Wnt4 mRNA is elevated in both $EpCAM^{+}$ (epithelial) and in $EpCAM^{-}$ (non-epithelial cells), Wnt5a levels only increase in non-epithelial cells.

In contrast to Wnts, PPARγ and its downstream molecule ADRP are decreased both in the EpCAM+ (epithelial) and in EpCAM- (non-epithelial) cell populations. These molecules are very important in adipogenesis in general and in the life of lipofibroblasts, the regulators of ATII maintenance in the lung.

Regulation of PPARγ expression was a canonical or β-catenin dependent process.

Wnt4 and Wnt5a both increase the mRNA level of S100A4 myofibroblasts marker and decrease PTHrP lipofibroblast marker. These results suggest activation of a lipofibroblast to myofibroblasts differentiation mechanisms during pulmonary senescence.

Increased myofibroblast presence in the aging lung can result in emphysema like structures, decreased respiratory surface are and in consequent breading difficulty.

Sirt1 and Sirt7 have elevated at mRNA level in mice, but only Sirt1 proteins were detected at increased levels in aged human lung tissues supporting our proposed mechanism of aging in the lung.

Additionally, human lung tissues exposed to resveratrol and/or Wnt4 resulted in PPARγ suppression and changes detected during pulmonary senesce.

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Kovacs, T., Feller, D., Ernszt, D. Rapp J., Sarosi, V., Kvell, K., and Pongracz, J. E. Epigenetic regulation of pulmonary senescence. To be submitted to Mechanisms of Aging (IF: 3.510).

The thesis based on the following conference presentations and posters

Presentations:

Kovács T, Kvell K, Willert K, Pongrácz J.E: The functional test of Wnt protein. First International Doctoral Workshop in Natural Sciences 2012.

Kovács T., Csöngei V., Ernszt D., Feller D., Pongrácz J.E.: Wnt4 promotes tissue destruction during lung aging via inhibiting PPARγ expression. Second International Doctoral Workshop in Natural Sciences 2013. ISBN: 978-963-08-7403-8 (First prize winner in oral presentation section)

Posters

Kovács T, Ernszt D, Pongrácz JE: The molecular pattern of aging lung. 9th János Szentágothai Interdisciplinary Conference and Student Competition, Pécs, Hungary, 3-4 May 2013 ISBN 978-963-642-519-7 (First prize winner in Medical Poster section)

Kovacs T, Csongei V, Feller D, Ernszt D, Bartis D, Pongrácz JE: Altered Wnt microenvironment during pulmonary senescence leads to drastic decline of the alveolar epithelial surface. ERS Lung Science Conference 2014 Estoril, Portugal. March 21-23, 2014

Csongei V, Feller D, **Kovacs T**, Bartis D, Helyes Z, Pongrácz JE: Three-dimensional human lung micro-cultures for in vitro studies of COPD. ERS Lung Science Conference 2014 Estoril, Portugal. March 21-23, 2014

Other publications

Bartis D, Csongei V, Weich A, Kiss E, Barko S, **Kovacs T**, Avdicevic M, D'Souza VK; Rapp R, Kvell K, Jakab L, Nyitrai M, Molnar TF, Thickett DR, László T, Pongrácz JE (2013): Down regulation of Canonical and Up-Regulation of Non-Canonical Wnt signalling int he carcinogenic process of Squamous Cell Lung Carcinoma. PLoS ONE 8(3):e57393. doi:10.1371/journal.pone.0057393 (IF: 3.730)

Ernszt D, Pap A, **Kovacs T**, Keller Zs, Fejes V.A. Gaál P, Werry JE, Nagy L, Pongracz JE, Kvell K: The missing link of thymic senescence (2014) Submitted to Nature Communications (IF: 10.742)

Lecture note

Miskei Gy, Rapp J, Kiss E, **Kovacs T**, Pongracz JE (2014): Basic and Complex Cell and Tissue Culture Techniques for Biotechnology Students. University of Pécs

Other posters

Feller D., Helyes Zs., Rapp J., Kun J., Ernszt D., Kovács T., Pongracz E. J.: Changes inexpression levels of Wnt signalling molecules in cigarette smoke- induced experimental model systems. 9th János Szentágothai Interdisciplinary Conference and Student Competition, Pécs, Hungary, 3-4 May 2013ISBN 978-963-642-519-7

Sípos G, Csöngei V, Kovács T, Kvell K, Pongrácz JE: Development of an in vitro 3D lung tissue model containing activated T-cells to study chronic obstructive pulmonary disease (COPD).9th János Szentagothai Inrterdisciplinary Conference and Student Competition, Pécs, Hungary, 3-4 May 2013 ISBN 978-963-642-519-7