Factors modifying extracellular vesicle release in normal and tumor tissue-derived organoid models

Ph.D. thesis

Gyöngyvér Orsolya Sándor

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
Zoltán Wiener, Ph.D., associate professor

Opponents:
Dr. Anna Sebestyén, Ph.D., senior research fellow
Dr. Krisztián Tárnok, Ph.D., senior lecturer

Head of the examination committee:
Péter Igaz, MD., D.Sc., professor

Members of the committee:
Krisztina Vellainé Takács, Ph.D., associate professor,
Balázs Mayer, Ph.D., senior research fellow

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1. Background

The two leading causes of tumor mortality are pancreas ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC). In both diseases, the 5-year survival rate is less than 15%. PDAC is the most common type of pancreas tumors. Several environmental factors play a role in its development, such as smoking, diabetes mellitus and chronic pancreatitis (CP). Most commonly, mutations in the *KRAS* and *TP53* genes are present in patient samples. NSCLC is a heterogeneous disease group, the most common subtype is lung adenocarcinoma (LUAD). LUAD is an epithelial-derived, glandularly differentiated, mucin-producing tumor type that develops in the distal airways. As with all lung tumors, smoking significantly increases the chance of developing it, but it is the most common lung tumor among non-smokers. The most common mutation is in the *TP53* gene in LUADs. One of the most important causes of the poor prognosis in both tumor types is the lack of early diagnosis. Thus, it is essential to better understand the effects of cellular biological processes leading to the development of the disease, especially changes in the tumor microenvironment.

Extracellular vesicles (EVs) are membrane surrounded particles released by cells, which can affect the function of target cells in several ways, e.g., with miRNA, proteins, lipids, thus providing a novel intercellular communication mechanism. As EVs transport biologically important molecules in a protected form, concentrated and isolated from enzymes of the body fluids, the detection of EVs holds a great potential for early tumor diagnosis. EVs have been shown to play a role in tumorigenesis in several tumor types, but the effect of tumor inducing factors on EVs is unknown.

Changes in the tumor microenvironment can significantly influence the viability and growth of tumor cells. The Wnt signalling pathway plays an important role in the development, survival, and metastasis of several tumor types. Changes in the extracellular matrix (ECM) and the presence of tumor-associated fibroblasts (CAF) are critical components of the tumor microenvironment, which have a

major impact on cancer cells. A better understanding of the impact of these processes on EV release can contribute to the development of EV-based diagnostics.

2. Objectives

The aim of my Ph.D. thesis was to study the effect of the microenvironment on the EV secretion intensity in different normal and tumor-derived organoid models, focusing on the ECM and Wnt protein production.

More specifically, we wanted to find answers to the following questions:

1. CP is an important/ significant risk factor for PDAC. Based on our research group's earlier results, a higher EV level can be detected in both CP and PDAC. Using a mouse pancreatic ductal organoid model, we were interested in whether microenvironmental changes (cytokines, ECM changes) common to CP and PDAC also affect EV production.

2. Our research group has previously found that Wnt activity affects EV release in intestinal adenoma. We wanted to investigate if this was a common phenomenon in various normal/healthy and tumor tissues.

3. For LUAD mouse models, a Wnt-producing microniche has been identified that is of central relevance to the proliferative capacity of tumor cells. In our experiments, we also wanted to confirm the presence of this niche in a human LUAD model.

4. We aimed to study how the observed cellular heterogeneity in Wnt secretion affects the number of dividing cells and EV release in different organoid models and whether EVs from fibroblasts influence the formation of tumor microniche.

5. Can cell populations be identified with different Wnt-producing microniche formation potentials in LUAD to form organoids with varying numbers of dividing cells?

3. Materials and methods

3.1. Cell cultures

We used A549 and H1975 non-small cell lung cancer cell lines for the experiments with immortalized normal lung epithel BEAS-2B cell line as control. For 3D cell cultures, we grew 20,000 cells in 25 µl growth factor-reduced, phenol red-free Matrigel droplets for 14 days to form spheroids.

3.2. Mouse pancreas ductal and lung organoid cultures

For isolating mouse pancreas ductal organoid, whole pancreta from C57Bl/6J mice were cut into small pieces and the pancreas tissue was digested. Next, we isolated pancreatic ducts under a microscope. Lungs from mice were digested and the cells were filteredthrough a 70µm cell strainer. The ductal and lung bronchiolar samples were washed with PBS and after centrifugation, the cells were embedded in growth factor-reduced, phenol red-free Matrigel and they were cultured in a chemically defined medium. The medium was changed every four days. The organoids were removed from the 3D matrix every 6-8 days and were mechanically split. In some experiments, organoids were treated with the Wnt activityinhibitor IWP2 or LGK974 or with the Notch signalling blocking molecules DBZ or DAPT. Furthermore, in some experiments, organoids were treated with IL-1β, IL-6, or TNF-α.

3.3. Mouse lung fibroblast cultures

We used lungs from C57Bl/6J mice to isolate lung fibroblasts. Briefly, we cut the lung tissue into small pieces, and after digestion, the cells were washed with PBS twice and they were then

cultured in fibroblast medium. In some experiments, fibroblasts were treated with LGK974.

3.4. Human bronchiolar and LUAD organoids and fibroblasts cultures

Surgically resected tumor samples and peripheral normal lung tissues from LUAD patients were cut and they were digested. After the digestion, the cells were washed with PBS twice, then the cells were embedded in growth factor-reduced, phenol red-free Matrigel. For selecting *TP53* mutant LUAD organoids, the medium containing 10μM nutlin-3 was changed every second day for ten days. Isolated fibroblasts were cultured in fibroblast medium.

3.5. Collagen-based organoid cultures

Mouse pancreas ductal organoids were removed from Matrigel, and they were washed with PBS twice. The cells were then embedded in collagen I, or in a varying combination of collagen I/Matrigel mixtures (25%, 50%, or 75% collagen I). In some experiments, pancreas ductal organoids were treated with IL-1β, IL-6, or TNF-α.

3.6. Detecting EVs with antibody-coated beads

Two days before isolating supernatant from cells or organoids, the medium was changed to fresh FBS-free medium. Cell debris, larger EVs and apoptotic bodies were removed from the supernatant by differential centrifugation. We used EV-free medium as a control with the same centrifugation steps. We then added anti-CD63 or anti-CD81 antibody-coated magnetic beads that had been blocked with 1% BSA to the supernatant. The percentage of positive beads was determined with a FACS Calibur instrument. In all experiments, cells labelled with Trypan blue were counted with a Burker chamber, and the results were normalized to cell number.

3.7. Nanoparticle Tracking Analysis (NTA)

Cells and organoids were prepared similarly as for FACS analysis. After the serial centrifugation, the supernatants were diluted and measured on a Zeta View Z NTA instrument.

3.8. EV isolation for functional experiments

Serum-free conditioned medium from cultured cells or organoids was collected after two days, then the supernatant was serially centrifuged and ultracentrifuged (UC). The EV containing pellets were then resuspended in PBS. Their EV number was assessed with NTA, and they were added to murine or human fibroblasts or human lung organoids.

3.9. Flow cytometry and cell sorting

Organoids or cell line-derived spheroids were removed from Matrigel, and they were digested with TrypLE. The single cell suspension was labelled with primary and then with secondary antibodies, and the positive cells were detected with Flow cytometry (FACS). In some experiments, organoid cell subpopulations were sorted with a fluorescent cell sorter (Sony SH800S) into tubes with medium or Qiazol lysis buffer (Qiagen) to establish organoid cultures or for RNA purification, respectively.

3.10. Immunocytochemistry

Cells or organoids were cultured on 4 or 8-well chamber slides, they were fixed, blocked and permeabilized in blocking buffer. They were then labelled with the primary antibody at 4°C overnight. After incubating the samples with secondary antibodies for 2 hours at room temperature, they were covered with ProLong Diamond antifade mountant containing DAPI. The samples were analyzed with a confocal microscope (Zeiss LSM800 and Leica TCS SP8), and images were evaluated by Zen 2.6 (blue edition) and ImageJ softwares.

3.11. Transmission electron microscopy (TEM)

After dissolving the EV-containing pellet after UC, a 2 µl droplet was dried on a grid. The EVs were then fixed with glutaraldehyde, stained with phosphotungstic acid and they were imaged with the help of dr. Péter Lőrincz (ELTE, Department of Anatomy, Cell and Developmental Biology) on a JEM-1011 transmission electron microscope.

3.12. RNA isolation and mRNA measurements from cells

Total RNA from cells and organoids was isolated with the miRNEasy Micro Kit, and RNA concentration was determined with a NanoDrop instrument. For mRNA measurements, 0.5µg total RNA was reverse transcribed with the SensiFAST cDNA Synthesis Kit. Quantitative PCR reactions were carried out with the SensiFAST SYBR Hi-ROX Kit using the SybrGreen method. For mouse pancreas ductal organoid measurements, we used TaqMan assays. The measurements were carried out on an ABI 7900HT Fast real-time PCR instrument, and the results were normalized for a housekeeping gene (*HPRT1*). In some experiments, heatmaps were produced with z-scores with the Heatmapper program (www.heatmapper.ca).

3.13. Sequencing

cDNA was amplified with the Phusion High Fidelity DNA Polymerase. The PCR products were isolated from 2% agarose gels, and they were sequenced with the help of dr. Csaba Bödör's research group (Semmelweis University, 1st Department of Pathology and Experimental Cancer Research) using an Applied Biosystems 3500 Genetic Analyzer instrument. Data was analyzed by the Chromas 2.6 software.

3.14. Statistical analysis

We used Microsoft Excel, SPSS version 25, and GraphPad softwares for statistical evaluation. Student's unpaired t-tests,

ANOVA test, Mann-Whitney U-test, or Kruskal-Wallis with Dunn post hoc test were applied with *$p<0.05$, **$p<0.01$, and ***$p<0.005$ significance levels. When analyzing fold change data, they were log10 transformed, and these transformed data were applied to one sample t-test.

4. Results

4.1. Collagen deposition results in a higher EV level in mouse pancreatic ductal organoid cultures

A recent comprehensive study identified that many microenvironmental factors, including changes in the PDAC ECM, such as the accumulation of collagen, are already present in CP, a major risk factor for the development of PDAC. Furthermore, based on our previous results, elevated EV levels can be detected not only in PDAC but also in CP patient blood plasma samples. To model changes in the microenvironment at the pre-tumor stage, mouse pancreatic ductal organoids were isolated. We proved the ductal origin of these organoids and the presence of EVs in their supernatants. Thus, we can conclude that our mouse pancreatic organoids proved to be a suitable model for our experiments.

Next we focused on factors with a known role in CP, such as the accumulation of collagen I, and the presence of IL-1β, IL-6, and TNF-α. When organoids were cultured in pure collagen I, they had an extensive cell death. In addition, we found no effect of IL-1β, IL-6, or TNF-α on the EV release. However, the addition of collagen I to Matrigel at different amounts resulted inhigher EV levels in the supernatants from ductal organoids. In addition, we found a correlation between the increasing collagen concentration and EV number. We found no significant change in the percentage of active caspase-3+ apoptotic cells and proliferating KI67+ cells when adding collagen to Matrigel. In addition, IL-6, TNFα, or IL-1β did not result

in a massive change of EV release when organoids were cultured in collagen/Matrigel either.

These results collectively provide evidence that not only mutations but also critical changes in the cellular microenvironment can lead to a striking increase in the EV level in pancreatic ductal cultures.

4.2. Wnt secretion inhibition decreases cell proliferation and EV release in normal mouse pancreatic ductal cells

Previously our research group found that the percentage of Wnt responding, proliferating cells and EV secretion were closely coupled in the intestine. To study whether this was a general phenomenon valid for other cell types as well, we first used mouse pancreatic ductal organoids. The vast majority of the organoid cells were positive for the Porcn enzyme, which is critical for Wnt protein secretion. Furthermore, we observed nuclear β-catenin in some cells, showing the activity of the Wnt pathway.

Blocking Porcn inhibits the post-translational modification of Wnt proteins, leading to the decreased secretion and activity of Wnt ligands. For our experiments, we used two commonly used PORCN inhibitors (IWP2 and LGK974). They reduced the proportion of the Ki67+ proliferating cells, but had no effect on the active caspase-3+ apoptotic cells. Furthermore, the inhibitors resulted in the lower RNA levels of Wnt targets. In parallel, they reduced the number of EVs in the supernatant of the organoids.

Collectively, inhibiting the activity of Wnt proteins had a critical effect on cell proliferation and EV secretion in normal pancreas ductal organoids.

4.3. Modulating the percentage of proliferating cells modifies EV release in mouse lung bronchiolar organoids

As the next model, we established bronchiolar organoids from mice. They contained all bronchiolar cell types, including

ciliated cells, secretory cells, basal cells, and club cells. Thus, these organoids represented a good model of the *in vivo* tissue environment.

When analyzing the Porcn+ cells, we observed a heterogeneity for Porcn positivity, and bronchiolar organoids contained less positive cells compared to the pancreas ductal organoids. In addition, only a subpopulation of the cells showed the proliferation marker Ki67, and some cells contained nuclear β-catenin, proving the activation of the Wnt signalling pathway. The organoids expressed more genes encoding Wnt ligands, and blocking Wnt secretion by the porcupine inhibitor LGK974 decreased the number of Ki67+ cells without affecting the number of apoptotic cells. These results suggested that specific organoid cell subpopulations critically contributed to establishing the special microniche for proliferating cells.

Interestingly, mouse organoid cells and fibroblasts had a characteristic Wnt ligand expression pattern, raising the possibility that fibroblasts are also critical in shaping the Wnt-dependent organoid cellular heterogeneity. Lung fibroblast-derived EVs increased the number of proliferating cells in the organoids. When blocking the secretion of Wnt ligands in fibroblasts with LGK974, it prevented the effect of EVs on organoid cell proliferation. Importantly, LGK974 did not affect the proportion of either Ki67+ proliferating or active caspase-3+ apoptotic fibroblasts, and it did not modify EV release from these cells. Thus, both Porcn+ organoid cells and Wnt activity coupled to fibroblast-derived EVs play a critical role in shaping the proliferating microniche in the organoids.

Modifying the microniche by applying LGK974 in organoids resulted not only in a decreased proliferation but also in a reduced RNA level of Wnt targets and in the lower EV secretion. In contrast, when blocking the Notch pathway, which is critical in many developmental programs, we found no difference in either the percentage of Ki67+ cells or EV release from the organoids. Thus, these results show that modulating specifically Wnt secretion or Wnt activity critically modifies the number of proliferating cells. It is also coupled to the change in EV release in bronchiolar organoids.

4.4. EV secretion intensity is dependent on the proportion of proliferating cells in human bronchiolar organoids

To test whether the percentage of proliferating cells correlates with the amount of released EVs in humans, we established bronchiolar organoids from the lung tissue. These organoids contained all the bronchiolar cell types. Similar to the mouse bronchiolar organoids, we found heterogeneity in PORCN expression among the cells, and we observed that only a subpopulation of the organoid cells was positive for KI67. In addition, β-catenin showed nuclear localization in some cells, indicating active Wnt signalling. When comparing the Wnt expression profile of organoid cells and fibroblasts isolated from normal lung tissue, we found that a Wnt gene group was characteristic for the epithelial cells. Inhibiting Wnt secretion decreased the RNA levels of the Wnt targets and the percentage of KI67+ cells in the organoids without changing the proportion of apoptotic cells. Similarly to mouse organoids, modulating the size of the active Wnt producing cell population resulted in a change in EV secretion. Thus, our data provide evidence that the size of Wnt producing cell subpopulation is coupled to an increased EV release in normal human bronchiolar organoids.

4.5. LUAD organoids contain a Wnt producing microenvironment that affects cell proliferation and EV release

Although the intratumoral heterogeneity, establishing Wnt-producing and Wnt-responding cells is critical for the progression of LUAD in a mouse model, the presence of such a microenvironment in humans has not yet been directly proven.

To study the role of the Wnt-producing microenvironment on EV secretion in LUAD, we first used commercially available cell lines. To test whether they show heterogeneity for Wnt production, we cultured cell lines of primary NSCLC origin and control lung epithelial cells in 3D conditions. Interestingly, we found no massive positivity for PORCN in any of the studied cell line-derived spheroids.

The addition of LGK974 did not modify the percentage of KI67+ cells and EV release in any of the cell lines 3D either.

Next, we isolated tumor organoids from patients according to previous methods. Tumor-derived samples give rise to organoids of cancer origin and normal organoids that often overgrow the tumor organoid. *TP53* is the most frequently mutated gene in LUAD. Thus, we applied nutlin-3 for the selection of *TP53* mutant organoids. Although none of the normal lung organoid cultures survived nutlin-3 treatment, many LUAD samples contained surviving organoids after this selection step. To further characterize the surviving organoid lines, the *KRAS* gene was sequenced for mutation hotspots (codon 12, 13, 61).

These organoids contained cells with differentiation markers, but LUAD organoids contained only a marginal number of KRT14+ cells compared to normal lung organoids, which is a previously published hallmark of organoids of tumor origin. Furthermore, we observed the nuclear localization of β-catenin in some LUAD cells, and LUAD organoids displayed a cellular heterogeneity for KI67 and PORCN, showing the presence of a Wnt producing microniche in human LUAD as well.

To study how the size of the proliferating LUAD cell population is regulated, we established fibroblast cultures from the tumor of LUAD patients (LUAD-F). Of note, LUAD cells and fibroblasts had a characteristic and only partially overlapping Wnt expression pattern. Importantly, LUAD-Fs secreted EVs, and these EVs increased the number of proliferating cells in LUAD organoids.

To further characterize the Wnt producing organoid microniche, we determined whether proliferating and PORCN+ cells accumulate in some specific cell subpopulations. We found that PORCN+ and proliferating cells are not coupled to specific cell markers in LUAD that can be explained by the lack of terminal differentiation of tumor cells to specific cell types.

Similar to the normal human bronchiolar organoids, we found that blocking Wnt secretion resulted in a reduced level of WNT target gene RNAs, a decreased proliferation and EV release without modifying the percentage of apoptotic cells.

Thus, our results indicate that the cellular heterogeneity established by both intra-tumoral Wnt-producing cells and fibroblast-derived EVs critically determines cell proliferation and the EV secretion intensity in LUAD organoids.

4.6. The CD133^{high} LUAD cell population gives rise to organoids enriched in PORCN+ cells and a higher EV release compared to that of the CD133$^{low/-}$ cells

Previous studies suggested that CD133 marks an aggressive cell population in lung cancer. To study whether this cell population has a different ability to create the Wnt producing microniche compared to other LUAD cells, we fluorescently sorted CD133^{high} and CD133$^{low/-}$ cells from LUAD patient-derived organoids. We found no difference in the Wnt target genes between the freshly sorted cells. Importantly, CD133^{high} cells formed bigger organoids, and these organoids had a higher *CD133* RNA level and more CD133+ cells even after two weeks. Thus, organoids maintain the difference in the CD133 expression pattern. CD133^{high} organoids had an elevated Wnt activity. Surprisingly, in contrast to CD133^{high} organoids, where all cells were positive for PORCN, only a subpopulation of CD133$^{low/-}$ LUAD organoid cells expressed this enzyme, suggesting that not all cells are able to secrete active Wnt ligands. The functional consequence of the difference in the size of the Wnt producing cell population was reflected by the lower number of KI67+ proliferating cells in CD133$^{low/-}$ cell-derived organoids as well. Interestingly, we found a higher EV concentration in the supernatants of CD133^{high} LUAD organoids that contained more proliferating cells.

Collectively, in contrast to CD133^{high} organoids, where the majority of cells both produced and responded to Wnt with proliferation, the CD133$^{low/-}$ organoids showed a heterogeneity for Wnt secretion. This resulted in a lower proliferation ratio and EV secretion intensity as compared to CD133^{high} cell-derived organoids.

5. Conclusions

- Based on our results, the higher EV secretion in tumor cells is only partly due to mutations. The presence of collagen I in the ECM for pancreas ductal organoids, which is a major risk factor for PDAC and is enriched in both PDAC and CP, leads to a higher EV level in the organoid supernatants.
- Our research group proved the the relationship between the size of cell populationresponding to Wnt proteins, proliferation, and EV release in intestinal organoids. According to our results, this finding is valid for pancreas ductal organoids, normal lung bronchiolar organoids, and LUAD organoids as well, thus, it is a general mechanism.
- For pancreas ductal organoids, the vast majority of cells were positive for the enzyme Porcn, which is responsible for palmitoylation and secretion of Wnt proteins, thus marking Wnt-producing cells. When inhibiting Porcn, both the percentage of proliferating cells and EV secretion were reduced.
- In normal lung bronchiolar and LUAD organoids, only a subpopulation of cells expressed the PORCN enzyme. Although the Wnt-producing microniche and its association with poor survival are already known in mouse LUAD models, we also proved its presence in human LUAD organoids in our experiments.
- In both normal bronchiolar and LUAD organoids, we observed a decrease in the proportion of proliferating cells and EV secretion when inhibiting PORCN.
- In our studies, CD133+ cells from LUAD organoids produced organoids with more PORCN+ and dividing cells, and increased EV release when compared to organoids formed by CD133- cells. These data may explain why, based on literature data, the CD133+ LUAD cell population has aggressive properties.

- We also proved that not only organoid cells but also Wnt containing EVs secreted by fibroblasts contribute significantly to the formation of the Wnt-producing and responsive microenvironment.

Based on the results of the dissertation, we can conclude that i) important changes in the microenvironment before PDAC tumorigenesis have a large effect on EV secretion of pancreas ductal cells, and ii) a correlation can be observed between the size of the Wnt-producing cell population, the percentage of dividing cells and EV release intensity in organoid models of several healthy and tumor tissues.

Since the EV-based diagnostics critically depends on both the amount and cellular source of EVs, our results may contribute to the development of these methods for PDAC and LUAD, which could significantly facilitate the detection of these tumors before specific symptoms appear.

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