The role of extracellular vesicles in the tumor-stroma communication in colorectal cancer

Ph.D. Thesis

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1. Background

Colorectal cancer (CRC) is one of the most frequent causes of cancer-related death in the Western countries. In the large proportion of patients, *APC* inactivation is a central initializing mutation in CRC tumorigenesis. This results in the continuous activation of the Wnt pathway, which leads to increased cell proliferation and loss of cell differentiation by intestinal epithelial cells.

Extracellular vesicles (EVs) are membrane-surrounded structures that transmit biologically important molecules from the releasing to target cells, thus providing a novel intercellular communication mechanism. Since EVs carry their cargo in a protected form and their secretion is generally increased in tumorigenesis, EVs hold a great potential for early cancer diagnosis.

Tumor-associated fibroblasts (CRC-F) are critical component of the tumor microenvironment which, when activated, contribute to tumor progression by producing various extracellular matrix elements (collagen) and molecules (TGFβ). The peri-tumoral fibroblasts (PTF), isolated from the normal colon near to the tumor tissue, are often used as the unactivated control cells for CRC-Fs. However, a recent publication comparing the expression profiles of PTFs and CRC-Fs found a low level of difference between the corresponding pairs. In the CRC tissue, TGFβ is a major activating factor of fibroblasts.

2. Objectives

The aim of my Ph.D. thesis was to study the communication between CRC cells and stromal fibroblasts via extracellular vesicles.

During the research, we wanted to find answers to the following questions:

1. Is the organoid technology suitable for EV research?

2. Which factors affect the EV production of the CRC cells?

3. Are the CRC cell-derived EVs affect the colon fibroblasts and if yes, what is the effect?

4. Are the colon fibroblast-derived EVs affect the CRC cells and if yes, what is the effect?

5. Is there any difference between the cargo of the EVs produced by various patient-derived organoids?

6. Is the active state of the fibroblasts affect the EV production of the cells and the cargo of the EVs?

3. Materials and methods

Cell culture

For the experiments we used HCT116, SW620, SW1222 and HT29 CRC cell lines and normal human colon fibroblasts (ATCC-1459), furthermore, we used Thp-1 cells as control. Cells were cultured in DMEM high glucose supplemented with 10% FCS. Before EV collection, cells were further cultured in either medium without FBS or containing 2.5% EV-free FBS.

Human organoid cultures

The tissue samples were cut into small pieces, and incubated in a digestion mix. From the digested sample, crypts and cells containing supernatant was isolated, than the crypt fraction was separate. The single cell containing fraction was used for fibroblast isolation. The isolated crypts were embedded into growth factor reduced and phenol red-free Matrigel and cultured in human organoid medium (HOM). In some experiments colonies or organoids were cultured in 24-well suspension plates. Hypoxia was generated with AnaeroGen bags, hypoxic condition was checked by anaerobic indicator.

Producing and culturing *Apc*-mutant mouse organoids

Normal intestinal crypts from C57Bl/6J or UBI-GFP mice. Approximately 500 crypts were embedded into Matrigel and cultured in small intestinal medium (SIM). sgRNA sequence for mouse Apc was published previously (sgRNA4, [Schwank, 2013]) and cloned into lentiCRISPR v2 into the BsmBI restriction sites according to Addgene's instructions. Apc-mutant

organoids were produced according to the previously published protocol [Sato, 2009] with some modifcations.

Collagen-based organoid cultures

Organoids were removed from Matrigel, embedded into collagen type I and cultured in HOM. When removing cells, collagenase II was added to the medium.

Isolation of Human Colon Fibroblasts (PTF and CRC-F)

Tumor and normal colon tissue were isolated and cut into small pieces and incubated in a digestive mix. After removal of tissue pieces, single cells were then centrifuged at 300 g for 5 min, washed twice in PBS and cultured in tissue culture plates in fibroblast medium containing 10% FBS.

Human EV detection by anti-CD63 or anti-CD81-coated beads

Two days before the EV isolation, the cell supernatant was changed to fresh EV-free FBS containing, or FBS free medium. Culture supernatants were collected, and after centrifuging at 300 g for 5 min and 2000 g for 20 min, EVs were bound to CD63 or CD81 antibody-coated beads that had been blocked with 0.1% BSA. After overnight rotation at 4 °C, beads were separated, then measured on a FACSCalibur instrument. In all experiments, cells were counted with Burker chamber and results were normalized to cell number.

Mouse EV detection by anti-CD81-coated beads

Anti-CD81 antibody was bound to magnetic bead, than EVs bound to beads were detected by PE-anti-CD81 antibody and the results were normalized to cell number.

EV isolation for RNA analysis

Cell or organoid culture supernatants were collected and serially centrifuged, and small EVs were isolated with ultracentrifugation (UC). For miRNA detection, EVs were separated by UC or by the addition of anti-CD63 and anti-CD81 coated beads and EVs were lysed in Qiazol Lysis Reagent. Alternatively, EV-derived total RNA was extracted with the ExoRNEasy Serum/Plasma Starter Kit.

qNano measurements

Culture supernatants were harvested after 48 h, serially centrifuged and the supernatant was applied to qNano analysis.

Nanoparticle Tracking Analysis (NTA)

Fibroblasts were cultured in FBS-free medium, the supernatants were harvested after 2 days. The supernatants were serially centrifuged and measured on a ZetaView Z- Nanoparticle Tracking Analysis instrument.

Proteomics analysis of EVs

CRC organoids were cultured in HOM and as a control, organoid-free Matrigel droplets were applied. EV pellet was resuspended in water and proteins were extracted using repeated freeze–thaw cycles followed by

miniaturized tryptic digestion as previously described [Osteikoetxea 2018]. Before the tryptic digestion, protein concentration was determined by the Micro BCA Protein Assay Kit. Proteins present in the Matrigel controls were removed from organoid sample-derived lists of proteins and these lists were further analyzed.

Liposome production and characterization

The liposomes had a mean diameter of 105 nm (the preparation and characterization of liposomes was carried out in collaboration with dr. Zoltán Varga, Research Centre for Natural Sciences).

TaqMan low density miRNA array

Normal colon fibroblasts were cultured with our without TGFβ. Fibroblast supernatants were harvested after 4 days, serially centrifuged, and the particle concentration was determined with NTA. EVs were isolated with anti-CD63 and anti-CD81-coated beads and EVs were lyzed in Qiazol. Total RNA with small RNAs was isolated with the miRNeasy Micro Kit, total RNA was reverse transcribed with Megaplex RT primers, the samples were amplified with Megaplex PreAmp Primers and then analysed with TaqMan™ Array Human MicroRNA Cards v2.0. Array cards were measured with an ABI 7900HT instrument. We then selected miRNAs that were absent in all the three controls and present in the TGFβ-treated samples. As background measurements, samples isolated from cell-free cultures were applied.

RNA isolation and RNA measurements

RNA and total RNA with small RNAs were isolated with the RNEasy Micro Kit and miRNeasy Micro Kit, respectively. In some experiments, EV-derived miRNAs were obtained using the ExoRNEasy Serum/Plasma Starter Kit. RNA concentration was determined by NanoDrop.

For miRNA measurements, total RNA was reverse transcribed with the TaqMan® Advanced miRNA cDNA Synthesis Kit. PCR reactions were then carried out with TaqMan® Fast Advanced Master Mix and TaqMan® Advanced miRNA Assays. Quantitative PCR reactions using the SYBRGreen method were carried out with the SensiFAST™ SYBR® Hi-ROX Kit on an ABI 7900HT Fast real-time PCR instrument.

Sequencing

cDNA was amplifed with Phusion High Fidelity DNA Polymerase. The DNA was then isolated from 2% agarose gel, purifed by the Gel Purifcation Kit and sequenced by the forward primers with Applied Biosystems 3500 Genetic Analyzer instrument. Data were analyzed by the Chromas 2.6 software.

Transmission electron microscopy

After ultracentrifugation, the pellet was resuspended in PBS and a droplet was dried on a formfar-carbon coated 300 mesh grid. The EVs were then fixed with 4% glutaraldehyde, stained with 2% phosphotungstic acid, and imaged with a MORGAGNI 268D transmission electron microscope (collaboration with dr. Zoltán Varga, Research Centre for Natural Sciences.)

Wound-healing assay

A scratch in confluent human colon fibroblast monolayer was created with a p200 pipette tip, then cells were treated with EV-free or EV-rich medium. Images were taken at different times with a Nikon Diaphot microscope. The cell-free areas were evaluated by the ImageJ software. In some experiments, EVs were isolated from CRC organoids cultured in hypoxia for 2 days.

Whole-mount staining

Organoids were cultured in 4-well chamber slides, fixed in 4% PFA, washed with PBS + 4% NaCl and blocked and permeabilized in blocking buffer. The following primary antibodies were applied at 4 °C overnight in blocking buffer: rat anti-vimentin, rabbit anti-lumican, rabbit anti-active caspase-3, rabbit anti-Ki67, rabbit anti-mucin2. After incubation, the samples were washed, and incubated with the secondary antibodies. The organoids were mounted in mounting medium containing DAPI and imaged with an Zeiss LSM800 confocal microscope.

Immunocytochemistry

Cells were fixed in 4% PFA, then blocked and permeabilized in blocking buffer. After washing, cells were incubated with primary antibodies overnight and then with secondary antibodies for 2 h at room temperature. After covering samples with ProLong Diamond antifade mountant countaining DAPI, they were analyzed with a Zeiss LSM800 confocal microscope.

Detection of SA-β-Galactosidase Activity

The determination of cellular senescence was carried out by the detection of Senescence-Associated-β-Galactosidase (SA-β-Gal) Activity. After the reaction, the samples were analyzed with light microscope.

Microarray experiments

RNA quality was determined with the Bioanalyzer Pico Chip and analyzed on Agilent 4 × 44 K human whole genome expression microarrays. Data were processed by the Feature Extraction Software 12.0.3.1 and then imported into Chipster (www.chipster.csc.f) and the standard Agilent one colour normalization method was applied.

Gene set enrichment and survival analysis

The gene expression data set was transferred to the Gene Set Enrichment Analysis software (http://www.broadinstitute.org/gsea) and the analysis was carried out with default parameters and gene permutation was applied. We used a modifed list of the kegg.v3.1.symbols gene set (http://www.broadinstitute.org/gsea/msigdb) with the addition of Wnt targets [Van der Flier LG, 2007], intestinal stem cell-specifc genes [Yeung TM, 2010], and exosome biogenesis genes. The exosome biogenesis gene set was created based on published data and genes with proved function in MVB biogenesis and exosome secretion were selected. The gene set is shown in the Supplementary Information.

For survival analysis, the GSE17537 and GSE14333 data sets were used, both containing patient survival data as well.

Statistical analysis

Student's paired or unpaired t test, Mann–Whitney U test, one-way ANOVA, and Tukey post hoc test or Kruskal–Wallis test with Dunn post hoc test were used with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$ signifcance levels. For evaluation, Microsoft Excel, SPSS version 25, GraphPad Prism and Sigma Plot softwares were used.

4. Results

As a first step of the research, we examined the EV production of different CRC cell lines in 3D Matrigel matrix, where the cells formed spheroids. We observed that CD63+ EVs could be detected from cell supernatant (SN) by the antibody-coated bead-based EV isolation method under both 2D and 3D culture conditions, however, in case of 3D matrix, the large EV marker Annexin V + events could not be detected from the SN. This may suggest, that the larger EVs cannot leave the 3D Matrigel matrix.

To study the CRC cell-derived EVs, we used organoid technology, to create 3D tissue cultures from CRC patient-derived samples. Importantly, all of our patient-derived organoid lines produced CD63+ and CD81+ EVs. The organoid cultures used in our experiments had a continuously Wnt and mutant p53 signaling pathway, but the KRAS pathway was not mutant. We proved, that CRC organoid-derived EVs contain miRNAs, however, the isolation method largely influences the detected profile of EVs and there is a high variation among samples. The antibody-coated bead-based method resulted in the lowest unspecific background, thus it considered to be the most specific method for study the miRNA content of EVs. We studied the protein cargo of EVs, and analyzing the EVs of three CRC organoid lines showed that only about 45% of the detected proteins were present in all samples, highlighting the large variance between individual patient-derived organoid samples

In the study to find factors that influence EV release from CRC cells we found, that the extracellular matrix protein collagen type I, which is important in the CRC progression, enhanced the EV secretion of CRC organoids. It is widely accepted, that tumor cells release EVs at a higher level compared to

normal cells. However, the molecular or genetic background of this is not yet known. Since CRC cells represent a late stage of tumorigenesis and carry a wide variety of mutations, we used a mouse model with well-defined genetic background. We introduced *Apc* mutation into wildtype (WT) small intestinal organoids by CRISPR-Cas9 and we selected *Apc*-mutant organoids, representing the adenoma stage of intestinal tumors, without adding the external Wnt-agonist R-Spondin1. Since WT intestinal stem cells are strictly dependent on R-Spondin1, only *Apc*-mutant organoids survive. Interestingly, we observed a massive increase in CD81+ EV secretion after *Apc* mutation. Gene Set Enrichment Analysis (GSEA) was carried out to test, whether altered expression of genes involved in the biogenesis of EVs (small EVs, multivesicular body biogenesis) was responsible for the enhanced EV production after the *Apc* mutation. However, in samples, carrying the *APC* mutation, the exosome biogenesis gene pool showed no positive enrichment. To test whether Wnt pathway activation may lead to an enhanced EV release, WT intestinal organoids were treated with Wnt3a or the GSK-3 inhibitor CHIR99021, known activators of this signaling pathway. Similar to *Apc* mutation, both Wnt3a and CHIR99021 resulted in an increased ratio of Ki67 + proliferating and decreased percentage of Mucin-2+ Goblet cells, which was paralleled by a lower RNA level of Mucin-2 and the enterocyte marker Alpi, and an increase in the Wnttarget stem cell marker Lgr5. Importantly, both Wnt3a and CHIR99021 resulted in a massively elevated EV release from organoids, similar to *Apc* mutation.

It is widely accepted, that the amount of stromal fibroblasts in the tumor tissue is negatively correlates with the CRC prognosis, thus it was crucial to study the colon fibroblasts. Since our data showed that several factors affect

the EV production of CRC and adenoma cells, we wanted to know, which factors affect the EV production of colon fibroblasts. For the studies, next to the normal colon cells we also used CRC patient-derived cancer-associated (CRC-F) and peritumoral (PTF) fibroblasts. Since the fibroblasts, as tumor-associated fibroblasts, are in an activated state in the tumor tissue, we wanted to know, if this active state affects the EV production of fibroblasts. We activated the NCFs, CRC-Fs and PTFs with TGFβ, and as previously published, TGFb reduced the proportion of the KI67+ proliferating cells and increased the intensity of aSMA expression, a general marker of activated fibroblasts. As expected, we observed an increased RNA level of FAP, ACTA2 (encoding aSMA), IL6, IL-11, and HBEGF, thus, proving that TGFβ treatment led to characteristic transcriptional changes in fibroblasts. By using the antibody-coated bead-based method, we did not find an increased CD81C or CD63 C EV release after TGFβ. Similarly, NTA, a widely used method for quantifying EV amounts, showed no difference in particle concentration and no shift in particle size distribution after treatment. Thus, these results indicate that TGFβ -induced fibroblast activation did not have a major effect on EV secretion.

Interestingly, however, activation of normal colon fibroblasts by TGFβ modified the miRNA cargo of fibroblast-derived EVs. Examining 377 miRNAs using a Taqman advanced miRNA Array, 209 miRNAs were detected in at least one of the samples and we found a change in the EV cargo with miR-101, 382, 424, and 642 present only in EVs released by TGFb-treated activated fibroblasts.

We examined the effect of the activated state of fibroblasts on their migration profile, so we performed a wound healing assay after TGFβ

treatment. Interestingly, TGFβ resulted in a larger wound area in a wound healing assay, showing the reduced migration of NCFs. however, we could not observe this in the case of PTF and CRC-F samples.

After that, we applied the organoid system to investigate the role of EVs in the tumor-stroma communication. First we tested the effect of CRC organoid derived EVs after differential UC using commercially available normal human colon fibroblasts. ATCC-1459 human colon fibroblast cells were treated with EV-enriched or EV-depleted medium prepared from supernatants of the 3D CRC organoids and microarray experiments were carried out. As expected, CRC-derived medium, independently of the EV content, resulted in an overall change in the gene expression of fibroblasts, but interestingly, CRC-derived EVs had no major effect on the transcriptional profile of fibroblasts. To test whether CRC organoid-derived EVs have a critical role under unfavorable conditions, we compared normoxia and hypoxia. CRC organoid-derived EVs did not induce an increased fibroblast motility in wound healing assays in either conditions Next, we purified EVs from human colon fibroblasts grown under normoxia or hypoxia and added them to CRC organoid-derived cells in either conditions. Interestingly, we observed an increased number of new organoids after EV treatment only when fibroblasts were cultured in which was not detected with EV-free supernatant. Importantly, EVs isolated from the acute monocytic leukemia patient-derived THP-1 cell line or liposomes had no effect, suggesting that the increase in organoid forming efficiency is connected to fibroblast EVs.

Overall, we demonstrated that communication between CRC cells and fibroblasts also occurs through EVs in tumor tissue

5. Conclusions

- We showed that small EVs are detectable in the supernatant of 3D organoids and spheroids, however larger EVs cannot leave the 3D matrix.

- We proved that CRC patient-derived organoids produce EVs, which can also be examined in the supernatant, thus demonstrating, that Matrigel-based organoid technology can be used for EV research in CRC.

- Critical factors in the tumorigenesis of CRC, such as *Apc* mutation or Collagen I accumulation, increase the EV production of tumor organoids. Furthermore, since the *APC* mutation is an early event in the CRC tumorigenesis, our data suggest, that this increased EV production is already present in the adenoma stage. We further demonstrated that the *Apc* mutation increases EV production by CRC cells through stimulation of the Wnt signaling pathway.

- Interestingly, although fibroblast-derived EVs stimulate the colony-forming ability of tumor cells in hypoxia in EGF-dependent CRC cases when external EGF is present, organoid-derived EVs did not result in a significant change in fibroblast activity.

- Activation of normal colon fibroblasts by TGFβ modifies the miRNA cargo of fibroblast-derived EVs, four miRNA (miR-101, 382, 424, and hsa-miR-642) appear only in activated normal fibroblast-derived EVs.

Our results highlight the importance of organoid technology in EV-characterizing research and demonstrate that communication between CRC cells and fibroblasts also occurs through EVs in tumor tissue. Our data provide valuable basis for research into the use of EVs as diagnostic marker or as a therapeutic drug delivery device.

6. Bibliography

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