# Novel paracrine communication mechanisms in the tumor promoting activation of stromal fibroblasts in human colorectal cancer

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Scientific background, major aims

Colorectal cancer (CRC) is the second leading cause of cancer related morbidity and mortality in Hungary. In the vast majority of CRC patients, mutations inactivating **APC** result in the continuous, external ligand independent regulation of the **Wnt pathway**. This leads to the uncontrolled proliferation of specific intestinal epithelial cell populations, such as stem cells, leading to adenoma formation. The accumulation of other mutations, such as *TP53*, *KRAS*, *SMAD4* etc drives then the progression of these adenomas to CRC.

**Extracellular vesicles (EVs)** represent a heterogenous group of lipid bilayer enclosed vesicles. The largest EVs are the apoptotic bodies, released by cells undergoing apoptosis. Microvesicles (MVs) are directly shed from the plasma membrane, while exosomes (EXs) are derived from the multivesicular bodies (MVB) of the endosomal compartment and they are released from cells upon fusion of the MVBs with the plasma membrane. However, due to difficulties in separating EVs according to their cellular origin, they are often categorized based on their size [1]. Thus, **large EVs** enriched for MVs (lEV) and **small EVs** (sEV) containing mostly EXs are separated by centrifugation and ultracentrifugation, respectively. EVs transfer a wide array of biologically important molecules, such as proteins, lipids, miRNAs when they target the recipient cells [2, 3]. While TSG101, CD63, CD9 and CD81 are often regarded as markers of sEVs, lEVs contain a high amount of phosphatidylserine (PS) in the outer membrane layer and they can thus be detected by Annexin V [4].

Importantly, the vast majority of the tumor EV data was obtained from 2D CRC cell line cultures. Clevers and his colleagues have shown that in the intestine, the $Lgr5^+$ crypt stem cells self-organize into continuously renewing organoids ("miniguts") in 3D matrix *ex vivo* [5, 6]. These "miniguts" recapitulate the basic features of the intestinal epithelium; they form crypts and villus-like structures and produce all the differentiated cell types, similar to the *in vivo* situation. They have also worked out an *ex vivo* culture system for long-term expansion of organoids from normal human colon, colorectal adenoma, adenocarcinoma etc [5, 7]. **Unlike the traditional cell lines, these 3D cultures maintain the cellular heterogeneity similar to the *in vivo* tumors and they provide an excellent and well-controlled, improved experimental system to dissect the underlying mechanism of diseases [8].**

**This project studied the role and mechanism of EV-based communication between tumor cells and stromal fibroblasts and the contribution of the critical *Apc* mutation. Since there are substantial differences between mouse and human intestinal cancers, we primarily focused on human CRC models. Furthermore, instead of traditional 2D cell cultures, we used the state-of-the-art 3D organoid technology that more reliably models human tumors.**

Results, experiments

We had the necessary **permission from the Hungarian Scientific Ethical Committee (TUKEB)** to collect clinical samples at the 1st Department of Surgery, Semmelweis University and the Uzsoki utcai Hospital, Budapest.

To study the biological significance of the tumor-derived EVs and to set up the basic experimental system, **we isolated fibroblasts** from CRC patients undergoing operation from the tumor tissue (cancer-associated fibroblasts, CAFs) and from the peritumoral normal tissue (peritumoral fibroblasts, PTF) by collagenase and dispase digestion. Importantly, PTFs are often considered as non-activated normal fibroblasts [9]. In addition, we purchased normal human colon fibroblasts (NCFs) from ATCC (CRL-1459). In agreement with previous publications [9], both NCFs, PTFs and CAFs were positive for α-SMA, thus, suggesting that this widely accepted fibroblast activation marker is not proper for distinguishing CAFs from other fibroblasts (**Publication 1**).

lEVs enriched for MVs (100-1,000 nm) usually have phosphatidylserine on their outer surfaces; thus, they can be detected with Annexin V. sEVs containing mainly EXs are usually CD81+ and/or CD63+ [1]. We isolated the lEV and sEV fractions from cell culture supernatants by differential centrifugation and ultracentrifugation [10]. Interestingly, while Annexin V+ lEVs were hardly detected, **we could identify CD63+ and CD81+ sEVs from both NCFs, PTFs and CAFs (Publication 1 and 3)**.

**To optimize EV detection and to characterize the EV production** of CRC cell lines 2D and 3D, SW480, SW1222, LoVo, Colo205, DLD-1, HCT116 and HT-29 cells were cultured. While the production of lEVs was determined by Annexin V staining, sEVs were bound to anti-CD63 or anti-CD81-coated magnetic beads and the proportion of positive beads was counted by flow cytometry. In addition, we used TRPS (qNano, Izon) to characterize the particle number and size distribution in 2D or 3D cell culture media. All the CRC cell lines produced both lEVs and sEVs in

2D cultures, but only sEVs were detected in 3D culture supernatants using 3D matrix. Interestingly, when spheroids were grown in Matrigel for 10 days and then further maintained in suspension cultures, we detected both lEVs and sEVs. However, these suspension cultures contained an elevated number of active caspase-3+ apoptotic cells compared to organoids in 3D matrices, suggesting that suspension cultures do not provide an optimal experimental system to study EV release. These data show that although CRC cells are able to secrete both types of EVs, only sEVs are released into the medium from 3D matrix. Similarly, **we observed only sEV release into the 3D culture medium when using CRC patient-derived organoid cultures (Publication 3).**

EVs are considered as transmission vehicles of proteins and miRNAs between cells and they hold a great promise in early cancer diagnosis. This assumption is based on the fact that i) tumor cells release EVs at a higher level compared to normal cells and that ii) cancer cell-derived EVs carry tumor-specific molecules (e.g. miRNAs) as cargo in a membrane-surrounded, protected milieu. **To characterize the cargo of CRC organoid-derived EVs**, we carried out protein analysis as the first step. Our mass spectrometric **proteomic analysis** of EVs from 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. To further characterize the cargo of CRC organoid-derived EVs, we measured 14 **miRNAs** in EVs isolated with three different methods. Whereas miR-484 or miR34a-5p were detected only by a commercial EV RNA isolation kit (ExoRNEasy Kit, Qiagen), most other miRNAs were present both in case of this kit or differential ultracentrifugation, although at different amounts. Interestingly, when using beads coated with anti-CD63 and anti-CD81, markers of most sEVs, we could detect only 8 miRNAs out of the 14 measured and this isolation method resulted in the lowest unspecific background in Matrigel samples without cells. Thus, CRC organoid-derived EVs carry proteins and miRNAs as cargo, however, the EV isolation method largely influences the detected profile and **there is a high variation among samples**. Since the bead-based isolation method produced the lowest unspecific background, we used this protocol in our other experiments when analyzing EV miRNA cargo. However, for functional assays the traditional ultracentrifugation (UC) could not be replaced with other isolation protocols (**Publication 3**).

According to the general view, the EV production increases during tumorigenesis, however, the contribution of individual mutations is largely unknown. To determine whether ***Apc* mutation, the first critical step in the majority of patients, has a significant impact on EV biogenesis or secretion**, we introduced an inactivating mutation into this gene in wild type (WT) intestinal organoids by the CRISPR-Cas9 technology. Since the EV production may be influenced by other genetic variations present in the human population as well, we used the genetically homogenous inbred C57Bl/6 (Jackson Laboratory) mouse strain. The successful Apc inactivation was proved by culturing organoids in R-Spondin1-free medium. Since WT intestinal organoids require this protein as a Wnt-agonist, only Apc-mutant adenoma organoids survive in R-Spondin1-free medium beyond 6 days. Using antibody-coated beads and flow cytometry, we detected a markedly elevated sEV release in the Apc-mutant adenoma cultures compared to the WT organoids (**Publication 3**).

Unfortunately, no single gene is responsible for EV release and in different cell types different EV pathways may operate. Exosomes (EX) are specific EVs of endosomal origin. To test whether the EX route is responsible for the enhanced EV secretion after *Apc* mutation, we selected 36 genes with proved function in multivesicular body formation (a specific endosomal compartment) and EX release (e.g. specific Rab proteins). This gene set was used in Gene Set Enrichment Analysis (GSEA, https://software.broadinstitute.org/gsea/index.jsp) on publicly available expression data derived from organoid libraries (GSE74843) [11]. Importantly, these organoid libraries contain only (tumor) cells of epithelial origin, thus excluding stroma-derived signals. Interestingly, the EX biogenesis gene set showed a negative enrichment in adenoma or CRC organoids compared to normal colon organoids, thus, suggesting that **the increased EV release after *Apc* mutation is not a consequence of the higher expression level of EX biogenesis genes** and alternative EV secretion pathways may function in this system (**Publication 3**).

Apc is a large protein with one of the most important functions to regulate the Wnt pathway. To test whether Wnt pathway activation may lead to an enhanced EV release, WT intestinal organoids were treated with Wnt3a or CHIR99021, known activators of the Wnt signaling pathway. Similar to *Apc* mutation, both Wnt3a and CHIR99021 resulted in an increased ratio of Ki67+ proliferating cells and an increased level in the Wnt-target stem cell marker Lgr5. Similar to Apc mutation, both Wnt3a and CHIR99021 resulted in a massively elevated EV release from organoids without an increased number of apoptotic cells. These results strongly support the hypothesis that **Apc mutation increases EV secretion via activating the Wnt pathway in the organoids. Furthermore, since *Apc* mutation is a very early event in intestinal tumorigenesis, our data suggest that increased EV release occurs already in the adenoma stage (Publication 3).**

To find further factors critically regulating EV release in CRC tumorigenesis, we focused on cytokines, growth factors and extracellular matrix components (ECM). IL-17, IL-22, TNFα and HGF have proved effects in CRC

tumorigenesis [12, 13] and we detected the expression of their receptors in CRC cells. However, they had no consequent effect on EV release from organoids. In contrast, when organoids were cultured in **collagen type I instead of laminin-rich Matrigel, the EV secretion was enhanced without a significant increase in apoptosis.** The effect of collagen I was proved not only in CRC organoids carrying a wide range of mutations, but in *Apc*-mutant mouse adenoma organoids as well. Interestingly, culturing in collagen resulted in the expression of some epithelial-mesenchymal transition (EMT) markers (vimentin, lumican). The importance of collagen I in CRC progression is also supported by our bioinformatical analysis on microarray data sets containing clinical data as well (GSE17537 and GSE14333), showing that high collagen I level is strongly associated with an early time point for cancer recurrence. **Collectively, not only mutations, but changes in the tumor microenvironment, such as the accumulation of collagen, result in an increased EV release from tumor cells (Publication 3).**

**To determine the effect of CRC patient organoid-derived EVs on fibroblasts**, cells were treated with EV-enriched or EV-depleted medium isolated from the CRC organoids and microarray experiments were carried out. As expected, CRC-derived medium, irrespective of their EV content, resulted in an overall change in the gene expression profile of fibroblasts. Interestingly, CRC-derived EVs had only a modest effect and only a small subset of genes (about 100 genes) showed a small (<2-fold) change. Furthermore, genes typically associated with fibroblast activation (ENC1, ST6GALNAC5, SEMA5A, TGFB2, TNFSF4 etc) [9] were not on the list of differentially expressed genes. In addition, we could not detect a major effect of CRC-derived EVs on fibroblast activation either under normoxia or hypoxia. In contrast to other studies where the authors use EVs derived from millions of cells and a large culture volume, thus increasing the risk for a significant amount of molecules co-purified with EVs in ultracentrifugation only by chance, we harvested EVs only from cc 200,000 cells and 50,000 fibroblasts were treated. We think that this EV:cell proportion approaches the physiological situation better and minimalizes the risk for co-purified contamination. However, we focused next on the analysis of fibroblast-derived EVs in the tumor-stroma communication (Publication 3).

Since we found that the number of Annexin V+ lEVs is much lower compared to the CD63+/CD81+ sEVs in NCF cultures, **we focused on the role of fibroblast-derived sEVs**. NCF-derived EVs were added to CRC organoid-derived cells and the organoid forming efficiency, a measure of stem cell activity, was determined. Interestingly, we detected an enhancement of stem cell activity in the presence of fibroblast-derived EVs in hypoxia, thus, suggesting that **stromal fibroblast EVs are more important in this cross-communication**. Of note, since our CRC organoids did not have KRAS mutations and they required EGF for culturing, we included recombinant EGF in the medium in these experiments (Publication 3).

Rab27a is a gene product involved in the secretion of EXs. Although Rab27a alone was shown to critically regulate EX secretion from breast cancer cells [14], its role is not very clear in other cell types. Furthermore, a large number of genes have been connected to EV secretion and the inactivation of individual genes rarely results in the drastic decrease of EV release. To avoid this problem, we applied the neutral sphingomyelinase inhibitor GW4869 that was published to block EV secretion in many cellular systems. Interestingly however, we found no effect in human fibroblasts at any of the used concentrations (5 and 20μM) for EV release. Since blocking the EV release from intestinal fibroblasts cannot be carried out with this tool, we focused on the specific protein cargo components of the EVs.

To analyze the mechanism how fibroblast-derived EVs exert their effects, we first studied WT intestinal organoids. The intestinal epithelium is continuously renewed by a small proliferating intestinal stem cell population (ISC), residing at the bottom of the intestinal crypts in a specific microenvironment, the stem cell niche. We showed that intestinal fibroblast-derived EVs are involved in forming the ISC niche in both mouse and human systems by transmitting Wnt and epidermal growth factor (EGF) activity. We demonstrated that intestinal fibroblast-derived EVs carry EGF family members, such as amphiregulin (AREG). Mechanistically, blocking EV-bound amphiregulin inhibited the EV-induced survival of organoids. Thus, **we proved the important role of fibroblast-derived EVs and amphiregulin as a novel transmission mechanism of factors in the intestinal stem cell niche (Publication 2).**

Fibroblasts in tumors show a characteristic gene expression profile and thus, these cancer-associated fibroblasts (CAFs) are considered to be in an activated state. TGFβ is critically involved in colon fibroblast activation [15]. To study the effects of EVs released by activated fibroblasts on CRC cells, first **we activated NCFs with TGFβ**. TGFβ reduced the proportion of the KI67+ proliferating cells and increased the intensity of αSMA expression, a general marker of intestinal myofibroblasts. Importantly, we found no change in the percentage of apoptotic cells. As expected, we observed an increased RNA level for fibroblast activation genes, thus, proving that TGFβ treatment led to characteristic transcriptional changes. However, we did not find an increased CD81+ EV release after TGFβ from NCFs, measured by a bead-based protocol or by Nanoparticle Tracking Analysis (NTA) that is a widely used method

for quantifying EV amounts and their size distribution. Thus, we found that **TGFβ-induced NCF activation did not have a major effect on EV secretion (Publication 1)**.

To determine **changes in the EV cargo after NCF activation,** we focused on miRNAs and we isolated EVs with anti-CD63 and anti-CD81-coated beads. The medium-scale screen, analyzing 377 miRNAs, detected 209 miRNAs in at least one of the measured samples. Interestingly, we found that four miRNAs (hsa-miR-101, 382, 424, 642) were present only in TGFβ-treated EV samples. Thus, **NCF activation changes the miRNA profile of the EV cargo (Publication 1)**.

Since AREG travels via fibroblast-derived EVs in the normal intestine, EVs may be important for EGF-dependent CRC organoids in the absence of external EGF family members. We added NCF-derived EVs to patient-derived organoid lines known to be dependent on EGF. Importantly, we observed a marked reduction in the percentage of proliferating organoid cells in the absence of AREG or EGF which was restored by NCF-derived EVs. In addition, pre-incubating EVs with a neutralizing anti-AREG antibody inhibited the effects of EVs, thus, proving the critical role of EV-bound AREG on CRC cell proliferation. Interestingly, we observed no difference in the percentage of KI67+ cells when CRC organoids were treated with identical numbers of EVs isolated from control or TGFβ-activated NCFs. Thus, **although TGFβ resulted in a change of EV miRNA cargo, this had no effect on CRC cell proliferation. However, both control and activated fibroblast-derived EVs induced CRC proliferation via AREG in those organoids that are dependent on EGF activity (Publication 1).**

To prove our findings in another model system, we used PTFs and CAFs. Similarly to NCFs, TGFβ reduced the proportion of the proliferating cells and increased the intensity of αSMA expression in both CAFs and PTFs. Importantly, TGFβ-induced fibroblast activation did not modify the amount of secreted EVs either in PTFs or in CAFs. Thus, **we found that TGFβ induced similar changes both in PTFs and in CAFs (Publication 1).**

Since we found no difference between our PTF and CAF cultures in their activation, we focused on CAF fibroblasts. Similarly to NCFs, we detected AREG on both control and TGFβ-treated CAF-derived EVs and they restored the proportion of KI67+ CRC cells in EGF-dependent organoids in the absence of exogenously added EGF family members. Importantly, we observed no difference when CAF cultures had been pre-treated with TGFβ and identical numbers of EVs were applied. Thus, **our results show that fibroblast-derived EVs primarily induce CRC proliferation via an activation-independent mechanism in EGF-dependent CRC organoids (Publication 1).**

Prox1 is a transcription factor that is expressed in the intestine only after a very high Wnt signaling intensity and it results in tumor progression. In a collaboration with Prof. Kari Alitalo's lab (University of Helsinki, Finland), we proved that Prox1+/Notch- cells have tumor stem cell properties and Notch turns off Prox1 in CRC [16]. **Thus, in addition to stroma-derived EVs, this serves as a novel mechanism for establishing the tumor stem cell population.** Interestingly, Prox1 regulated stem cell differentiation also in muscles via the Notch patway (**Publication 4**).

Personnal: The project was carried out with the planned personnel. The project financed a Semmelweis PhD Scholarship for the full-time scientist, resulting in a lower personnel cost compared to the original plans. However, this fact allowed us to cover more reagents, enabling the more successful implementation of the project.

Scientific papers from this grant:

**Publ 1.** Oszvald Á, Szvicsek Z, Pápai M, Kelemen A, Varga Z, Tölgyes T, Dede K, Bursics A, Buzás EI, **Wiener Z.** Fibroblast-Derived Extracellular Vesicles Induce Colorectal Cancer Progression by Transmitting Amphiregulin. Front Cell Dev Biol. 2020 Jul 7;8:558. doi: 10.3389/fcell.2020.00558. eCollection 2020. IF: 5.201

**Publ 2.** Oszvald Á, Szvicsek Z, Sándor GO, Kelemen A, Soós AÁ, Pálóczi K, Bursics A, Dede K, Tölgyes T, Buzás EI, Zeöld A, **Wiener Z**. Extracellular vesicles transmit epithelial growth factor activity in the intestinal stem cell niche. Stem Cells. 2020 Feb;38(2):291-300. IF: 6.022

**Publ 3.** Szvicsek Z, Oszvald Á, Szabó L, Sándor GO, Kelemen A, Soós AÁ, Pálóczi K, Harsányi L, Tölgyes T, Dede K, Bursics A, Buzás EI, Zeöld A, **Wiener Z.** Extracellular vesicle release from intestinal organoids is modulated by Apc mutation and other colorectal cancer progression factors. Cell Mol Life Sci. 2019 Jun;76(12):2463-2476. IF: 6.496

**Publ 4.** Kivelä R, Salmela I, Nguyen YH, Petrova TV, Koistinen HA, **Wiener Z**, Alitalo K. The transcription factor Prox1 is essential for satellite cell differentiation and muscle fibre-type regulation. Nat Commun. 2016 Oct 12;7:13124. IF: 12.124

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