The role of extracellular vesicles in the normal intestine and intestinal tumorigenesis

Doctoral Thesises

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1. Introduction

The intestinal epithelium is continuously renewed by a rapidly proliferating stem cell population, residing at the bottom of the intestinal crypts in a specific microenvironment, the stem cell niche (SCN). The intestinal stem cell population (ISC) can be characterised by the expression of the Lgr5 protein. This recptor amplifies Wnt-signalling. ISCs critically depend on niche factors such as EGF-receptor ligands, originated from the surrounding cells of the microenvironment, such as intestinal fibroblasts, or Paneth cells.

Colorectal cancer is among the most frequent cancer types in the developed world. In more than 80% of CRC patients, mutation in the *APC* gene is an initializing genetic change, leading to the continuous and ligand-independent activation of the Wnt pathway. In addition, the oncogenic activation of KRAS leads to the independency of the adenoma cells from external epidermal growth factor (EGF) activity. The surrounding microenvironment forming cells such as fibroblasts of the stroma can help tumour growth via secreting niche factors that can further enhcance the growing capabilites of the tumour. Activation of fibroblasts for example by the citokine TGFbeta can elevate this tumour supporting capability even further. Low frequencies of the EGFR-Erk axis (of which the KRAS is frequently mutated) are usually accompanied by high number of cancer associated fibroblasts and elevated level of TGFbeta.

Extracellular vesicles (EVs) are membrane-enclosed structures,
secreted by both normal and cancer cells. The largest EVs are the apoptotic bodies released by cells undergoing apoptosis. Microvesicles (MVs) are directly shed from the

plasma membrane, while exosomes (EXs), the best characterized and smallest EV subpopulation, are derived from the multi-vesicular bodies (MVB) of the endosomal compartment and they are released from cells upon fusion of the MVBs with the plasma membrane. EVs are able to transport a huge variety of cargos such as lipids, proteins and nucleic acids.

We hypothesise that niche factors secreted by fibroblasts can be transferred via the extracellular vesicle pathway.

2. Objectives

1) Do intestinal fibroblast-derived EVs alter proliferation of intestinal organoids when all niche factors are present?
2) Can any of the niche factors be replaced by fibroblast-derived EVs?
3) Can we detect the niche factors on EVs?
4) Do EVs alter proliferation if niche factors are dispensable due to tumorigenic mutations?
5) Do CAF-derived EVs alter proliferation of CRC organoids?
6) Do activation of fibroblast cultures change their tumour supporting ability?

3. Methods

Briefly, we cultured human intestinal normal and cancer associated fibroblasts and murine intestinal normal fibroblasts, and EVs were isolated from the culture supernatants. The required niche factors (EGF, Noggin, R-Spondin, Wnt) were replaced one by one by EVs, and the organoids cultures were characterised.

EV-isolation

EVs were isolated by differential ultracentrifugation. Only the smallest EV subpopulations were used, isolated by the highest, 100.000g centrifugation step. EVs were washed with PBS after isolation.

Organoid cultures

As a model for the normal intestinal epithelia we used RD organoid culture systems, derived from normal murine small intestine or colon, or from human normal colon. These organoids self-organise info continuously renewing organoids, after they are embedded into Matrigel and treated with niche factors, such as EGF, Noggin and R-Spondin. Colon organoids require Wnt-proteins as well, since both murine and human colon lack Wnt-secreting Paneth-cells. For some of our experiments, organoids isolated from *Lgr5-EGFP-IRES-Cre*^{ERT2} mouse strain were used. For modeling colorectal cancer, we used patient derived CRC organoids. These organoids require EGF and certain inhibitors.

Live/Dead organoid ratios

By simple brightfield microscopic observation live and dead organoids can be distinguished. Live organoids form a continous epithelial layer, while dead organoids fall into single individual cells.

Immunocytochemistry

Our human and murine fibroblast cultures were tested for the fibroblast marker alphaSMA.

In the organoid cultures, proliferating cells ratios were determined by KI67 immunocytochemistry, and apoptotic cells ratios were determined by active Caspase3 immunocytochemistry. The EGF-receptor activating

effect of EVs were determined by immunolabeling for the phosphorylated form of EGF-receptor.

Real Time PCR

Quantitative real time PCR was used to determine the origin (epithelieal or stromal) of EGF-receptor ligands. Specific primers were designed by us, *GAPDH* or *HPRT* genes were used as housekeeping genes for control

Flow cytometric assays

For detecting EVs from the supernatant, larger EV populations were centrifuged down, so only the smallest EVs remained in the supernatant. Then the supernatant was incubated with EV-specific magnetic beads. The EVs captured by the beads were labeled against the small EV markers (such as CD63 or CD81) and then the beads were measured by flow cytometry.

For detecting EGF-receptor ligands on the surface of EVs, two type of bead assays were used. We used either magnetic beads coated by antibodies against specific EV markers or latex beads that captures every EV type. EVs were incubated with either of the beads, and were labeled against different EGF-receptor ligands. After that, beads were measured by flow cytometer.

Western Blot

Simple Western Blotting method was used for the detection of EGF-receptor proteins from the ultracentrifugated EV pellet.

4. Results

According to our results, EVs do not carry any molecules with BMP antagonising or Wnt amplifying activity. Replacing either Noggin or R-Spondin proteins for murine

normal small intestinal organoids with EVs resulted in a massive organoid death.

HCF-derived EVs can replace Wnt proteins in the intestinal SCN. EVs replacing Wnt proteins can elevate human and murine colon organoid survival ratios to Wnt-treated organoid levels. The novelty of our results is the observation that Wnt proteins can be transmitted in the normal colon SCN by fibroblast-derived EVs without any additional treatments or wound. It needs to be pointed out that our experiments proved that EVs can replace Wnt proteins, however, we did not analyse the presence of EV-bound Wnt-proteins.

Replacing EGF by fibroblast derived EVs can restore live intestinal organoid ratios to the control level. The simple observation that small intestinal and colon organoids (regardless if of murine or human origin) cannot survive without exogenous recombinant EGF shows that the epithelium does not produce enough EGF on its own. Our results were the first to show that normal fibroblast derived EVs contribute to the normal, non-tumorous intestinal SCN. With our work we proved that HCF- and MIF-derived EVs can replace EGF for murine small intestinal and colon and, importantly, for human colon organoids as well.

After these results, we focused on EGF-family proteins. We found that replacing EGF with EVs restores KI67+ proliferating cell populations and at the same time decreases active Caspase3+ apoptotic populations. *Lgr5+* cell numbers are restored, too, and the EGF-receptor is phosphorylated, suggesting that some of the EGF-family members are present with correct orientation and activity on the surface of EVs. This hypothesis was confirmed by using the EGF receptor inhibitor Gefitinib. When

intestinal organoids were treated with Gefitinib, neither recombinant AREG nor EVs were able to restore living organoid ratios. These results suggest that the EGF replacing effect is mediated primarily through the stem cells. To our knowledge, it has not been previously reported that EVs can have such a significant impact on the normal intestinal stem cell niche, and because of that our focus was set on EGF-family members. Importantly, EGF-family members were expressed primarily by stromal fibroblast cells and not by the organoids, containing epithelial cells. By using specific antibodies against different EGF-family members, we proved that AREG is present in a significant amount on EVs. The presence of AREG on EVs was further validated by Simple Western method. When fibroblast-derived EVs were incubated with neutralising anti-AREG antibody, EVs lost the EGF-replacing ability and organoid death ratios could not be restored to control levels anymore. All of these observations lead to the conclusions that HCF derived-EVs carry a significant amount of AREG on the surface with the correct orientation, and that this EV bound AREG is able to replace recombinant EGF for intestinal organoid cultures.

After the previous results we focused on whether the transmission of AREG can be utilised by colorectal cancer (CRC) cells as well. To answer this question, we used patient derived organoids and cancer associated fibroblasts. Our CRC organoids were sensitive for the removal of recombinant EGF from the culturing media, decreasing KI67+ cell numbers, and a marked reduction of their size was also observed. This points out that several CRC tumours rely on extra-tumoral EGF secretion, which can drive the growth of the tumour. *KRAS*, the most

frequently mutated EGF-signalling pathway component has a mutation in around 50% of CRC tumours [103].

The role of the EGF-Erk pathway in CRC progression is well documented, and the *KRAS* gene is one of the most frequently mutated components. These findings suggest that our CRC organoid lines rely on external sources of EGF-receptor ligands. In CMS4 type, which is accompanied by lower *KRAS* and *BRAF* mutation frequencies, the tumour tissue is characterised by a high number of CAFs and strong TGFβ signalling. Based on these findings, we replaced recombinant EGF with HCF- and CAF-derived EVs, and in some of our experiments, the fibroblast cultures were pre-treated with TGFβ.

Based on our data, both HCF- and CAF-derived EVs should be taken into consideration when specific therapies are developed against CMS4 type CRC. This subtype is particularly difficult to treat, with the worst 5-year overall survival (62%) and relapse-free survival (60%) of the four subtypes. CMS4 subtype mRNA expression profile is characterised by a high expression of genes associated with mesenchymal, activated EMT and activated TGFβ pathway phenotypes, with the highest stromal infiltration properties as well.

Both HCF and CAF-derived EVs were able to replace recombinant EGF with KI67+ cell numbers restored to the control levels. AREG was detectable on the surface of CAF- derived EVs as well according to our latex-bead based flow cytometric assay results. The importance of AREG in colorectal cancer has already been proven; AREG can be used as a prognostic factor in colorectal cancer [104]. In several experiments, inhibiting EGF-receptor or AREG resulted in a massive decrease of the number of proliferating cells. The limitation of our studies

was the small number of patient-derived organoids we could use.

Pre-treating either HCF or CAF cultures with TGFβ did not modify the EGF replacing effect of the EVs. These fibroblasts could have been already activated, and TGFβ only further activated them. However, this activation did not lead to an increased EV production. All these results suggest that stroma cell-derived EVs have a major role in maintaining intestinal stem cell homeostasis. Furthermore, they are important contributors to CRC progression via transmitting AREG for CRC cells at an early stage of the tumorigenesis when the *KRAS* or *BRAF* genes are not yet mutated.

5. Conclusions

Major conclusions of our studies are as follows:

1. HCF or MIF derived EVs do not alter the proliferation of normal intestinal organoids, when all the niche factors are present.

2. HCF-derived EVs can replace Wnt proteins in the intestinal stem cell niche.

3. HCF-derived EVs can replace EGF in the intestinal stem cell niche through the EGF-receptor.

4. HCF-derived EVs have a direct impact on Lgr5+ stem cells.

5. The EGF-replacing effect is mediated through the EGF-receptor.

6. HCF-derived EVs carry the EGF-receptor ligand AREG on their surface.

7. HCF-derived EVs have no effect when EGF is dispensable.

8. HCF- and CAF-derived EVs support the growth of EGF-dependent CRC organoids.

9. TGFβ does not modify the effects of EVs either in EGF-dependent or in EGF-independent 3D models.

10. CAF-derived EVs can replace AREG in the tumour microenvironment.

6. Bibliography of the candidate's publications

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*Shared first autorship

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