THE ROLE OF EXTRACELULAR VESICLES IN THE NORMAL INTESTINE AND INTESTINAL TUMORIGENESIS

PhD thesis

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Contents

List of Abbreviation

AREG = amphiregulin

CAF = cancer associated fibroblast

CRC = colorectal carcinoma

EGF = epidermal growth factor

EV = extracellular vesicle

HCF= human colon fibroblast

MIF = mouse intestinal fibroblast

NCF = normal colon fibroblast

SCN = stem cell niche

SI = small intestine

1. Introduction

1.1. Histology of the small and large intestine

The small intestinal epithelium is organised into a huge number of crypt-villus units. Villi are pocket-like protrusions of the intestinal wall that sticks out of the epithelium into the gut lumen, helping to maximise the absorptive surface. A villus is covered by a single cell epithelial layer, composed of postmitotic non-dividing cells. Crypts, better known as Lieberkühn-crypts are pocket-like out buddings, covered by a single-cell layer as well. However, many cells in a crypt are actively dividing, ensuring the continuous replenishment of the intestinal epithelium. Under the epithelial layer, the stromal parts of the intestine can be found, comprised of lymphocytes, fibroblasts and capillaries embedded into extracellular matrix proteins. Villi are present only in the small, but not in the large intestine [1]. The whole gut epithelium is constantly renewed in every 3-5 days [2]. This requires a high number of new cells that is provided by the constantly dividing stem cells, residing at the bottom of the crypts.

1.2. Cell types of the small and large intestine

Many different cell types with distinctive functions build up the epithelial layer of the intestine (Figure 1):

1) Enterocytes, the most numerous cell type have a primary role in absorption. They express many catabolic enzymes, which are secreted into the gut lumen [3].
2) Enteroendocrine cells, secreting gastrointestinal hormones, such as secretin [4].
3) Goblet cells, producing mucus [3].
4) Tuft cells, the IL-25 producing chemosensory cells which have a function in the immune response [5].
5) Transit amplifying cells, a rapidly dividing cell population, ensuring the progenitor cells for the terminally differentiated cells.
6) The +4 cells, termed according to their position in the intestinal crypts counted from the crypt bottom.
7) Paneth cells with an important role in maintaining the stem cell niche, participating in the antimicrobial defence as well [6,7].

8) Stem cells at the bottom of the crypt, also known as Cycling Crypt Base Cells (CBC cells).

All the above-mentioned cell types are daughter cells of stem cells.

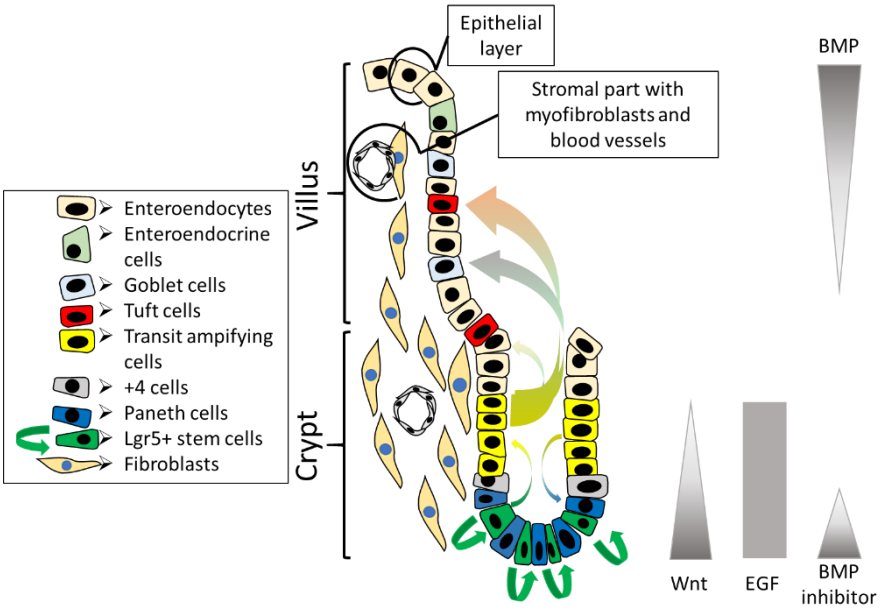

Figure 1 Cell types and niche factor gradients along the intestinal crypt-villus unit

1.3. Epithelial cells are produced by LGR5+ CBC stem cells located at the crypt base

Many candidate cell populations have been considered for stem cell functions such as the +4 cells or cells of the transit amplifying zone.

The +4 cell designation reflect the relative position of these cells from the bottom of the crypt. They are at a quiescent, G0, non-dividing state. These cells are also known as label retaining cells or LRCs, since they retain BrdU-labelling, which also confirms their quiescent state [8]. It is still difficult to define the exact phenotype of the +4 cells, since all of their previously accepted markers were shown to be expressed in LGR5-high CBC cells as well [9]. The +4 cells have a dual role: while they are secretory progenitors (precursors for Paneth cells and enteroendocrine lineages) [10], they can also revert to a stem cell phenotype upon tissue injury or selective ablation of LGR5+ CBC cells [11] .

Transit amplifying cells are undifferentiated progenitors between the states of terminally differentiated phenotype and stem cells. These cells act as an intermediate station for the stem cell-terminal cell pathway. They proliferate rapidly: they divide every 12-16 hours

completing around five divisions while they migrate towards the tip of the villus [12]. Their daughter cells can be found in the villi within 24 hours and after 48 hours they are shed to the gut lumen [13]. After they reach the crypt-villus border, they rapidly differentiate into one of the intestinal epithelial cell types, such enterocytes or tuft cells.

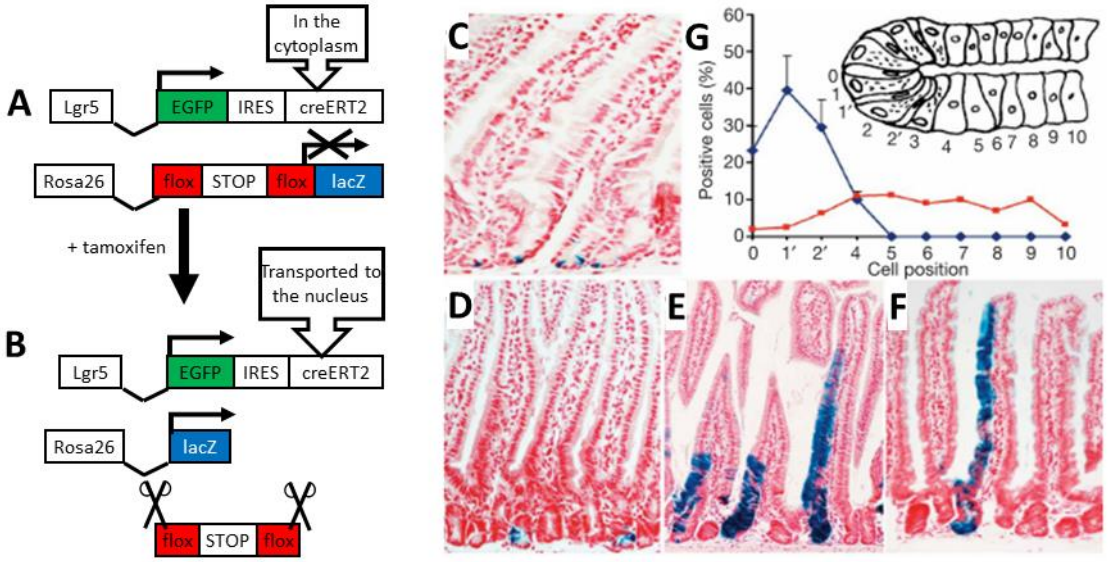

Figure 2 The transgenic mouse model used by Barker et al [14] and modified by the author
A) The transgenic mouse expresses EGFP and creERT2 proteins under the Lgr5 promoter. The CreERT protein is localised to the cytoplasm and it has no access to the floxed transcriptional stop cassette. Thus, lacZ expression is blocked
B) Tamoxifen treatment translocates the CreERT protein to the nucleus. The cre enzyme cuts out the stop cassette irreversibly and lacZ starts to be expressed. Since the Rosa26 is a constitutive promoter (i.e.: "always on"), all and only the daughter cells will express the lacZ reporter gene.
D-F) Histological analysis of lacZ activity in small intestine 1 day (C) 5 days (D) and 60 days (D) after the induction of CreERT with tamoxifen
C) Intestinal section from an Lgr5-EGFP-IRES-creERT2/ Rosa26-lacZ mouse 12h after tamoxifen injection.
G) Blue cell frequency at specific positions relative to the crypt bottom (blue line) compared to long-term DNA-label-retaining cells obtained in adult mice after irradiation (red line).

The "every-day" stem cells are the CBC cells with the specific expression of the gene *LGR5*, demonstrated by Barker N and his co-workers [14]. Figure 2A shows that after crossing the *Lgr5*$^{EGFP\text{-}IRES\text{-}CreERT2}$ mouse strain with a *R26R-lacZ* reporter strain, they found that all cells along the intestinal epithelial layer are the descendants from the LGR5+ crypt base columnar cells. By the *Lgr5*-restricted expression of the inducible

Cre^{ERT} enzyme, this could be selectively activated only in *Lgr5* expressing cells. The Cre enzyme was translocated to the nucleus after tamoxifen injection, where it cut out the floxed transcriptional stop cassette, thus, resulting in the transcription of the LacZ reporter gene driven by the constitutive promoter *Rosa26*. Since this genetic change is irreversible, thus, all daughter cells of the proliferating *Lgr5*+ cells continued lacZ production, regardless of cell type. In case the genetic change occurs in a cell with stem cell features, lacZ is present in differentiated daughter cells and newly produced stem cells as well, thus, creating a ribbon of genetically labelled cells. This so-called lineage tracing experiment was an elegant proof to terminate the long-lasting debate about the identity of stem cells, proving that Lgr5 is indeed a stem cell marker in the murine intestine. A major problem with LGR5 protein as a marker for human intestinal stem cells is that mRNA and protein levels are too low to use them as a robust marker. For these reasons, Laurens et al proposed to use *OLFM4* as a marker for LGR5+ stem cells [15].

However, the discovery of the Lgr5+ stem cells still left the question open whether the stem cell fate is a hard-wired or determined by the local microenvironment. The stem cell phenotype is modified by a special microenvironment, the stem cell niche (SCN). The SCN is formed by the Paneth cells and the stromal cells by expressing surface proteins and producing soluble factors [16]. Paneth cells are only present in the small intestine, they are absent in the large intestine, where only so-called Paneth-like cells can be found [6]. The stem cell phenotype is critically influenced by the SCN: progenitors and other differentiated cell types are able to replenish the selectively ablated LGR5+ cells [11] if they get inside of the SCN. The ability to reverse to a stem cell phenotype is quite likely performed by the progenitors contacting Paneth cells, which possess a high amount of membrane bound Wnt proteins, a critical ligand family for stem cells [17]. If a cell is released from the stem cell niche, it loses the stem cell phenotype with the ability to regain it under the influence of the stem cell niche factors [18].

All these findings conclude that i) the stem cell phenotype is maintained and restricted by the stem cell niche, ii) these stem cells express the stem cell marker LGR5 and iii) other, more differentiated cells can revert to the stem cell state under specific conditions.

1.4. Key factors of the intestinal stem cell niche

Table 1. Stem cell niche factors in the intestinal stem cell niche

The most important secreted niche factors in the intestinal stem cell niche	
Protein	**Function**
Wnt-proteins	**Maintaining stem cell phenotype** [19]
R-Spondins	**Wnt-agonist amplifying the Wnt signalling intensity and duration** [20]
EGF-receptor ligands such as EGF	**Transmitting a mitogenic signal** [21]
BMP-antagonists such as noggin	**Inhibiting differentiation** [22]

The key factors participating in the intestinal stem cell niche are summarized in Table 1. These factors are the most important ones, which all contribute to forming the intestinal SCN through different ways. Their exact role and the signalling pathways they influence is detailed below one-by-one.

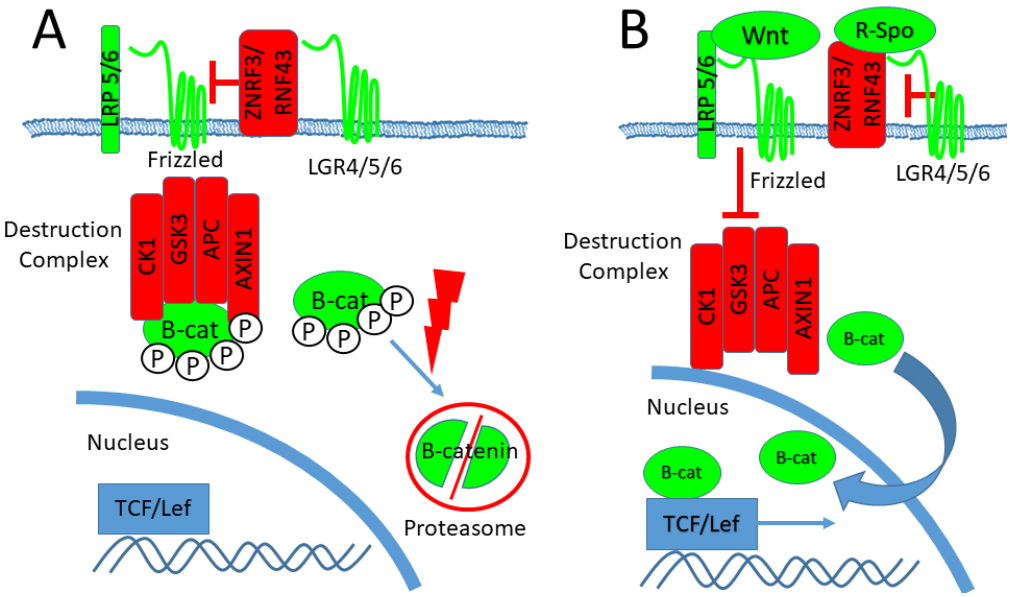

Figure 3 The canonical Wnt-β-catenin pathway
A) If Wnt proteins are not present, the intracellular destruction complex is active, and phosphorylates cytoplasmic β-catenin through several steps. This process marks β-catenin for proteasomal degradation. Wnt target gene expression is not initiated.
B) Extracellular Wnt proteins bind to the Frizzled-Lrp receptor complex, which in turn inactivates the destruction complex, β-catenin is not degraded, and can be translocated to the nucleus where it acts as a transcriptional co-factor, initialising Wnt target gene expression. R-Spondins help the Wnt-signalling to be strong and sustained, by inhibiting Znrf3/Rnf43 surface proteins. In the absence of R-Spondins, Znrf3/Rnf43 proteins quickly inhibit the Wnt-Frizzled-Lrp complex, and Wnt signalling will be only short lived and transient.

The canonical Wnt-pathway (Figure 3) is one of the most important signalling pathways in the intestine. The products of Wnt target genes promote a stem cell phenotype and cell proliferation. Lack of extracellular Wnt-proteins inactivates the pathway. The intracellular β-catenin destruction complex is a major component of the Wnt-pathway. The destruction complex, containing GSK3β, CK1, AXIN2 and APC proteins marks cytoplasmic β-catenin for proteasomal degradation, thus preventing it from entering the nucleus. Extracellular Wnt-proteins can inactivate indirectly the destruction complex by binding to the surface-bound Wnt-receptor Frizzled-proteins. This effect is quite weak and short-term since surface bound ZNRF3 and RNF43 proteins render the Wnt-Frizzled complex inactive almost immediately. R-Spondins help to maintain a long and sustained Wnt-signal by inhibiting ZNRF3 and RNF43 proteins [23]. Because the destruction complex is inactivated, cytoplasmic β-catenin levels can elevate, and β-catenin can be

transported to the nucleus where it promotes Wnt-specific target gene expression by acting as a cofactor after binding to TCF/LEF transcription factors [24]. Such Wnt target genes are for example *LGR5*[14], *C-MYC* [25], or *ASCL2* [26]. If Wnt is absent, the destruction complex remains active and phosphorylates cytoplasmic β-catenin on multiple sites, marking β-catenin for further ubiquitination and proteasomal degradation. When β-catenin levels are reduced, and Wnt-target gene expression is turned off. One of the central components of the destruction complex is the APC protein. If APC is mutant, a key phosphorylation step cannot be performed β-catenin is marked for degradation, and the Wnt-pathway will be constitutively, ligand independently activated, transmitting constant signal which promote stem cell phenotype and cell proliferation. Indeed, the initiation step for the vast majority of colorectal cancers is the loss-of-function mutation of both alleles of the *APC* gene [27].

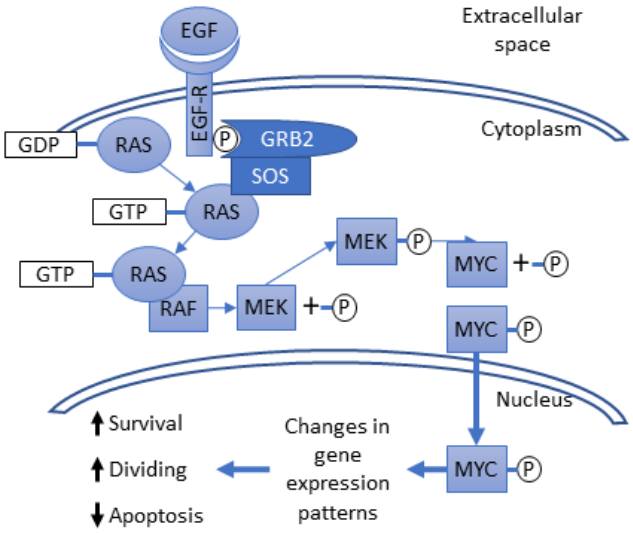

Figure 4 The mitogenic EGF-Erk axis signalling pathway.
Extracellular EGF proteins activate the EGF-receptors. This initiates a cascade of intracellular phosphorylation steps, which results in gene expression changes. The EGF-Erk signalling axis' main function is to increase surviving and division while simultaneously decreasing apoptosis. RAS and RAF proteins are often mutated in different tumour types, resulting in a constantly activated signalling, which leads to uncontrolled proliferation.

The EGF-ERK axis (Figure 4) transmits mitogenic and anti-apoptotic signals to the nucleus. After the extracellular EGF-family proteins bind to their receptors, gene expression patterns change, enhancing cell survival, division and inhibition of apoptosis. An excellent example for the gene activating transcription factors is MYC. Components

of the EGF-signalling pathways, such as KRAS, often undergo gain-of-function mutations during colorectal cancer tumorigenesis, resulting in a constantly activated mitogenic signal [28].

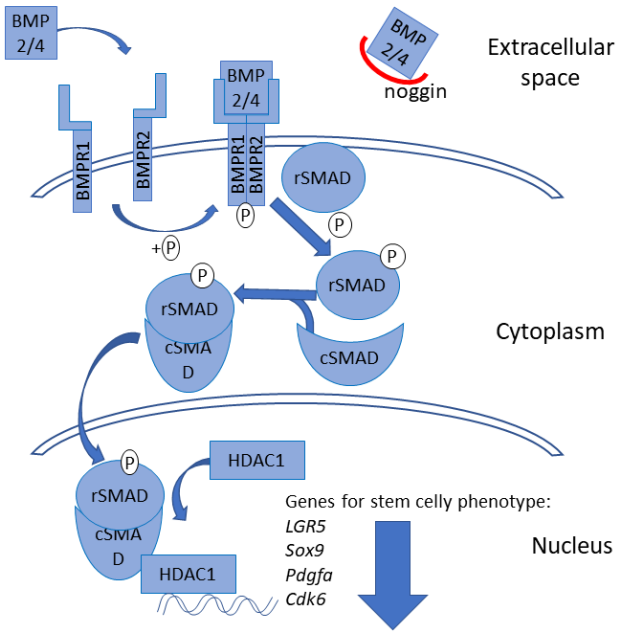

Figure 5 BMP signalling helps differentiation.
After extracellular BMP proteins activates their respective receptors, the receptors mobilise members of the SMAD protein family, which form complexes. These SMAD complexes then can be translocated to the nucleus, where they decrease stem cell promoting factor expression, and by that help cell differentiation.

One of the main functions of the BMP pathway (Figure 5) is to stimulate the differentiation of progenitor cells into their terminally differentiated cell types. After the ligand bind to its receptor, a series of phosphorylation steps recruit the r-SMAD/c-SMAD complex to the nucleus, where it regulates gene expression. The significance of this pathway is well represented by a series of experiments, when the *Bmpr1a* receptor was knocked out in a mouse model. These studies showed the expansion of the stem cell and transit amplifying cell populations, leading to the formation of polyps [29]. BMP-signalling not only helps the differentiation processes, but it restricts the stem cell phenotype to crypt bottom as well. This is achieved by recruiting HDAC1 proteins and epigenetically silencing *Lgr5*, *Sox9*, *Pdgfa*, *Cdk6* and *Cdca7* [22]. In the crypt bottom, however, BMP-signalling is undesirable, thus, BMP-antagonists, such as gremlin 1/2 or noggin, help to neutralize BMP-signalling. These BMP-antagonists are primarily secreted by myofibroblast underlying the crypt epithelium [30].

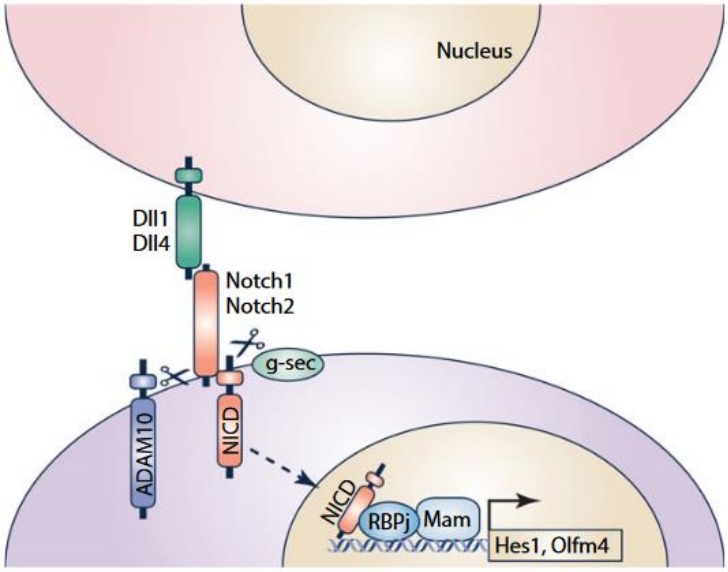

Figure 6 Notch signalling mechanism in the intestine [31]
Notch signalling involves the engagement of two cells. After the receptor-ligand interaction, Notch-receptor is cleaved, and the Notch intracellular domain (NCID) is translocated to the nucleus, where it acts a transcriptional co-factor, and activates Notch target genes.

The Notch pathway's (Figure 6) primary role is to help stem cells not to differentiate, and to maintain a stem cell phenotype. It is one of the pathways that relies mostly on juxtracrine contacts [32]. If a cell with Notch-receptor gets into contact with a Notch-ligand carrying cells, the intracellular part of the Notch-receptor (NICD) is released by proteolysis, transported to the nucleus where it acts as a transcriptional cofactor [33,34]. If Notch signalling is blocked in rodents, an expansion of goblet and enteroendocrine cells can be observed [35,36]. In mouse intestinal adenoma models with Apc mutation ($Apc^{min/+}$), overexpression of the NCID had many additional tumours and a worse overall survival compared to Apc^{min} only mice [37].

1.5. Formation and classification of colorectal cancer types

Colorectal cancer is a complex, heterogeneous disease group. Sporadic or inherited mutations result in aberrant cell divisions and the formation of polyps that slowly progress to the malignant carcinoma stage and ultimately to metastases.

About 80-90% of colorectal cancer cases are formed through the classical adenoma-carcinoma sequence. After the formation of a polyp or adenoma (Figure 7), progression to invasive carcinomas occurs slowly within 10-15 years. Transformed stem cells are formed after mutation of both *APC* alleles that results in constant activation of the Wnt pathway, leading to unrestricted cell division. After this initiating mutation, other typical genes affected by mutations are *KRAS* or *BRAF*s (both are essential activating components of the EGF pathway), or genes responsible for maintaining epithelial traits which results in the induction of epithelial-mesenchymal transition. The loss of the genome guarding *TP53* results in chromosome instability, the genome will be severely damaged, and the tumour produces metastases throughout the body. The sequential mutations in these and other genes was first suggested by Vogelstein and Fearon [38]. With an elegant experiment Barker N. et al. proved that the Lgr5+ stem cells are the cell-of-origin for long-term intestinal tumorigenesis [39]. This proves the validity of the bottom-up-model, hypothesising that the tumour spreads from the crypts to the intercryptic region.

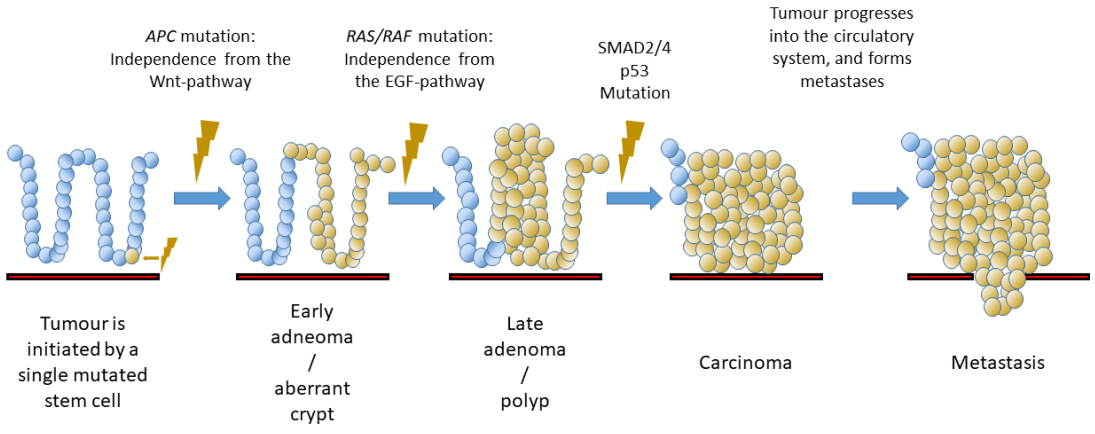

Figure 7 Schematic pathway of classical colorectal cancer carcinogenesis model CRC is originated from a single cell with APC mutation. This hyperproliferative cell then develops into a polyp, which progresses to the adenoma state. After gaining other tumorigenic mutations, the adenoma slowly develops into a carcinoma, which then form metastases in other organs.

Inflammatory Bowel Disease (IBD) is a major risk factor for CRC by promoting a constant inflammation in the bowel. Tumours caused by this inflammation share many similarities with the adenoma-carcinoma pathway (Figure 8). Common genetic alternations are the constant activation of the Wnt-β-catenin pathway, mutated *KRAS* and *TP53* genes and involvement of the TGF-β pathway and mismatch repair genes. However, the timing of the mutations is different between the two pathway [40]. Importantly, some colorectal cancer samples show a serious inflammatory infiltration, without any prior IBD.

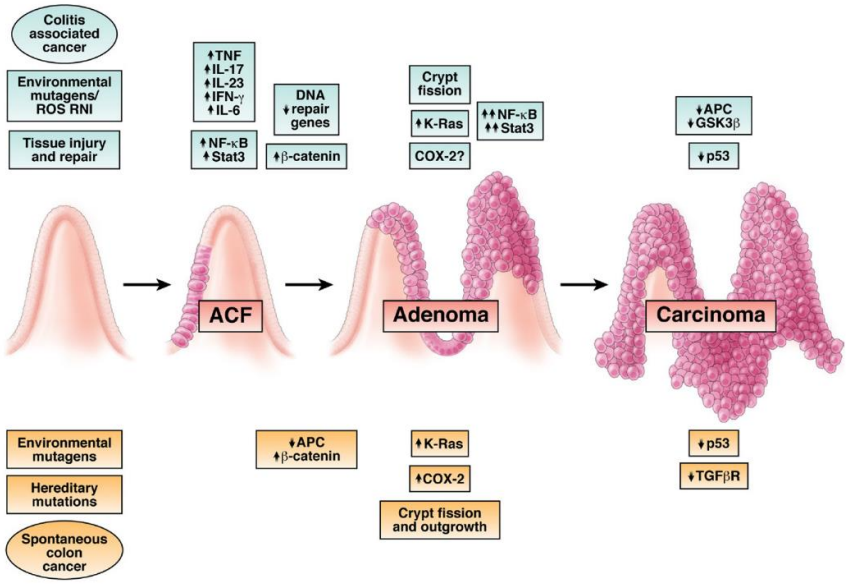

Figure 8 Mechanisms of colitis associated cancer and classical colorectal cancer development [41]
During colitis associated cancer (CAC, upper panels) production of proinflammatory cytokines is induced, which can lead to the mutation of proto-oncogenes and tumour-suppressor genes. This can lead to genomic instability through several possible ways. Sustained inflammation facilitates tumour progression by activating the proliferation and antiapoptotic properties of pre-malignant cells. CAC has several common features with CRC.

In these cases, the tumour is initiated by a local chronic inflammation. Activated inflammatory cells produce reactive oxygen species and nitrogen intermediers that can damage the DNA and induce mutations [42,43]. The inflammation is capable of creating a pre-malignant state by the aberrant crypt foci (ACF), and inhibiting β-catenin degradation even when both *APC* alleles are wild-type: for example, TNF-α secreted by activated macrophages is able to elevate cytoplasmic β-catenin levels [44].

The major mediator for these processes is NF-κB. Canonical NF-κB pathway can be activated by many stimuli, such as by TNF-α, IL-1β or by different pathogen associated molecular patterns [45]. After these ligands bind to their respective receptors, NF-κB is released from an inhibitory complex and it is transported to the nucleus as a homodimer to initiate gene expression pattern changes [46].

This process does not need to follow the bottom-up model, since tumours can be formed by terminally differentiated cells through this pathway. Schwitalla and colleagues proved that mutated *APC* gene or elevated β-catenin levels are not enough to induce a stem cell phenotype outside of the stem cell niche. However, when one of them is combined with the activated NF-κB pathway, it can lead to an extreme activation of the β-catenin pathway [47]. This model is the top-down hypothesis (Figure 9).

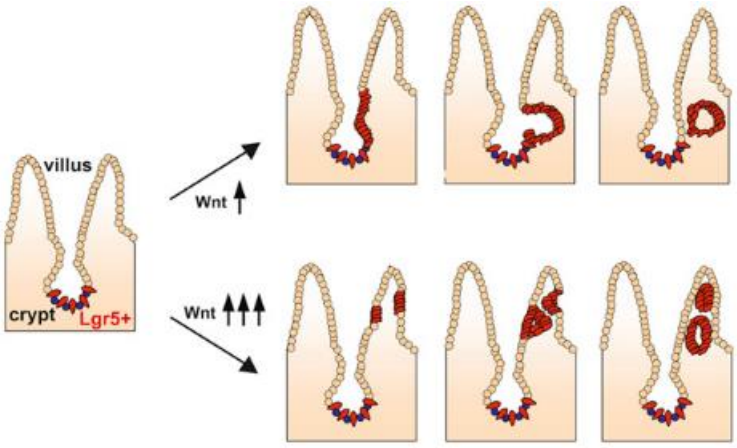

Figure 9 Comparison of bottom-up and top-down models of colorectal cancer formation [47]
For modest Wnt-activation (by loss of APC) the mutation needs to occur in an Lgr5+ cell, otherwise the cell is shed off. The mutated Lgr5+ cell is invaginated and invade the sub-epithelium, representing the bottom-up model. Top-down model suggests that Lgr5- cells can reacquire Lgr5 expression if β-catenin signalling is enforced, and by that these dedifferentiated cells can have the same properties as a crypt stem cell.

The third pathway for colorectal cancer tumorigenesis is the serrated neoplasia pathway described by Jass and Smith [48]. The most important features include mutated *BRAF* or *KRAS* genes, a high degree of epigenetic instability and the hypermethylated state of the CpG islands in tumour suppressor gene promoters [49]. Because of the hypermethylated CpG islands, this phenotype is also known as the CpG island methylation phenotype (CIMP). Hypermethylation can affect for example the mismatch repair gene *MLH1*, *MSH2*. After the epigenetic silencing of these genes, a high degree of sporadic

chromosomal instability can be observed [50]. Mismatch gene silencing results in microsatellite instability (MSI) as well [51]. The hereditary disease Lynch-syndrome, leading to a predisposition to colorectal cancer, is also caused by the silenced mismatch repair genes [52]. Colorectal cancer can develop from terminally differentiated cells through the serrated neoplasia pathway as well. The aberrantly elevated expression of the BMP-antagonist gene *GREM1* is able to disturb the intestinal differentiation processes, to force terminally differentiated cells to acquire stem cell phenotype and these cells are then able to proliferate again independently of the SCN [53].

1.6. The consensus molecular subtypes (CMSs) of colorectal cancer

Since CRC is an extremely heterogeneous disease, creating a comprehensive categorization method can help to choose the correct treatment for patients as early as possible. In 2015, several groups contributed to develop the four consensus molecular subtypes (CMS1-4) for colorectal cancer [54].

CMS1: approximately 14% of all CRCs are considered to be of the CMS1 subtype. The precursor lesion is a serrated polyp type. Typically, the oncogenic *BRAF* V600E mutation and CIMP are present, DNA mismatch repair genes are usually inactivated and the tumour microenvironment is characterised by lymphocytic infiltration. Since the CIMP phenotype causes the silencing of many mismatch repair genes, MSI occurs in this subtype: an average of 47 mutation per 1 million base pairs was detected as compared to microsatellite stable (MSS) CRC with 2.8 mutation per 1 million base pairs. CMS1 tumours are generally well curable if they are detected at an early stage prior dissemination. The reasons for this are the high number of infiltrating CD8+ anti-tumour cytotoxic T-cells and the low amount of tumour supporting cancer-associated fibroblasts (CAFs). The average five-year survival rate for CMS1 is about 73%.

About 39% of CRC cases falls into the **CMS2** group. This type is equivalent to the adenoma-carcinoma pathway. Chromosomal instability results in aneuploidy, a high number of indel mutations and loss/gain of chromosomal fragments. Since the mutation rate is relatively low, it is reasonable to think that those few mutations affect important genes. Usually these patients are diagnosed at stage III and their five-year survival rate is 77%.

CMS3 type is also known as the metabolic subtype and about 13% of all colorectal cancer cases falls into this category. Chromosomal instability and low copy number alternations are the main features with a moderate level of MSI. Around 30% of CMS3 cases are hypermutated, with 68% *KRAS* mutation, the highest among all colorectal cancer cases. Analysis of mRNA expression patterns revealed that 9 out of the analysed 10 metabolic pathways are dysregulated. *KRAS* mutations render all anti-EGF-receptor monoclonal antibody treatments ineffective, making it difficult to cure these patients. In addition, there are no characteristic, easily targeted mutations. The five-year survival of these patients is 75%.

CMS4 like CMS1 develops from a serrated neoplasia. The level of TGFβ decides whether a serrated neoplasia will develop into either the CMS1 or CMS4 phenotypes. Higher TGFβ tends to shift tumour development toward CMS4. In contrast to CMS1, CMS4 tumours are MSS with low levels of methylation. The tumour microenvironment is inflammatory, with an excessive amount of tumour supporting CAFs, high activity of the TGFβ pathway, intensified vascularisation and epithelial-mesenchymal transition. This tumour type is usually diagnosed at a late stage with the worst prognosis. The five year survival rate is only 62% [52,55,56].

1.7. Research models of intestinal stem cells and colorectal cancer

Initially, for modelling intestinal cancer diseases and stem cells, conventional two-dimensional cell cultures were used. Two options are available for these kind of model systems: normal, non-transformed tissue samples, and samples isolated from tumour tissues. Since these cells are adherent by their nature, culturing them is only possible on extracellular matrix protein- (such as collagen or laminin) treated adherent tissue culture plastic surfaces. The main problems with conventional cell cultures include the lack of cellular heterogeneity and the lack of three-dimensional tissue structure. As briefly described in this literature overview, tumour tissues are composed of many different cell types, and their ability to grow in three dimension is important considering the increased number of cell-cell contacts they can form, and by that increasing intercellular communication.

In contrast to 2D cell cultures, a novel and currently state-of-art 3D organoid system contains all cell types of epithelial origin from the original tissue, and by using laminin-

rich Matrigel as a three dimensional scaffold for modelling the extracellular matrix (ECM), they have the ability and possibility to grow in all directions. It is possible to add different ECM proteins to the Matrigel, further improving the model. These organoid cultures are genetically stable and they can be genetically modified representing their other major advantages for research[57,58].

1.8. Intestinal stroma associated fibroblasts

Fibroblasts reside under the epithelial cell layer and are major components of the stroma. Contrary to epithelial cells, fibroblast are not attached to a basement membrane, but they are embedded in the extracellular matrix [59]. Fibroblasts are heterogenous cells, and most of them are of mesenchymal origin [59].

Previously it was thought that after activation by different stimuli such as wound or inflammation, resting or quiescent normal fibroblasts (NFs) start to express the marker αSMA, they undergo morphological changes, and they start to secrete large amounts of growth factors (Figure 10). They help wound healing by inducing epithelial stem cells divide more rapidly [60]. The expression of αSMA is considered a trait of cancer associated fibroblasts (CAFs) as well while normal, resting or quiescent fibroblasts are inactive and αSMA negative. Therefore, it was expected that αSMA positive activated CAFs could be distinguished from αSMA negative inactive normal colon fibroblasts (NCFs). However, this concept has been challenged by a recent publication where the authors found that NCF-CAF pairs often had the same αSMA expression levels [61]. Nevertheless, it is generally accepted that αSMA positive fibroblasts are activated. αSMA negative fibroblasts are difficult to culture, since the cell isolation procedure tends to activate them, and they sense the wound event such as the rupture of the continuous epithelial tissue.

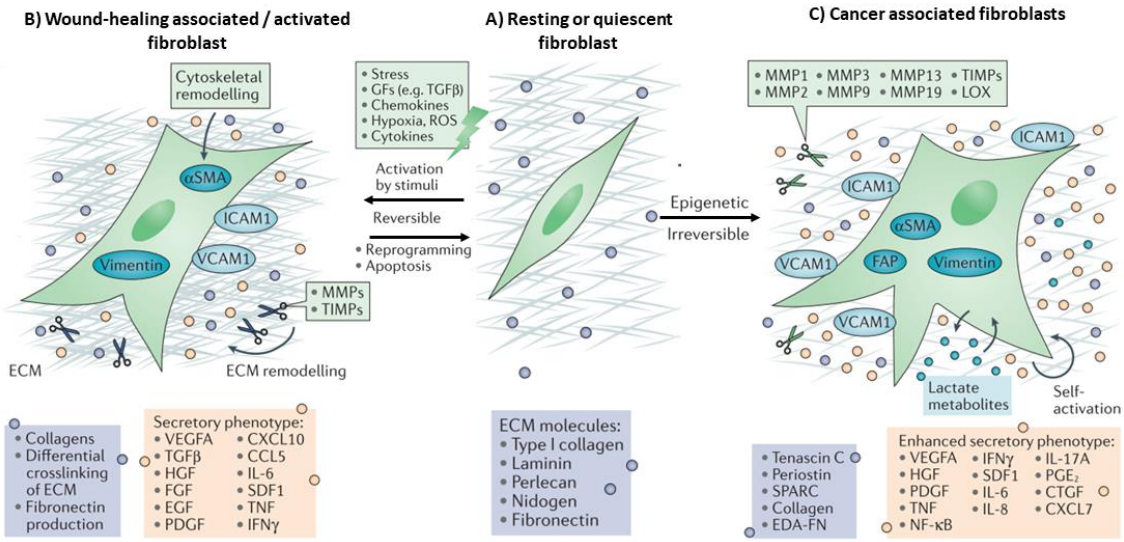

Figure 10 Properties and changes of secretome of differently activated fibroblasts. Quiescent fibroblast (A) have a spindle like morphology and considered αSMA negative. Activation (B), such as a wound will change the morphology to a stellate one, and the fibroblasts start to secrete growth factors and chemokines and remodel the extracellular matrix as well. This activation is reversible. Fibroblasts along the cancer tissues (C) can be activated irreversibly through epigenetic changes. Their secretory and ECM remodelling capabilities are even more enhanced. These properties together will help and support tumour growth. Kalluri 2016, modified [62]

Intestine associated fibroblasts contribute to the formation of the SCN by secreting factors and by that they limit stem cells to the crypt base. Such secreted factors include the EGF-family proteins [60,63,64] and the Wnt-family members WNT2B or WNT4 [17]. Intestinal myofibroblasts have been described to promote wound healing via expressing AREG, a member of the EGF-family proteins. AREG is expressed primarily by myofibroblasts in the intestine [65]. All these findings suggest that intestinal fibroblasts have an important role in the homeostasis of the normal intestinal epithelium.

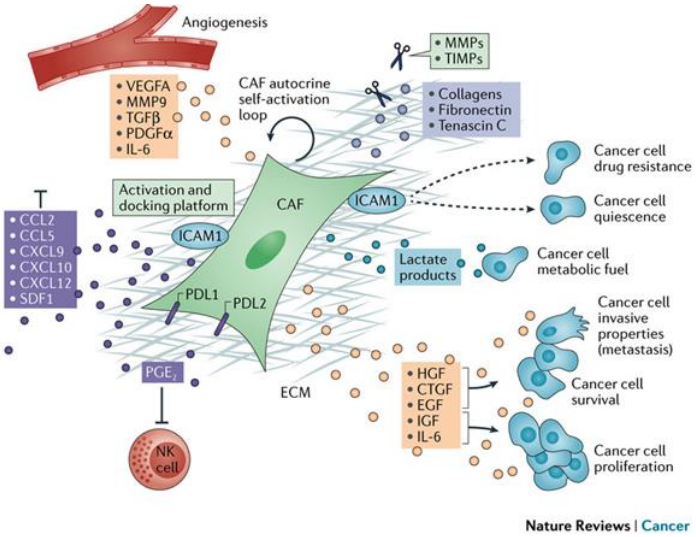

Figure 11 Role of fibroblasts in cancer. Cancer associated fibroblasts support tumour growth through numerous ways: producing growth factors, inflammatory cytokines, remodelling the extracellular matrix, complementing the tumour cells' metabolic pathways, and enhancing drug resistance. (Kalluri 2016 modified [62])

Fibroblasts are major contributors of the colorectal cancer progression as well (Figure 11). CMS4, characterised by a high number of tumour supporting fibroblasts, has the worst five-year survival rate among the four CMS subtypes. In addition to secreting growth factors, cancer associated fibroblasts can support tumour growth through numerous ways. For example, CAFs are able to express metabolic pathway-related proteins which complement the tumour's metabolic pathways, thus, removing toxic metabolites and supporting tumour growth [66]. Patients with high expression of stroma-associated genes have an overall worse prognosis [67].

1.9. Extracellular vesicles and their role in cancer progression

Extracellular vehicles (EVs) are lipid-bilayer surrounded structures, secreted by virtually all cell types through evolutionary conserved ways [68]. EVs can carry vastly diverse cargos, such as proteins, lipids, and nucleic acids (Figure 12). The first encounter of EVs was described in 1946 by Chargaff and West. During their experiments they have found that platelet-free plasma exhibited clotting properties. This ability was significantly reduced after a high-speed centrifugation step. They concluded that human plasma has a coagulation component, which can be sedimented by a high-speed centrifugation step, and appears as a small sediment at the bottom of the centrifuge tube [69]. After this discovery, the sediment was further analysed an EVs were described by others [70,71].

EVs are considered as important intercellular communication tools and with their better understanding, they could be potentially used as novel biomarkers or even therapeutic tools. The former is an attractive concept, considering that EVs are present in every body fluid types [72], thus, providing an easily available, non-invasive method for sample collection. EVs as intercellular messengers have vastly diverse functions. EVs can carry mRNAs that can be translated into proteins in the target cells [73]. EVs can also transport proteins in their active form [74].

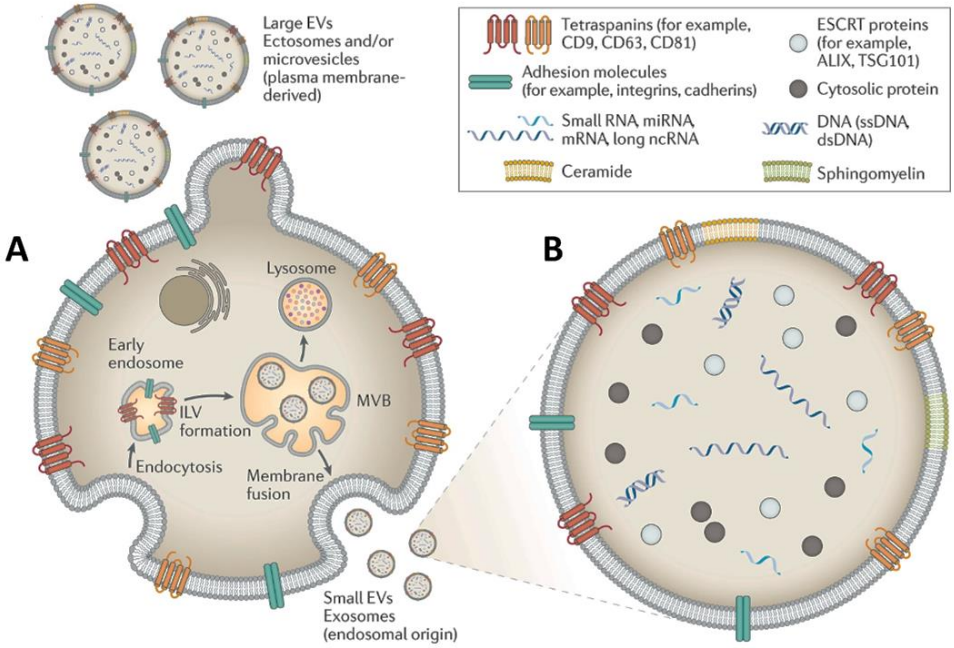

Figure 12 Extracellular vesicle cargo types and formation methods [75].
EVs can be formed by shedding or by the exocytosis of multivesicular bodies (A). EVs can have a diverse cargo, such as different nucleic acids, lipids and proteins (B).

EVs can be classified based on their size, biogenesis or their cargo type etc. Previously three main EV populations were distinguished that could be separated from each other by using gradient differential centrifugation steps:

1. exosomes, < 200nm in diameter, formed by the exocytosis of multivesicular bodies
2. microvesicles, 200-1000nm in diameter, formed by shedding from the cell membrane
3. apoptotic bodies, >1000nm in diameter, formed by blebbing during apoptosis

Since no established molecular markers are available for the different EV subpopulations, recently, the International Society for Extracellular Vesicles (ISEV) suggested an operational terminology based on EV size which I will use in my thesis [76]:

1. small EVs, sEVs: diameter <200nm, isolated by 100,000g ultracentrifugation
2. middle sized EVs, mEVs: diameter ~200-800nm, pelleted by 12,500g centrifugation
3. large EVs, lEVs: 800nm<diameter, pelleted by 2000g centrifugation

According to our results [77], Matrigel with organoids allows the sEV population to be released into the supernatant. sEVs are isolated by 100,000g ultracentrifugation, the average EV diameter is ~200nm and EVs of MVB origin are primarily enriched in this fraction. They can be characterised by a high positivity for the CD81/CD63 markers. However, these markers can be present in other EV subpopulations as well [78].

The role of EVs, such as fibroblast-derived ones in normal homeostasis is a yet unexplored field. However, research on EVs in cancer is a rapidly growing and evolving field of biology. It is getting recognised that EVs are a major part of tumour secretome, and they can have an impact on tumour formation. They are able to contribute to the pre-metastatic niche formation by carrying an organ-specific integrin pattern on their surface, ultimately leading to tissue-specific metastases [79]. Hypoxic lung cancer cells can initiate vascularisation through exosomes, thus, improving the oxygen supply [80]. Cancer cell-derived exosomes can trigger the differentiation and activation of fibroblasts into myofibroblasts and CAFs, creating a tumour permissive or even supportive microenvironment for themselves [81,82]. Cancer cells further improve their chance to survive and grow by secreting immunomodulating exosomes, which have the ability to inhibit the anti-tumour natural killer cell activity [83]. Communication between cancer cells and CAFs is bidirectional and CAFs can secrete tumour supporting EVs as well, by packing specific miRNA cargo into the EVs [84,85]. Metastatic capacity of cancer cells can be increased by fibroblast-derived EVs in breast cancer animal models [86]. Fibroblast-derived EVs are able to enhance endocrine therapy resistance by transferring mtDNA that can induce oxidative phosphorylation in recipient breast cancer cells [87]. Finally, CAF-derived EVs can also induce drug resistance in colorectal cancer models [88].

2. Objectives

In our projects we focused on how intestinal stromal fibroblasts communicate with intestinal epithelial cells via EVs. As fibroblast models, we used i) commercially available human colon fibroblasts, ii) isolated murine intestinal fibroblasts and iii) patient- derived CAFs. EVs were isolated from fibroblast conditioned media by differential ultracentrifugation. For modelling the intestinal epithelium and intestinal stem cells, we employed the best currently available model system, the three-dimensional organoid model system. We studied EV mediated communication between stromal cells and epithelial cells both in normal and tumour models.

Our specific aims were to answer the following questions:

1. Do intestinal fibroblast-derived EVs alter the 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 the proliferation if niche factors are dispensable due to tumorigenic mutations?
5. Do CAF-derived EVs alter the proliferation of CRC organoids?
6. Does activation of fibroblast cultures change their tumour supporting ability?

3. Results

3.1. Murine and human intestinal fibroblasts secrete EVs

First, we wanted to characterise our fibroblasts cultures, and to find out, if they secreted EVs. We isolated EVs from both murine and human intestinal fibroblast culture supernatant. Magnetic beads covered with antibodies against the EV marker CD63 or CD81 were incubated with the EVs, labelled against the markers, and were measured by flow cytometry. Ultracentrifuged pellets were also analysed by scanning electron microscopy to determine the presence of EVs, and Tuneable Resistive Pulse Sensing method and Nanoparticle Tracking Analysis were used to determine the diameters of the EVs.

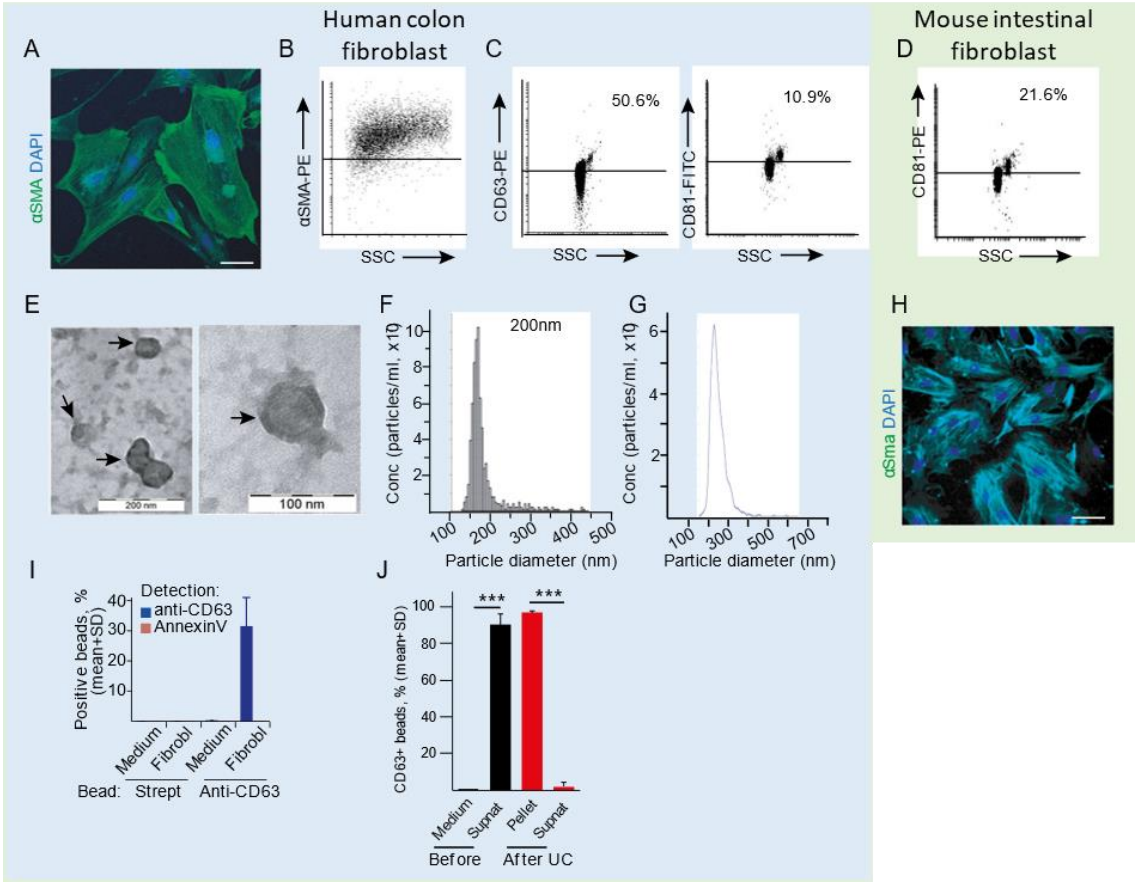

Figure 13 Human and murine intestinal fibroblasts secrete extracellular vesicles. Human (blue) and murine (green) results are indicated
A) αSMA immunocytochemistry labelling of human colon fibroblasts (HCFs).
B) Flow cytometric analysis of HCF αSMA levels.
C) Positive percentages of anti-CD63 or anti-CD81 coated magnetic beads, incubated with HCF-derived supernatant.

D) Determining the specificity of the anti-CD63 magnetic beads, compared to non-specific Streptavidin coated beads.
E) Representative transmission electron microscopic (TEM) image of HCF derived small EVs
F and G) Diameter distribution of HCF derived EVs, determined by TRPS (F) and NTA (G) methods
H)) αSMA immunocytochemistry labelling of murine intestinal fibroblasts (MIFs)
I)Positive percentages of anti-CD81 coated magnetic beads, incubated with MIF-derived supernatant
J) Probing the pellet and the supernatant for EVs after ultracentrifugation by flow cytometric analysis of antibody-coated beads.
Scale bars: 50 μm, Student t-test was used for statistical analysis P<0.05, ** P< 0.01, *** P< 0.005, n.s., not significant*

Our normal human colon fibroblast (HCF) and murine intestinal fibroblast (MIF) cultures were positive for the fibroblast marker αSMA (Figure 13A-B and H). Since only smaller EVs can be released from the Matrigel used for culturing organoids, we focused on EVs pelleted by 100.000g ultracentrifugation. Both HCFs and MIFs secrete EVs with a peak diameter between 150-200nm, determined with Tuneable Pulse Resisting Sensing (Figure 13F) and Nanoparticle Tracking Analysis (Figure 13G). They are highly positive for the markers CD63 or CD81 (Figure 13C, D, I). The presence of EVs was also confirmed by electron microscopy (Figure 13E). Ultracentrifugation successfully sedimented the vast majority of EVs (Figure 13J).

3.2. HCF- or MIF-derived EVs do not alter organoid viability when all the niche factors are present

Our next question was if intestinal fibroblast-derived EVs could alter organoid viability when all the critical niche factors were present. The survival rate of murine small intestinal organoids can be determined by microscopic observation. Dead organoids break down to individual single cells and the continuous epithelial layer is disrupted (Figure 14B). Live organoids create new crypts (termed budding organoids) and the cells form a continuous epithelial layer (Figure 14A). Since murine small intestinal organoids are relatively easy to isolate and maintain, we used them as our primary system for modelling the intestinal epithelium. These organoids require the niche factors EGF, R-Spondin1 and noggin. We isolated EVs from normal human colon fibroblast culture supernatant by ultracentrifugation, and they were added to the complete media of the organoid cultures. For all our experiments we used a constant fibroblast cell number and incubation time.

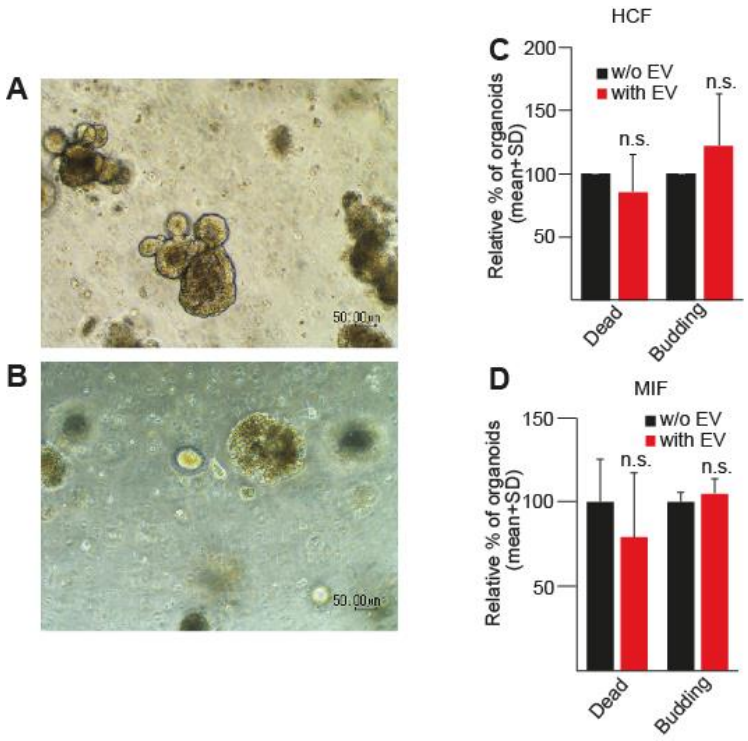

Figure 14. Human normal intestinal fibroblast derived extracellular vesicles have no additional effect in the stem cell niche
Representative microscopic images of murine small intestine derived organoids: live one (A) and dead one (B)
C) and D) Dead/Budding organoid ratios after incubating murine small intestinal organoids with every niche factor and human colon fibroblast or murine intestinal fibroblast derived EVs
*n = 3, mean + SD, analysis of variance and Tukey post hoc test, *** P < 0.005)*

Adding HCF or MIF derived EVs to complete organoid culture media did not significantly alter the dead/live organoid ratios (Figure 14).

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

In contrast to SI organoids where Paneth cells produce Wnt proteins, colon organoids critically depend on external Wnt factors in cultures due to the lack of this cell type. Therefore, we chose colon organoids to study the effect of effects of Wnt. Removing Wnt-proteins from the culture media significantly reduced the ratio of live organoids, while replacing Wnt-proteins with HCF derived EVs restored live organoid ratios (Figure 15).

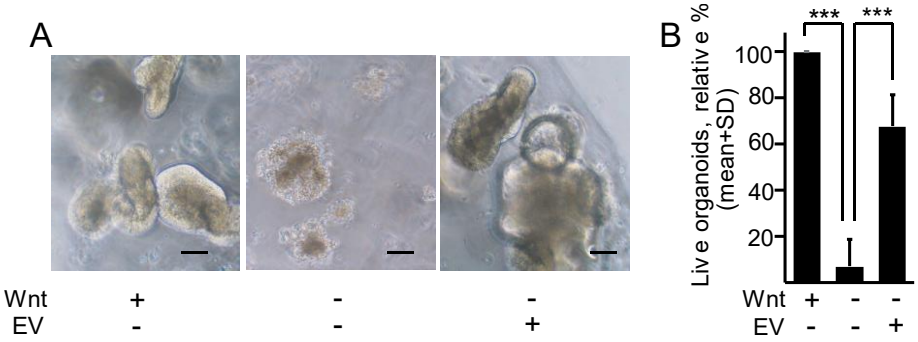

Figure 15 HCF-derived extracellular vesicles can replace Wnt-proteins
A) Representative microscopic images of human normal colon organoids, with or w/o WNT or HCF derived EVs. Scale bars: 20µm
B) Ratios of live organoids normalised to WNT-treated samples.
*(n = 3, mean + SD, analysis of variance and Tukey post hoc test, *** P< 0.005)*

3.4. HCF-derived EVs can replace EGF in the intestinal stem cell niche via the EGF-receptor

Next, we were interested in whether fibroblast derived small EVs can replace other essential SCN factors. For this purpose, we used HCF and MIF fibroblast cultures as a source for EVs, and small intestinal organoids isolated from C57Bl/6J mice as an intestinal epithelial model. EVs were isolated using differential ultracentrifugation.

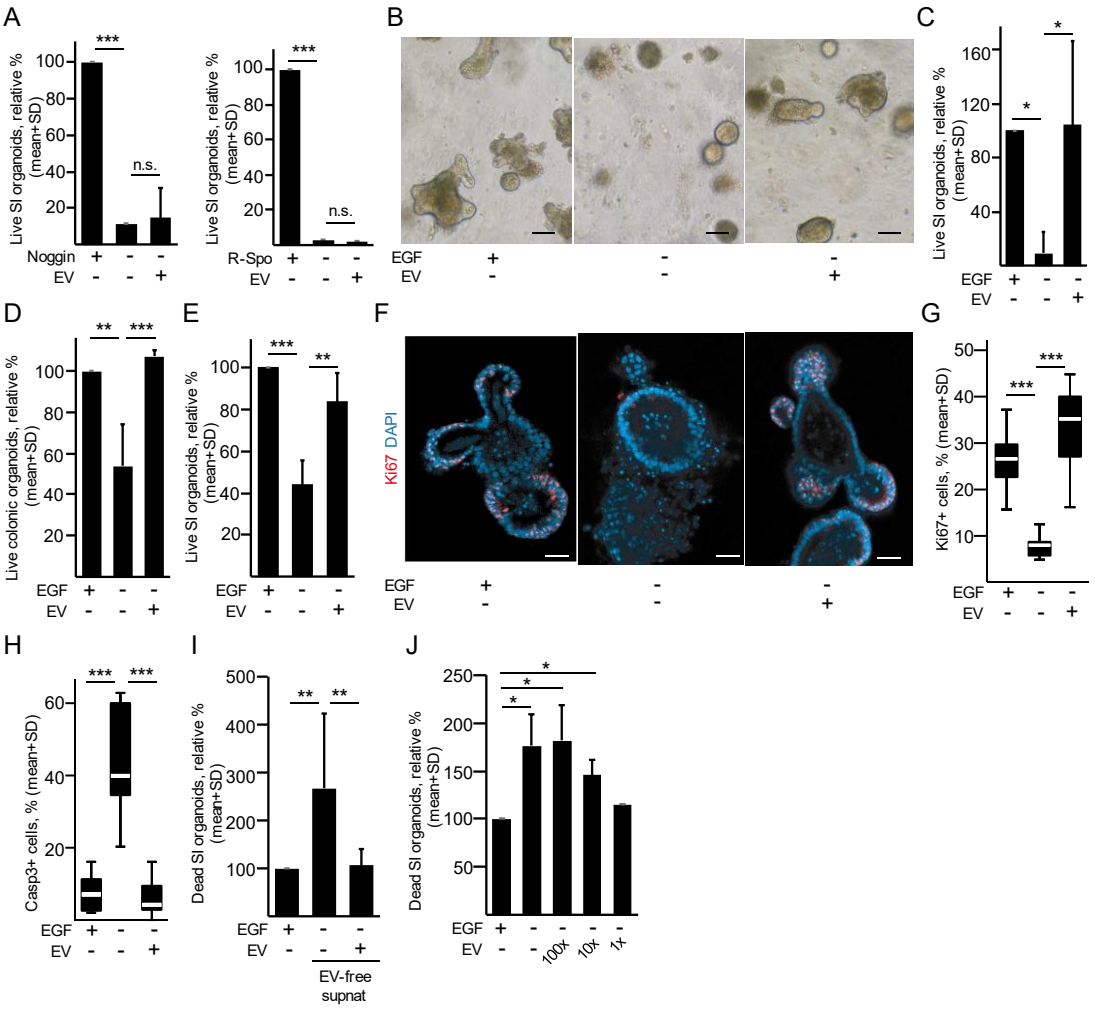

Figure 16 Fibroblast-derived extracellular vesicles (EVs) transmit epidermal growth factor (EGF) activity in the intestinal stem cell niche.
A) Surviving small intestinal (SI) organoids four days after removing noggin or R-Spondin1 and/or adding human colon fibroblast (HCF)-derived EVs (n = 3).
B and C) Representative images (B) from SI organoids when EGF was removed or HCF-derived EVs were added and the quantification at day 4 (C) (n = 5).
D) Proportion of living colonic organoids with the indicated treatments. EVs were pelleted from HCF supernatants (n = 3).
E) Quantification of the living SI organoids at day 4 when EGF was absent or mouse small intestinal fibroblast (MIF)-derived EVs were added (n = 3).
F and G) Representative images (F) and the quantification of KI67+ proliferating cells (G) from SI organoids in the absence of EGF or the presence of HCF-derived EVs at day 4 (n = 10-12 from three experiments).
H) The percentage of active Caspase-3+ apoptotic cells in SI organoids with the indicated treatments at day 3 (n = 10-12 from three experiments).
I) The relative percentage of dead SI organoids when HCF-derived supernatant was applied after ultracentrifugation (UC). Note that UC removes EVs from the supernatant. EGF or the UC-pelleted EVs with the EV-depleted UC supernatant were added to some organoids (n = 3).
J) Dead organoid rate when HCF-derived EVs were applied at different dilutions

(1×,10×, 100×) in the absence of EGF (n = 3).
*Kruskal-Wallis test and Dunn post hoc test (G, H) or analysis of variance and Tukey post hoc test (A, C, D, E, I, J) were used. Scale bars: 50 µm, *P < 0.05, **P < 0.01, ***P < 0.005, n.s., not significant*

Removing noggin or R-Spondin1 from murine SI organoid culture medium significantly reduced the ratios of live organoids. Replacing either of the above factors by HCF-derived EVs could not restore this ratio (Figure 16A). However, when we replaced EGF with HCF-derived EVs, we observed a significant rescue effect (Figure 16B-C). This rescue effect was observed when EGF was replaced with HCF-derived EVs in a murine colon organoid model system (Figure 16D). MIF-derived EVs had this rescue effect as well on small intestinal organoids (Figure 16E). This rescue effect is indicated by restoring the KI67+ proliferating and by reducing the active Caspase3+ apoptotic populations (Figure 16F-H). When we applied the ultracentrifuged EV-free supernatant of the fibroblast in the absence of EGF, the ratio of dead organoids increased (Figure 16I). Similarly, dilution of EVs increased the ratio of dead organoids ratios (Figure 16J).

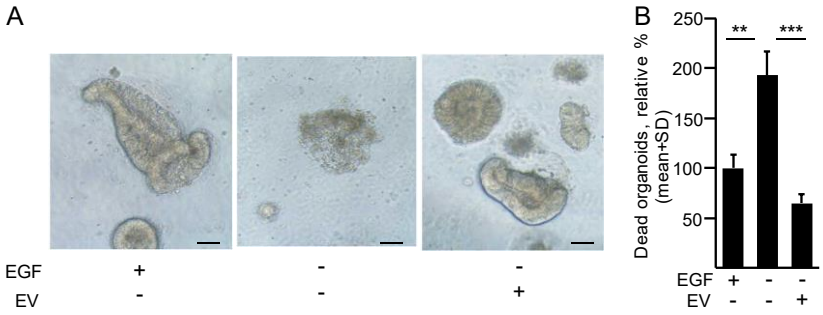

Figure 17 Extracellular vesicles can replace epidermal growth factor in human intestinal colon organoids as well.
A) Representative microscopic images of human colon organoids, after treatment with/without EGF or HCF derived EVs, scale bar = 25µm
*B) Dead organoid ratios (n=3) Mean+SD are shown. ANOVA with Tukey post hoc test was carried out, ** = p<0.01, *** = p<0.005*

The EGF-replacing effect of HCF-derived EVs was also confirmed by using normal human colon organoids (Figure 17). Removing EGF significantly increased the ratio of dead organoids and replacing EGF with HCF-derived EVs could reduce the ratio of dead organoids to the control level. Since we found that EVs had the most striking effect when EGF was absent, we focused on their EGF-replacing effect.

3.5. HCF-derived EVs had an impact on stem cells in the absence of EGF

To further analyse the EGF-replacing effect of intestinal fibroblast derived EVs, next we isolated small intestinal organoids from the *Lgr5-EGFP-IRES-Cre*^{ERT2} mouse strain. In these mice, an EGFP reporter gene is cloned after the *Lgr5* promoter, resulting in EGFP expressing intestinal stem cells. However, the transgenic Lgr5 locus is often silenced and thus, Lgr5+ stem cells lose their green fluorescent signal. To solve this problem, we treated the organoid cultures with the Wnt-pathway agonist CHIR99021 that is known to expand the EGFP-expressing stem cell population [89].This compound is a direct inhibitor of GSK3 protein, and by that, an inhibitor of the destruction complex, amplifying Wnt-signalling and *Lgr5* expression. HCF derived EVs were tested for their EGF replacing effect.

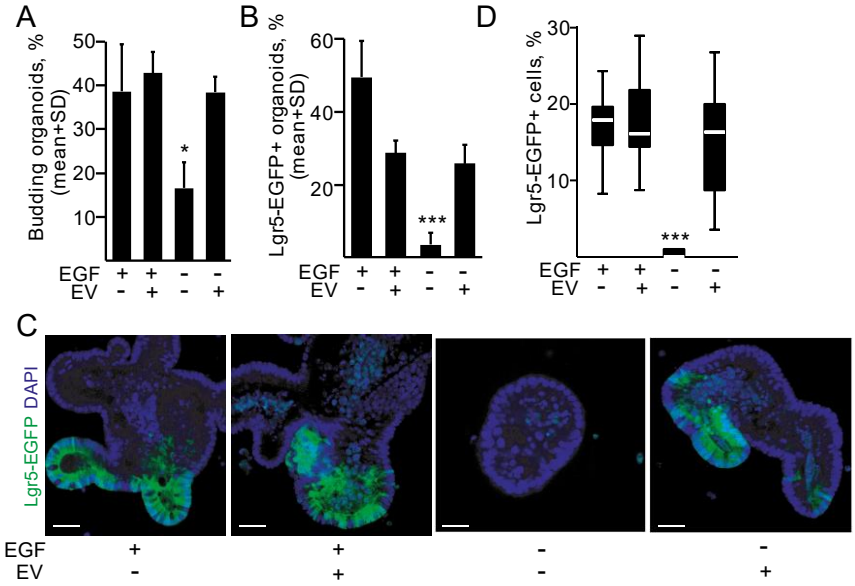

Figure 18 Human colon fibroblast derived EVs can help Lgr5+ stem cell surviving when epidermal growth factor lacks
A) Ratios of budding organoids containing two or more crypts at day 4 after treatment (n = 4)
B) Lgr5-EGFP+ containing organoid ratios (n=4)
C) Representative confocal microscopic images, taken on treatment day 4, scale bars = 50µm
*D) Lgr5-EGFP cell ratios compared to all cells (n= 10-11 from three experiments) Analysis of variance and Tukey post hoc tests (A, B) or Kruskal-Wallis and Dunn post hoc tests (D) were used. * and *** indicate P < .05 and P < .005, respectively, compared with all other groups. Mean + SD (A, B) or minimum, Q1, median, Q3, and maximum (D) are shown*

Removing EGF from murine small intestinal culturing media resulted in a massive loss of crypt forming ability, (Figure 18A) and a significant reduction of Lgr5-EGFP+ containing organoid ratios (Figure 18B). The ratio of Lgr5-EGFP cells compared to all cells per organoid was reduced as well (Figure 18D). Replacing EGF with HCF-derived EVs could restore these effects (Figure 18A-D).

3.6. EGF-replacing effect is mediated through the EGF-receptor

Our next question concerned whether this EGF-replacing effect was mediated through the EGF-receptor. To answer this question, we applied the competitive EGF-receptor inhibitor Gefitinib. HCF-derived EVs and C57Bl/6J mouse-derived small intestinal organoids were used in our experiments.

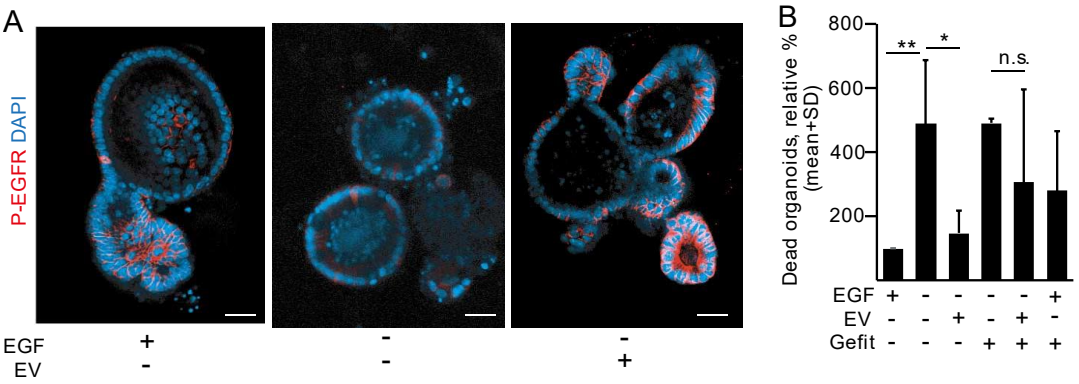

Figure 19 Epidermal growth factor replacing effect is mediated through the EGF-receptor.
A) Representative confocal microscopic images, showing phosphorylated EGF-receptor specific immunostaining of normal murine small intestinal organoids, with the indicated treatments.
*B) Dead organoid ratios after the indicated treatments, counted on treatment day 4. Gefitinib was applied on the organoids 30 min before adding either EGF or EVs (n=4). ANOVA and Tukey post hoc tests were used. Mean+SD are shown. * = $p<0.05$, ** = $p<0.01$, n.s. = not significant*

Culturing murine small intestinal organoids with complete, EGF containing medium leads to the activation of EGF-receptor in the crypts, indicated by the phosphorylation of the receptors. After removing EGF from the media, this effect is lost. However, replacing EGF with HCF-derived EVs leads to the phosphorylation of EGF-receptor (Figure 19A). Blocking EGF-receptor with the competitive inhibitor Gefitinib increases dead organoid ratios, even in the presence of EGF or EVs (Figure 19B).

3.7. HCF-derived EVs carry EGF-receptor ligands on their surface

To further investigate the EGF-replacing activity of the EVs, we were interested if EVs carried EGF-receptor ligands on their surface. First, we analysed the expression of EGF-receptor ligands in HCF cells and in mouse small intestinal organoids by reverse transcription quantitative PCR. After that, we isolated small EVs from HCF culture supernatant, and incubated CD63-specific magnetic beads with them. Magnetic beads were labelled with primary antibodies, specific for different EGF-receptor ligands, and they were analysed by flow cytometry.

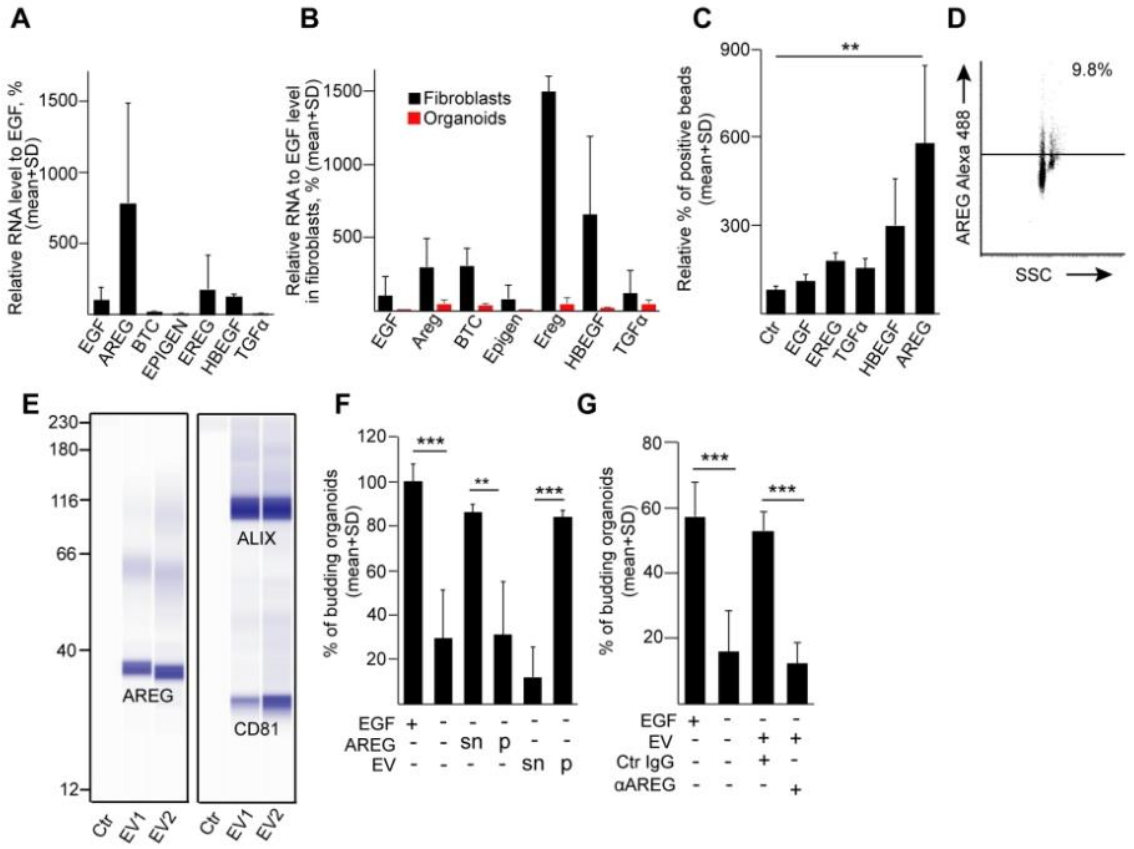

Figure 20 Human colon fibroblast derived extracellular vesicles carry epidermal growth factor receptor ligands on their surface
A) Relative mRNA levels of different EGF-family members, normalised to EGF protein, in HCFs n=3
B) Comparison of EGF-family members expressions between MIFs and murine normal small intestinal organoids. HPRT normalised EGF mRNA levels in MIFs were taken as a baseline 100%, n= 3
C) Relative percentages of positive anti-CD63 coated magnetic beads, after incubated with HCF-derived EVs, and labelled with indicated antibodies. EVs were pelleted by ultracentrifugation. n= 3
D) Representative flow cytometric image of C)

E) Simple Western (WES) analysis of two HCF-derived ultracentrifugated EV preparation (EV1 and EV2) for AREG (34kDa) and for EV markers CD81 (29kDa) and ALIX (108 kDa). Control sample was prepared with cell-free medium.
F) Percentages of crypt forming, budding organoids. Samples were prepared: either AREG containing medium or EV containing supernatants were ultracentrifuged down, and either the pellet or supernatant was used. As a control, regular EGF-containing full medium was used (n=3)
G) Percentages of crypt forming, budding organoids. Samples were prepared. HCF derived ultracentrifuged EVs were either incubated with neutralizing anti-AREG or control antibodies. n=3
*ANOVA and Tukey post hoc tests were used. Mean+SD are shown. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ n.s. = not significant*

In the intestine, EGF family members are primarily expressed by fibroblasts, and not by epithelial cells (Figure 20A-B). HCF-derived EVs carry EGF-receptor ligands on their surface and AREG (AREG) was present at a detectable level, determined by antibody-coated bead capture based flow cytometry (Figure 20C-D). The presence of AREG as well as sEV-related markers in the pelleted EV pellet after ultracentrifugation were confirmed by the capillary-based Simple Western Blot analysis (Figure 20E). AREG can replace EGF in the intestinal stem cell niche, and as another experiment, we added AREG to the medium, and the effects of the supernatants and pellets after ultracentrifugation were tested in SI organoid cultures. Interestingly, this supernatant, but not the pellet, had a rescue effect on organoid death, showing that exogenously added AREG does not co-purify with medium components (Figure 20F) and blocking surface bound AREG by a neutralizing antibody prevents the EGF-replacing rescue effect of HCF derived EVs in a murine small intestinal organoid system (Figure 20G).

3.8. HCF-derived EVs do not alter organoid growth if EGF is dispensable

Our research group previously established *Apc*-mutant mouse small intestinal organoids that can be considered as an early adenoma model. Importantly, these organoids grow independently from exogenous niche factors. Next, we wanted to test if HCF derived EVs had any additional effect when EGF is dispensable.

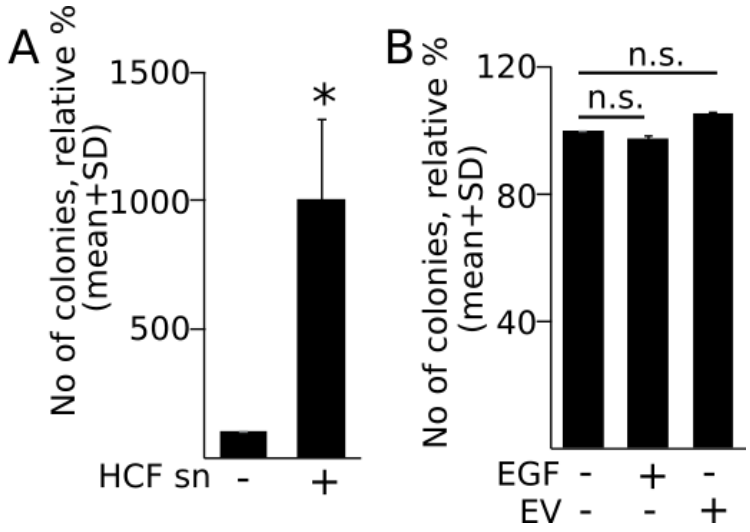

Figure 21 Fibroblast-derived EVs have no role in Apc-mutant organoids.
A) Relative percentages of colonies initiated by single cells derived from dissected organoids, incubated with or without human colon fibroblast derived supernatant (n=3).
B) The relative proportion of Apc-mutant organoids in the presence and absence of EGF or fibroblast-derived EVs n=4.
*Paired t-test (A) and ANOVA with Tukey post hoc test (B) were used. Mean ± SD are shown, * = $p<0.05$, n.s. not significant*

Interestingly, HCF-derived supernatant without any purification or centrifugation step had a major impact on the colony forming ability of Apc-mutant murine intestinal organoids (Figure 21A). However, this effect is not caused by either EGF or EVs. HCF-derived EVs or exogenous EGF did not alter the number of *Apc*-mutant colonies (Figure 21B).

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

Our research group has previously established and characterised three patient derived colorectal cancer organoid lines. Importantly, these organoids depended on EGF activity and lacked mutations in KRAS [77]. First, we were interested if HCF-derived EVs could replace AREG in the tumour microenvironment, using the patient-derived organoids as a model for colorectal carcinoma.

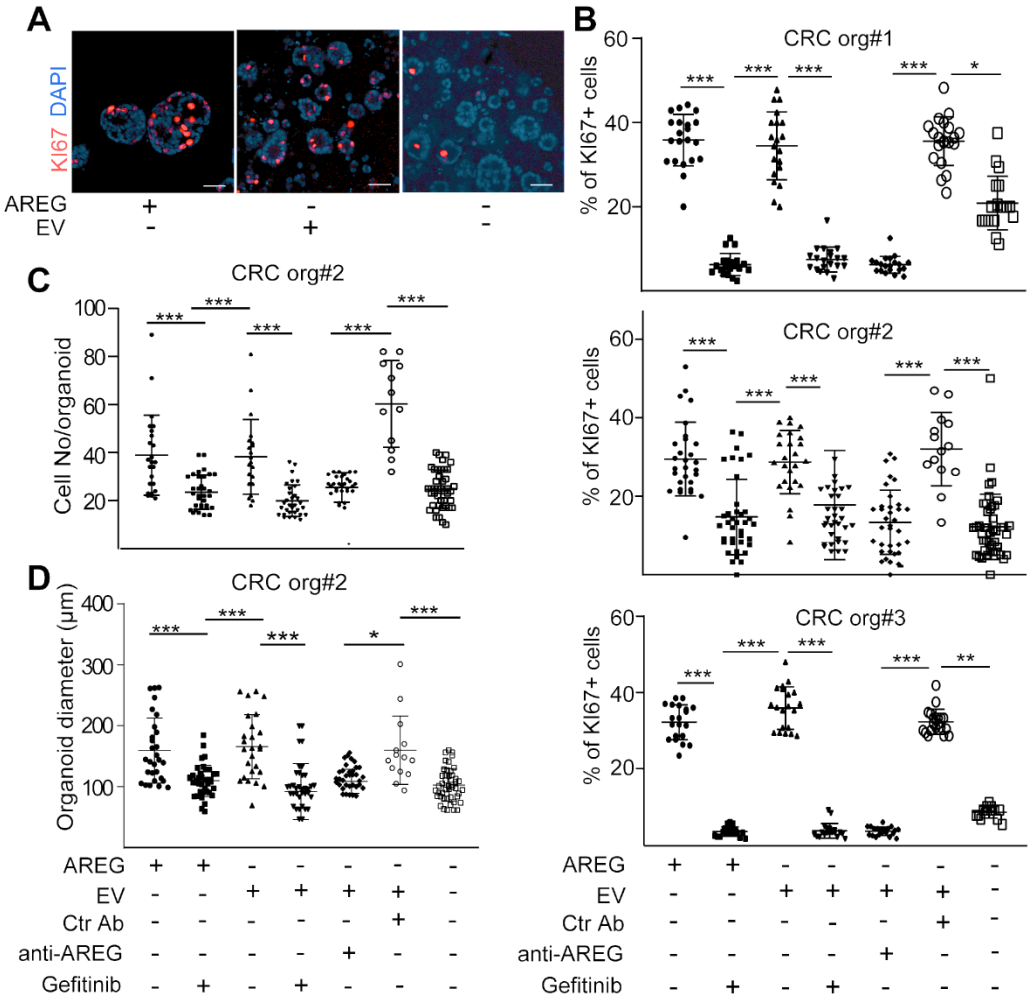

Figure 22 HCF-derived EVs induce the proliferation of CRC organoid cells via carrying AREG.
A-B) Representative confocal microscopic images (A) and the quantification of KI67+ cells in CRC patient-derived organoid lines with the indicated treatments. Note that 2.5x10^7 HCF-derived EVs were applied.
*C-D) Cell number (C) and diameter (D) of CRC organoids. Kruskal-Wallis and Dunn's multiple comparison tests were used (B, C, D) with *p<0.05, **p<0.01, ***p<0.005. Scale bars: 100 µm (A).*
Confocal microscopic images were quantified from three experiments for each organoid line for B, C, and D.

We observed a marked reduction of KI67+ cells when AREG was absent from the culture media compared to AREG containing full culture media. Replacing AREG with HCF-derived EVs to the media restored the number of KI67+ cells (Figure 22A-B). In addition, blocking EGF receptor by the EGF receptor inhibitor Gefitinib or pre-incubating EVs with a neutralizing anti-AREG antibody inhibited the effects of EVs (Figure 22B-D).

3.10. No functional difference in EV effect when activating fibroblasts either in EGF-dependent or in EGF-independent 3D models

Next, we were interested if fibroblasts activated by TGFβ, released EVs with a modified function in the tumour microenvironment. TGFβ is an important factor converting HCFs to CAFs. For this purpose, we pre-incubated HCF cultures with TGFβ prior to EV isolation. The isolated EVs were applied in the three CRC organoid lines, KI67+ and active Caspase3+ cell ratios as well as AREG positivity of the EVs were determined. As a negative control, we used the EGF independent SW1222 CRC cell line. The SW1222 cells form 3D organoid-like structures and the larger "megacolonies" contain lumens [90,91]. Importantly, the presence and the number of lumens correlate with CRC stem cell activity and differentiation. Furthermore, megacolonies highly resemble organoids [90] and these cells do not require EGF family members.

The effect of TGFβ-treated HCF-derived EVs did not differ from EVs isolated from non-treated HCFs; the KI67+ and active Caspase3+ cell ratios were the same among control and TGFβ-treated samples (Figure 23A-B). TGFβ treatment did not change the AREG positivity of HCF-derived ultracentrifuged EVs, as detected by a latex-bead based flow cytometric assay (Figure 23 C). As expected, recombinant AREG had only a marginal inducing effect on the proportion of KI67+ cells in SW1222-derived megacolonies and neither control nor TGFβ-activated HCF-derived EVs modified the ratio of proliferating cells (Figure 23D-E). These data suggest that EVs released by fibroblasts have no effect on CRC proliferation when EGF activity is dispensable (Figure 23D-E).

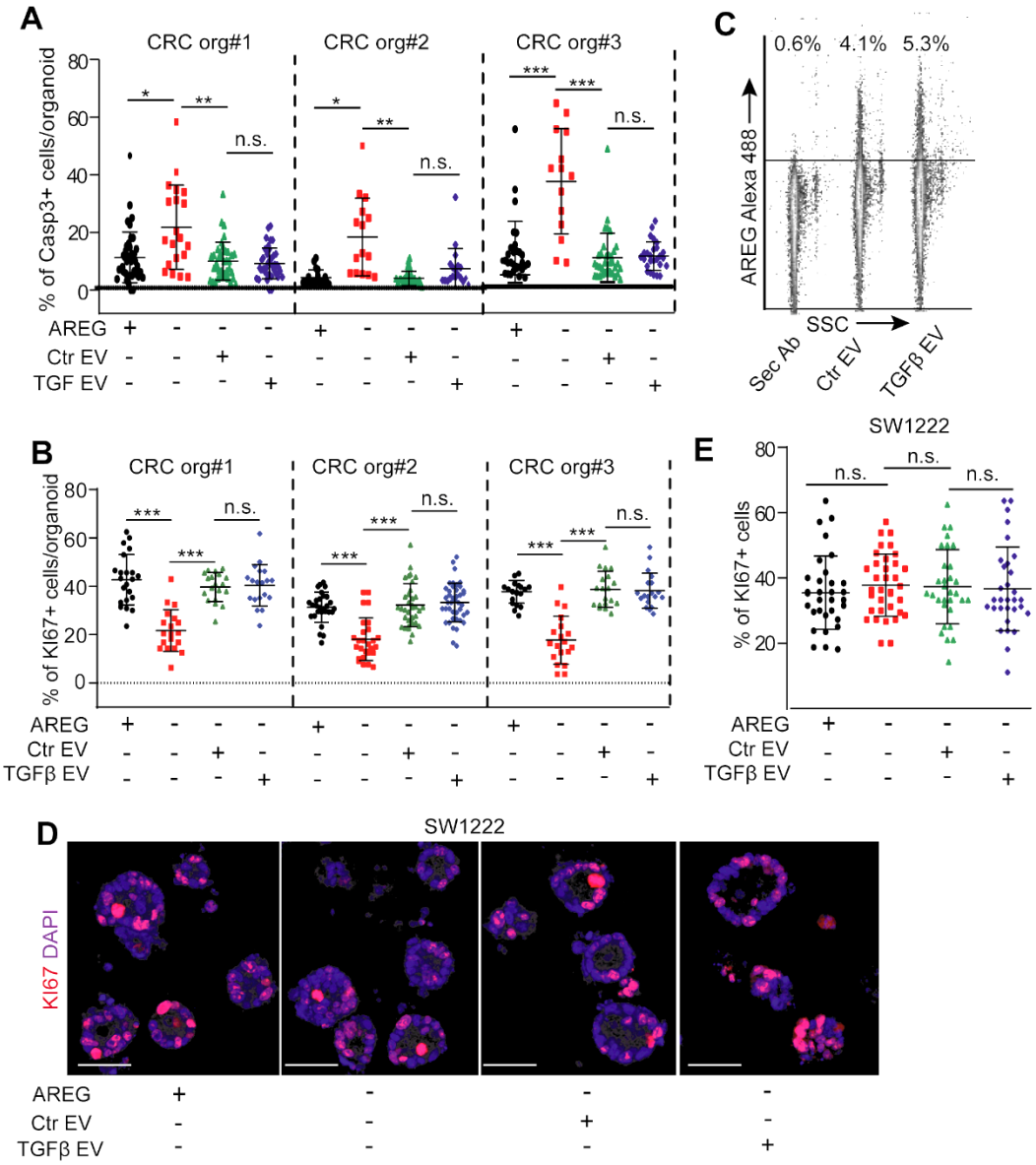

Figure 23 TGFβ does not modify the effects of EVs either in EGF-dependent or in EGF-independent 3D models
A) The percentage of active Caspase3+ cells in CRC patient-derived organoid lines in the presence or absence of AREG, control or TGFβ-treated HCF-derived EVs.
B) The effect of HCF-derived EVs on CRC organoids. HCFs were pre-treated with TGFβ (TGFβ EV) or they were cultured untreated (Ctr EV) before collecting EVs.
C) Detecting AREG on EVs isolated from control (Ctr EV) or TGFβ-treated HCFs (TGFβ EV) and bound to latex beads (flow cytometry).
D-E) The percentage of KI67+ cells in SW1222 cell-derived colonies in the presence of the indicated treatments. Representative confocal microscopic images (D) and their quantification (E). EVs were isolated from control or TGFβ-treated (4 days) HCF cultures. Kruskal-Wallis and Dunn's multiple comparison tests were used (A, B, E). Confocal microscopic images were quantified from three experiments for A, B and E. Scale bars: 50 μm (D).

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

After the analysis of the EV based relationship between HCFs and CRC epithelial cells, we were interested in the effects of CAF derived EVs. For this purpose, we used CAFs isolated from the CRC tumour samples.

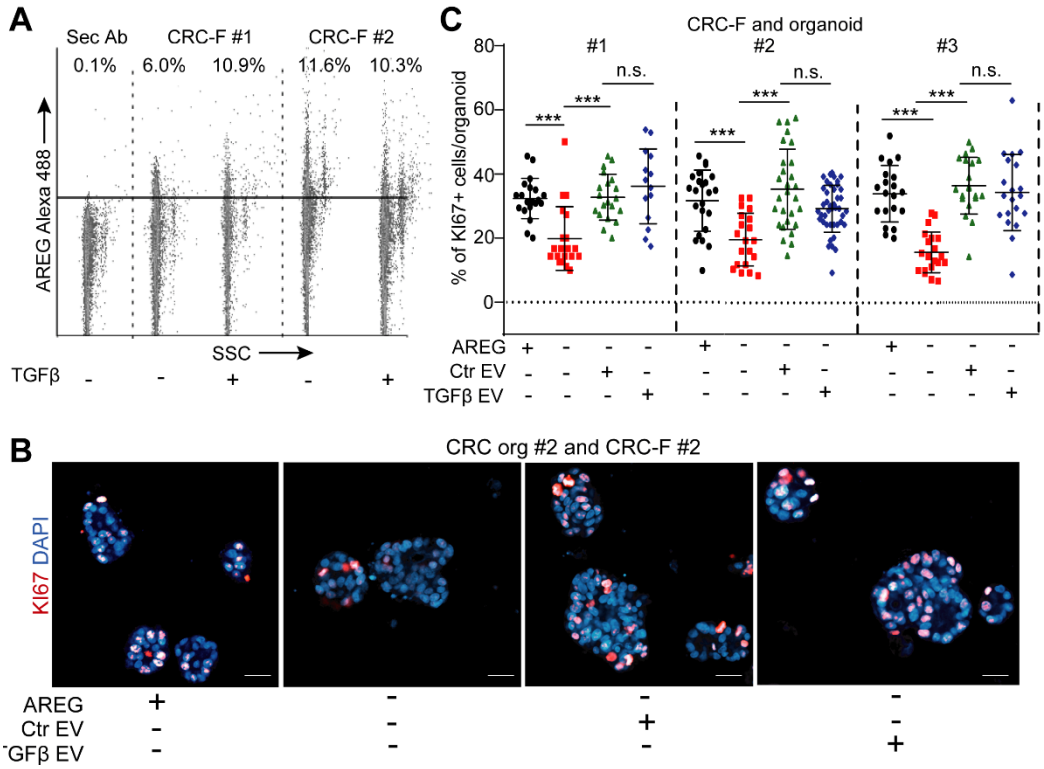

Figure 24 CRC-F cell-derived EVs increase the percentage of proliferating CRC organoid cells independently of pre-treatment with TGFβ.
A) Detecting AREG on ultracentrifugated EVs bound to latex beads and isolated from control or TGFβ-treated CRC-F cells (flow cytometry).
B-C) The percentage of KI67+ CRC organoid cells with or without AREG or EVs derived from control or treated CRC-Fs (paired samples). Confocal microscopic images (B) and quantification (C). Kruskal-Wallis and Dunn's multiple comparison tests (C) were used. Scale bars: 50 µm.

Similarly to HCFs, we detected AREG on both control and TGFβ-treated CAF-derived EVs by flow cytometry (Figure 24A), and these EVs restored the proportion of KI67+ CRC cells in EGF-dependent organoids in the absence of exogenously added EGF family members (Figure 24B-C). Importantly, we observed no difference when CRC-F cultures

had been pre-treated with TGFβ and identical numbers of EVs were applied (Figure 24B-C).

4. Discussion

In our studies we proved that EVs are major components of the intestinal stem cell niche and that these EVs represent a novel way to transport essential niche factors in the intestinal SCN. First, we characterised our human and murine intestinal fibroblast cultures, which were positive for αSMA and they secreted a significant amount of CD81+ and CD63+ EVs. When Noggin or R-Spondin was replaced with EVs, the ratio of live organoids was severely reduced, suggesting that EVs do not carry any molecules with BMP antagonising or Wnt amplifying activity. Replacing one of these niche factors with EVs resulted in a massive organoid death.

Wnt proteins are hydrophobic essential factors in the intestinal SCN. It has been previously shown that intestinal cell plasma membranes constitute a reservoir for Wnt proteins and Wnt3a is transported mainly in a cell-bound manner through cell division and not by secretion [17]. It has been proved that the EV-based transfer is one of the possible mechanisms for Wnt protein transmission and that they are able to travel via multivesicular body-derived exosomes [92]. The relevance of this finding has been showed in many diseases, such as pulmonary fibrosis or heart diseases [93–95]. In the intestine, macrophage-derived EVs carry Wnt proteins to help the repair of the intestine after damage [96]. When culturing normal colon organoids, addition of lipid components to the culture media greatly enhances organoid surviving, since it promotes the solubility of Wnt proteins. Lipid components are usually supplied by adding fetal bovine serum. However, we lose the well-defined composition of the media by using serum. For this reason, liposomes can be applied instead of serum [97]. It is still under intensive debate which cell types produce the largest amount of Wnt-proteins in the colon. Cells of the epithelium do not produce sufficient amount of Wnt-proteins(colon organoids composed of epithelial cells cannot survive without supplemented recombinant Wnt proteins), thus, raising the question which other cells serve as sources for these essential niche factors and how these factors are transported. According to our results, HCF-derived EVs can replace Wnt proteins in the intestinal SCN. EVs replacing Wnt-proteins can elevate colon organoid survival ratios to Wnt-treated organoid level. The novelty of our results is the observation that Wnt proteins can be transmitted in the normal colon SCN by fibroblast-derived EVs. 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: there

are numerous members of the Wnt-protein family, their transmission via EVs have already been well described.

EGF receptor ligand family members are synthesised as membrane-bound molecules and can then be cleaved by proteases and released. EGF family proteins such as AREG can act in a paracrine, autocrine and juxtracrine manner [98,99]. Importantly, membrane-bound AREG can also activate EGF receptors before its cleavage [100]. It has been previously reported that AREG can travel via exosomes, however, this was proven with an AREG-transfected MDCK cell line. These AREG carrying exosomes were able to stimulate human breast cancer cells, proving their physiological activity, and they can be five times more efficient than recombinant soluble AREG [101]. Other papers have also proved EV-bound AREG, however these papers used cell lines, and the effects of the EVs were not always tested [102–104]. It has been previously shown that intestinal fibroblasts produce EGF family members, however, the method of transmission was not analysed before. Our results differ in that we used a model system as close to the normal intestinal homeostasis as currently possible. 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. This also raises the question what other sources are for EGF-receptor ligands and how these ligands are transported. Our results were the first to show that normal fibroblast derived EVs contribute to the normal, non-tumorous intestinal SCN by transferring amphiregulin and thus increasing the proliferation of stem cells. 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 findings, we focused on EGF-family proteins. We found that replacing EGF with EVs restored KI67+ proliferating cell populations and at the same time decreased active Caspase3+ apoptotic populations. *Lgr5*+ cell numbers were restored, too, and the EGF-receptor was phosphorylated, suggesting that some of the EGF-family members were 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 Blotting 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.

The role of the EGF-Erk pathway in CRC progression is well known, and the *KRAS* gene is one of the most frequently mutated components. 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, so next we investigated 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. After 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 [106]. 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. Summary

The exact mechanism and types of intercellular communication between stromal fibroblast and the intestinal stem cell niche are still not fully understood. The intestinal stem cell niche is formed by many niche factors that help the intestinal stem cells to divide, not to differentiate and to maintain a stem cell phenotype. Extracellular vesicles (EVs) are membrane surrounded recently recognized players of intercellular communication that carry diverse cargo types. In our project, we isolated EVs by differential ultracentrifugation from intestinal fibroblast conditioned media. By using the best currently available *in vitro* model system, the three-dimensional intestinal organoid culturing method, we tested if EVs could replace important factors in the intestinal stem cell niche.

We found that intestinal fibroblast-derived EVs are able to replace Wnt proteins and EGF-receptor ligands, but not BMP-antagonists or the Wnt signal amplifying R-Spondin. Replacement of EGF-receptor ligands with EVs restored the number of i) KI67+ proliferating, ii) active Caspase3+ apoptotic, and iii) *Lgr5*+ stem cells to control levels. This EGF replacing effect was mediated through the EGF receptor, which became phosphorylated when intestinal organoids were treated with EVs. When inhibiting EGF receptor with Gefitinib, organoid survival ratios were significantly reduced even in the presence of EGF or EVs.

HCF-derived EVs carry EGF receptor ligands on their surface, and AREG is present in a significant amount. When EVs were incubated with neutralising anti-AREG antibodies, the rescue effect was not observed, suggesting a correct orientation of the EV-bound AREG. HCFs and CAFs could support the growth of EGF-dependent colorectal cancer organoid lines, but not the proliferation of CRC cells independent from external EGF activity. Pre-treating fibroblast cultures with the fibroblast activating cytokine TGFβ did not modify this replacing ability.

In summary, our results show that fibroblast-derived EVs have a major role in forming the intestinal stem cell niche and providing EGF activity for CRC cells with normal EGF receptor signalling pathway via transporting AREG. This finding may help the scientific and medical community to develop better regenerating and anti-tumour strategies based on EVs.

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8. Bibliography of the candidate's publications

8.1. List of publications used for the thesis

Oszvald, Ádám*; Szvicsek, Zsuzsanna* ; Pápai, Márton ; Kelemen, Andrea; Varga, Zoltán ; Tölgyes, Tamás ; Dede, Kristóf ; Bursics, Attila ; Buzás, Edit Irén ; Wiener, Zoltán

Fibroblast-derived extracellular vesicles induce colorectal cancer progression by transmitting amphiregulin

*Shared first autorship

FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY 8 Paper: 558, 15 p. (2020)

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Extracellular vesicles transmit epithelial growth factor activity in the intestinal stem cell niche.

STEM CELLS 38: 2 pp. 291-300., 10 p. (2020)

8.2. List of publications not used for the thesis

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The growth determinants and transport properties of tunnelling nanotube networks between B lymphocytes

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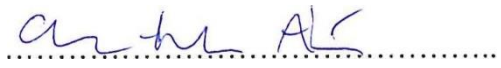
Statement of originality

I, Adam Oszvald-Haibach, Neptun-code i6z901, hereby confirm that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Budapest, 2020.08.31.



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