CROSSTALK BETWEEN THE MICROBIOME AND CANCER CELLS BY QUORUM SENSING PEPTIDES

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Highlights

• Some quorum sensing peptides promote colon cancer cell invasion and angiogenesis.
• Quorum sensing peptide Phr0662 influences tumor progression by EGFR targeting.
• Cytokine profiles confirm the peptide's stimulatory effect on metastasis in vitro.
• The microbiome–mammals crosstalk may explain the microbiome's effect on health.

ABSTRACT

To date, the precise role of the human microbiome in health and disease states remains largely undefined. Complex and selective crosstalk systems between the microbiome and mammalian cells are also not yet reported. Research up till now mainly focused on bacterial synthesis of virulence factors, reactive oxygen/nitrogen species (ROS/RNS) and hydrogen sulphide, as well as on the activation of exogenous mutagen precursors by intestinal bacteria. We discovered that certain quorum sensing peptides, produced by bacteria, interact with mammalian cells, *in casu* cancer cells: Phr0662 (*Bacillus* sp.), EntF-metabolite (*Enterococcus faecium*) and EDF-derived (*Escherichia coli*) peptides initiate HCT-8/E11 colon cancer cell invasion, with Phr0662 also promoting angiogenesis. Our findings thus indicate that the human microbiome, through their quorum sensing peptides, may be one of the factors responsible for cancer metastasis.

1. INTRODUCTION

Studies of the human microbiome revealed an individual and age-related diversity of microbes, occupying different habitats like skin, mouth, mammary gland, vagina and gut [1-5]. The beneficial effects of the gastrointestinal microbiota are currently being exploited in daily life by the use of probiotics. These products, most often positioned as functional foods, claim to restore the gut microbiota composition, possibly preventing gut inflammation or other intestinal or systemic disease phenotypes [6]. The most commonly studied organisms for probiotic therapies in the treatment of gastrointestinal diseases include organisms of the genera *Bacillus*, *Enterococcus*, *Escherichia*, *Faecalibacterium* and *Propionibacterium*. *Enterococcus faecium*, together with *Bacillus subtilis* and *Lactobacillus* spp., were investigated as probiotics for acute gastroenteritis, while *Streptococcus* spp., *B. subtilis* and *E. coli* were explored for their use as probiotics in irritable bowel syndrome; a reduced duration or decreased abdominal pain of both gastrointestinal diseases was established. Manipulation of the microbiota with rationally selected pre- or probiotics can inhibit pathogens, strengthen epithelial barrier functions and supply the host with key nutrients (*e.g.* vitamins) [7]. Despite these claimed health benefits, it has also to be cautioned that certain safety aspects should be taken into account when using probiotics: taxonomic identification of the probiotic strain is necessary to avoid pathogenicity, as well as inhibiting the risk of infection and antimicrobial resistance [8]. The use of probiotics is indeed already associated with diverse side effects, *e.g.* bacteremia, fungemia and gastrointestinal ischemia; critically ill patients and immune-compromised individuals are the most-at-risk populations [9].

However, the interactions with the host can affect metabolic, neurological, inflammatory and immunological functions as well, and also the development of cancer can directly or indirectly be promoted [10]. For example, the progress of colorectal cancer can directly be initiated by DNA-

damaging superoxide radicals or genotoxins, both produced by gut (mucosa-associated) bacteria. Indirectly, bacteria can induce cell proliferation or pro-carcinogenic pathways by T-helper cells or Toll-like receptors, respectively [11]. Human microbiome studies have revealed significant differences between cancer patients and healthy controls regarding the relative abundance of certain microbes. In colon cancer patients, an increased population of *e.g. Escherichia coli* was observed in feces, inducing colitis and colibactin synthesis and thereby promoting inflammation and cancer [12].

Although the quorum sensing process within gram-positive bacterial colonies is already extensively described in literature, the direct link between quorum sensing peptides and tumor development remains unexplored. Recent investigations revealed that the quorum sensing process is activated in the human gut: a set of acylhomoserine lactone (AHL) molecules, *i.e.* signaling molecules produced by gram-negative bacteria, were identified in human feces of gastrointestinal disease patients as well as healthy subjects [13]. Moreover, bacterial quorum sensing molecules are likely to play a role in bacterial colonization of mucosa, thus requiring quorum sensing-mediated biofilm formation [14]. Finally, Casula and Cutting showed the germination of *Bacillus subtilis* spores in the murine gastrointestinal tract, thereby probably requiring signaling peptides for quorum sensing pathway activation [15]. Although thus not yet investigated, it is very likely that also quorum sensing peptides are found in the human intestinal tract.

In this study, we utilize a collagen invasion assay, transcriptome assay, Chick Chorioallantoic Membrane (CAM) assay, cytokine profiling and phospho-receptor tyrosine kinase array to investigate the influence of quorum sensing peptides on mammalian cancer cells. Our preliminary observations unexpectedly reveal that quorum sensing peptides stimulate metastasis behavior of human colon cancer cells, thereby opening new perspectives on the role and applications of the

microbiome on the guest's health, with the possibility of translating these findings into other biological and applied medical fields as well.

2. MATERIALS AND METHODS

2.1. Cell culture

An epithelial subclone from human ileocecal colorectal adenocarcinoma cells (ATCC$^{®}$ CCL-224), *i.e.* HCT-8/E11, was grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (V/V) fetal bovine serum (FBS), 1% (w/V) L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 5% CO_2.

2.2. Collagen type I invasion assay

Morphology changes of HCT-8/E11 cells were investigated using previously described methods [16]. In brief, 10 000 cells were seeded per well in a 48-well plate, containing 150 µl of collagen type I gel per well, thereby investigating cell morphology 24 hours post-treatment (Leica DMI3000B phase contrast microscope). Peptide solutions (Supplementary Table 1, all from GL Biochem, Shanghai, China) were prepared using ultrapure water (with or without DMSO), obtaining final peptide concentrations of 1 µM, 100 nM and 10 nM after 1:10 dilution using growth medium; the placebo sample solely contained ultrapure water. Two independent morphology 'scorings' were obtained for each of the 3 replicates; peptides were found positive if collagen-invasion or cell-stretching at minimum 2 consecutive concentrations was established at minimum 2 out of the 3 replicates.

2.3. Human Transcriptome Array

Peptide (EDF-analogue: 10 nM, Phr0662 and EntF-metabolite: 100 nM and 1 µM) treated cells were analyzed in duplicate (independent treatment and analysis) for whole transcriptome expression using Affymetrix GeneChip Human Transcriptome Array 2.0. by AROS Applied Biotechnology A/S (Aarhus, Denmark); RNA expression was compared with placebo (ultrapure water) treated samples. Data analysis was performed using Transcriptome Analysis Console (Affymetrix) and MetaCore (Thompson Reuters) software programs.

2.4. Chick Chorioallantoic Membrane (CAM) assay

The Chick Chorioallantoic Membrane (CAM) assay was performed as described by Sys *et al.*: 6 days after (pre-treated, 100 nM) tumor cell transfer to the fertilized eggs, CAM was microscopically scored and histologically examined after H&E staining [17,18]. For quantification of microscopically observed neovascularisation, an average CAM score was calculated: the number of blood vessels in the 1 mm diameter ring around the 2 mm radius tumor centre was determined. Significant differences were evaluated using the Mann–Whitney U test.

2.5. Cytokine profiling

Cytokine expression was investigated using the Human XL Cytokine Array kit (R&D Systems, Abingdon, United Kingdom), following the instructions of the supplier. In brief, cell supernatant was obtained after 24 hours of incubation with Phr0662 (100 nM) and 102 different cytokines analyzed in duplo using spotted capture antibodies, followed by incubation with detection antibodies and chemiluminescent visualization. Membranes are finally exposed to X-ray films for 1-10 minutes. The mean (blank corrected, n = 2) pixel density is calculated using ImageJ

software and compared with placebo treatment. Significant differences were evaluated using the independent samples t-test.

2.6. Phospho-receptor tyrosine-kinase array

A human phospho-receptor tyrosine kinase (RTK) array kit (R&D Systems, Abingdon, United Kingdom) was used to detect phosphorylation levels of 49 different RTK after Phr0662 treatment (100 nM) of HCT-8/E11 cells, following the manufacturer's instructions. Cell lysates were prepared after 5 minutes of peptide incubation and incubated with capture antibodies (in duplo). Previous experience with the receptor tyrosine kinase assay showed that a 5 minutes exposure time is a generally acceptable exposure time to obtain a sufficient degree of receptor activation/phosphorylation without dropping down to basal phosphorylation levels due to phosphatase activity. Incubation was followed by treatment with detection antibodies and Horseradish Peroxidase for chemiluminsescent visualization. X-ray films were again analyzed for mean (blank corrected, n = 2) pixel density and compared with placebo results using the ImageJ software program. Significant differences were evaluated using the independent samples t-test.

3. RESULTS

3.1. Epithelial to mesenchymal (EMT)-like transition and related cell migration activities

To investigate the potential role of chemically diverse quorum sensing peptides in oncology [19, Wynendaele et al., unpublished results), we explored their morphologic effect on a human epithelial colon cancer cell line, *i.e.* HCT-8/E11. Three quorum sensing peptides, or metabolites thereof, were found to significantly induce tumor cell invasion through a collagen type I extracellular matrix: Phr0662 (ERNNT, 98.24%), an EntF-metabolite (SNLVECVFSLFKKCN, 80.63%) and an EDF-analogue (NWN, 98.40%) (Figure 1), while others, *e.g.* cAM373

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(AIFILAS, 97.82%) and iAD1 (LFVVTLVG, 97.74%) [20], both originating from *Enterococcus faecalis*, do not induce invasive characteristics. This epithelial to mesenchymal (EMT)-like process (*i.e.* observation of invasive cellular extensions) is one of the main mechanisms involved in colorectal cancer metastasis, establishing metastatic disseminations with potential life-threatening consequences [21].

Peptide Phr0662 is synthesized by *Bacillus* species [22]. The quorum sensing peptide designated Extracellular Death Factor (EDF), originally described by Kolodkin-Gal *et al.*, is responsible for the *mazEF* and *chpBIK*-mediated cell death in *Escherichia coli*. This intestinal commensal bacterium synthesizes this EDF peptide during logarithmic growth stages when stress-situations are induced [23,24]. Our study now enlarges the role of EDF to mammalian cells. EntF is synthesized by *Enterococcus faecium*, a commensal bacterium in healthy humans and animals, which is responsible for a number of nosocomial infections [25]. *Enterococcus faecium*, together with *Enterococcus faecalis*, is the primary source of reactive oxygen species (ROS), causing genomic alterations which are correlated to colorectal cancer [26]. However, based on the results of this study, its quorum sensing peptide (or its metabolite) can contribute to metastatic tumor behavior as well.

The microscopically observed invasive characteristics were further corroborated by whole transcriptome analyses on both placebo and peptide-treated tumor cells (Table 1). RNA extraction was performed on a heterogenous mixture (*i.e.* cells with and without induced invasive cellular extensions) instead of on isolated HCT-8/E11 cells, and only two duplicates were performed in this exploratory study, so the standard (parametric) ANOVA p-values are only given for the sake of completeness but are not meant to conclude any statistical significance (requiring more data and non-parametric evaluation). This exploratory study was meant to observe trends based on the mean fold-change. The up- or downregulation of certain genes

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supported the phenotypic observations of cellular invasiveness: *e.g.* the upregulation of histone cluster 1 H4 (*HIST1H4A-F/H-L*) and *CXorf61* (Cancer/Testis Antigen 83 (*CT83*) or Kita-Kyushu Lung Cancer Antigen (*KKLC1*)) is associated with cell cytoskeleton remodelling. Histone H4 is involved in different biological pathways through its link with β-arrestin 1, activin A and Notch1. Via β-arrestin 1, Histone H4 is linked to cell migration and metastasis in colon cancer *in vivo* [27]. The Histone H4-activin A link is again associated with cell migration and cytoskeleton remodeling (via Smad3) and cell proliferation (via Smad4) in colon cancer [28,29]; in normal human epithelial cells, activin A can induce EMT as well. Finally, via the link with Notch1, in colon cancer, it causes metastasis and cell invasion; a deregulation of the Notch signaling pathway thus affects EMT and tumor aggressiveness [30,31]. Through its link with micro-RNA 520h (*miR-520h*), *CXorf61* upregulation is associated with increased tumor cell mobility and enhanced *in vitro* cell invasion activity [32]. Moreover, downregulation of micro-RNA 558 (*miR-558*) is associated with a bad prognosis of colon cancer outcome, indicating tumor metastases and thus EMT-like behaviour [33]. Micro-RNA 644a (*miR-644a*) functions by directly binding to its target site in the 3' untranslated region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, thereby significantly repressing their expression [34]. Downregulation of *miR-664a* thus upregulates β-actin levels, leading to increased cell motility and cell migration [31].

3.2. Angiogenesis confirms metastatic potential of quorum sensing peptides

Downregulation of microRNA 222 (*miR-222*), as observed 24 hours after Phr0662 and EDF-analogue peptide addition, is associated with tumor growth reduction (through PUMA) and apoptosis induction [35], next to a bad cancer prognosis [33]. MicroRNA 222 is also highly expressed in endothelial cells, possessing anti-angiogenic properties through its targets c-kit

(mast/stem cell growth factor receptor kit, CD117), p27Kip1, p57Kip2 (cyclin-dependent kinase inhibitor 1C) and cyclin G1 [36]. The downregulation after quorum sensing peptide addition thus initiates angiogenesis. This angiogenic effect was analyzed for Phr0662, seen the highly changed transcriptomic profile of the cancer cells after 24 hours of treatment. Our Chick Chorioallantoic Membrane (CAM) assay results confirmed these pro-angiogenic properties (Figures 2a and 2b): its addition clearly promoted neovascularisation, thereby facilitating metastasis of the tumor. Without tumor cells, the addition of the peptide alone did not promote neovascularisation, confirming the crosstalk between the peptide and the cancer cells (*i.e.* indirect angiogenic effect) (Figure 2c). Histological evaluation of the membrane confirmed the invasive tumor cell properties through the chorion into the mesoderm, induced by the quorum sensing peptide (Figure 2d).

Cytokine screening after Phr0662 treatment of the cancer cells revealed pro-angiogenic effects as well (Figure 3): the quantitative increase of the vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), interleukin 6 (IL-6) and stromal cell-derived factor 1 (SDF-1α or CXCL12) all indicated an induction of angiogenesis by the investigated peptide [37]. IL-6 was also found to drive tumor initiation and subsequent growth and metastasis, with a poor clinical outcome observed in colorectal cancer patients. Through its link with STAT3, it promotes the expression of VEGF and FGF, supporting the rapid vascularization required for tumor growth and metastasis [38,39].

3.3. Phr0662 targets the epidermal growth factor receptors EGFR and ErbB2

Receptor Tyrosine Kinase (RTK) screening of Phr0662-treated HCT-8/E11 cells revealed a 2 times higher ErbB2 (HER2/neu) and a significant increase in epidermal growth factor receptor

(EGFR, HER1 or ErbB1) phosphorylation compared to the placebo cell samples (Figure 4). A comparable activity is observed for the Phr0662 quorum sensing peptide and the autocrine ligand TGF-α, 5 minutes after HCT-8/E11 treatment.

The EGFR is overexpressed in many types of cancers, especially colorectal cancer, and is significantly associated with tumor-node-metastasis and a more aggressive clinical tumor progression [40]. This regulation of cellular functions is achieved by activating different intracellular signaling cascades: phosphatidylinositol 3-kinase (PI3K/Akt), the ras/raf/MEK/mitogen-activated protein kinase (MAPK) and the signal transducer and activator of transcription (STAT) pathways. EGFR signaling regulates the synthesis and secretion of different angiogenic growth factors as well, including VEGF; overexpression of ErbB2 also leads to increased angiogenesis [41].

4. DISCUSSION

Despite our small quorum sensing peptide set, our results suggest that some quorum sensing peptides can exert (part of) their effects through the epidermal growth factor receptor (EGFR), thereby activating the Ras/raf/MEK/MAPK, PI3K/Akt and STAT intracellular signaling cascades [41], leading to an altered gene transcription and finally tumor metastasis (Figure 5). β-arrestin 1, which is linked to HIST1H4 and thus possibly upregulated after peptide treatment, can activate the EGFR pathway as well, thereby playing a pivotal role in colon cancer metastasis [27]. Other mechanistic plausible hypotheses, such as the possibility of quorum sensing peptide activation of G-protein coupled receptors (GPCR), followed by an induced EGFR phosphorylation (*i.e.* EGFR transactivation) [42], however still need to be investigated. Next, Smad proteins (*i.e.* Smad 2, 3 and 4) can be stimulated through Activin A binding with its ActRII receptor, linking both cell

cytoskeleton remodeling and cell migration to quorum sensing peptide presence [28,43]. The upregulation of *HIST1H4* can be associated with increased Notch1 stimulation, again connected to cancer metastasis, together with angiogenesis and NF-κB production [30,44]; NF-κB in turn contributes to the progression of colorectal cancer by regulating cell proliferation, angiogenesis and tumor metastasis [45]. Micro-RNA's block mRNA translation and thus impede the synthesis of specific proteins by recruiting the micro-RNA-induced silencing complex (miRISC) to target mRNAs [46]; downregulation of micro-RNA 222 and 644a thus increases KIT and β-actin protein expression, respectively, thereby promoting endothelial cell proliferation and migration (angiogenesis) [36,47] and tumor cell invasiveness and motility [34]. β-actin can be upregulated through the IL-6 receptor pathway as well: the increased IL-6 expression after quorum sensing peptide treatment, as observed with our cytokine array, activates β-actin phosphorylation, again promoting tumor cell migration [48]. Both the transcriptome outcomes and cytokine results thus correspond well and are visually confirmed by the collagen invasion and CAM assays. A good correlation is observed for VEGF as well: an increased IL-6 pathway activation leads to VEGF upregulation [49], which is again observed in our cytokine array; VEGF can then trigger the VEGF receptor (VEGFR) pathway in endothelial cells, leading to altered gene transcription and subsequently angiogenesis. The pro-angiogenic cytokines SDF-1α and HGF both activate the Ras/raf/MEK/MAPK, PI3K/Akt and STAT intracellular signaling cascades through CXCR4 and MET receptor binding, respectively. These cytokine-receptor axes are involved in tumor progression, angiogenesis and metastasis [50,51]. Future investigations concerning the identification of quorum sensing peptide targets, should however be performed to confirm our initial findings and to have more mechanistic insights on the effect of these and other quorum sensing peptides on colon cancer cells.

These exploratory *in vitro* results thus indicate metastasis-promoting characteristics for some quorum sensing peptides. Assuming the *in vivo* presence of these molecules influenced by the individual microbiota composition, our findings may potentially have a great impact on the patient's health, with effects on cancer metastasis. Exploiting our findings, it would be interesting to consider how we can prevent these pro-metastatic effects? First, it may be important to adjust the patient's life style, including diet and hygiene measures. The influence of food pattern cannot be neglected, *e.g.* switching from a low-fat, plant (non- or limited digestible) polysaccharide-rich diet to a high-fat, high-sugar diet can shift the structure of the gut microbiota within a single day [52,53]. Appropriate hygiene measures will diminish the risk of unwanted infections.

6. REFERENCES

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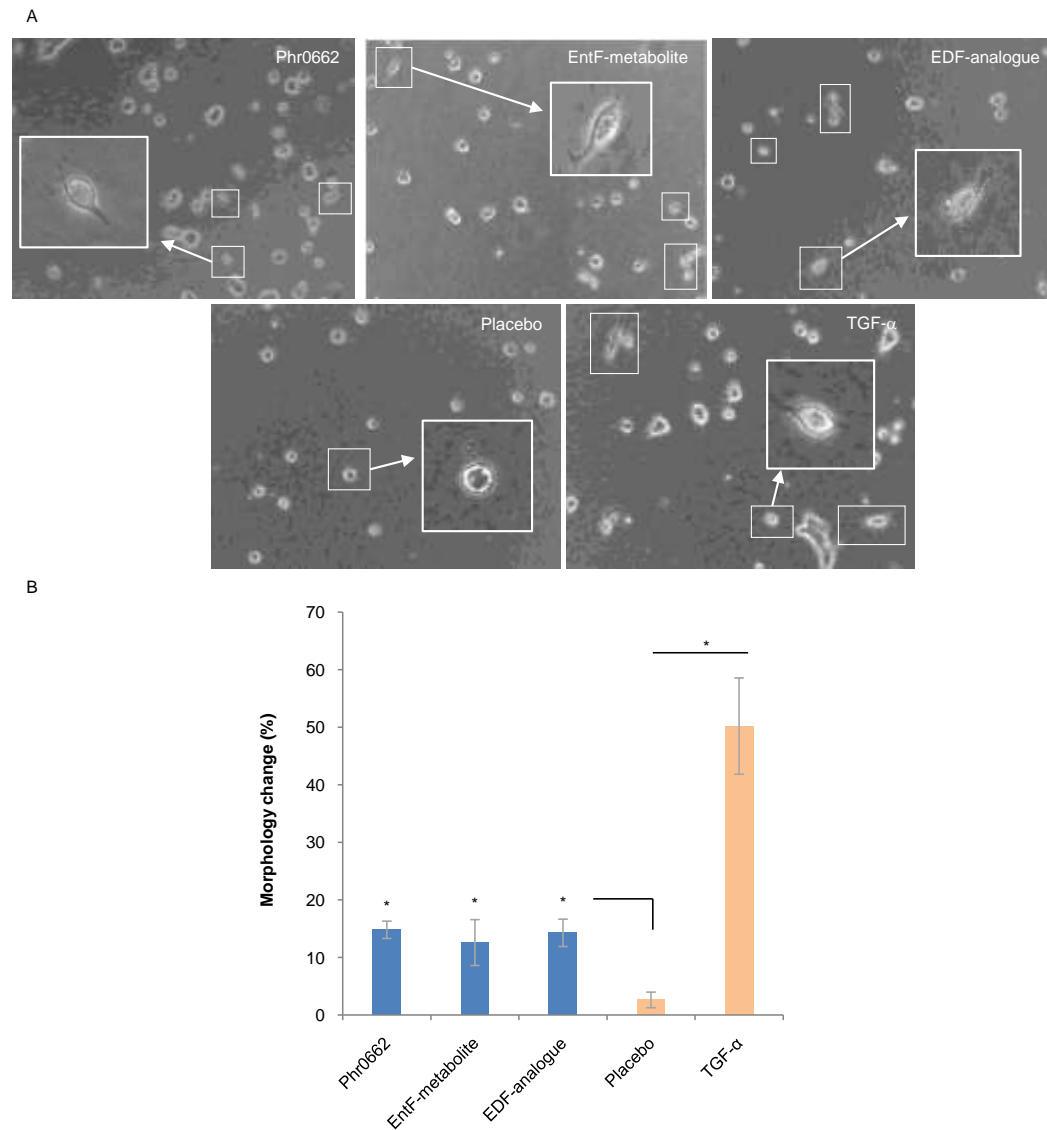
7. FIGURES

Figure 1: Collagen invasion of 3 quorum sensing peptides at 10 nM. (A) Morphologic changes of HCT-8/E11 cells, observed 24 hours post-treatment with Phr0662 (ERNNT), EntF-metabolite (SNLVECVFSLFKKCN) and EDF-analogue (NWN). Placebo sample serves as negative control and TGF-α (0.1 µg/ml) as the very strong positive control. Samples were additionally tested at 100 nM and 1 µM, showing invasion at these concentrations as well (data not depicted). **(B)** Mean (n = 3) percentage of cancer cells with induced morphology changes: a significant difference is observed between the peptide and placebo treatments (* $p < 0.05$ (Mann-Whitney U test)). Error bars represent SEM values.
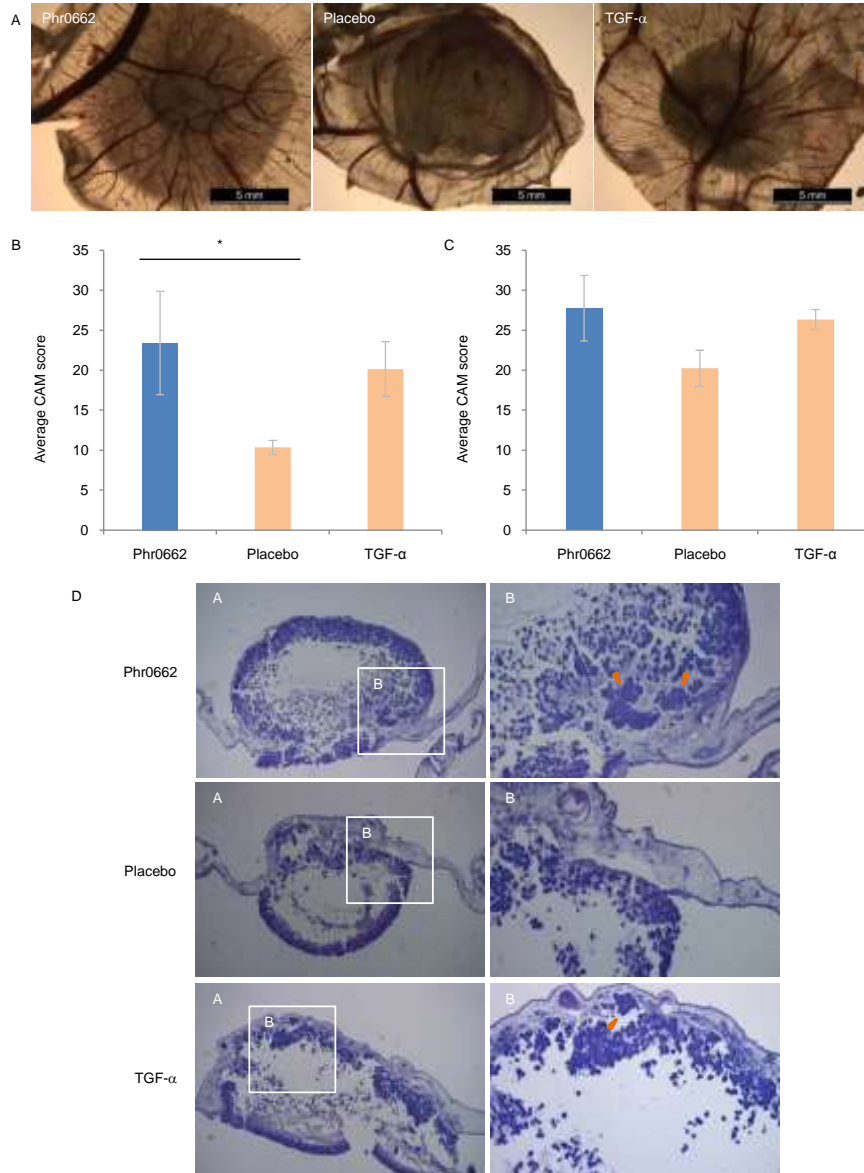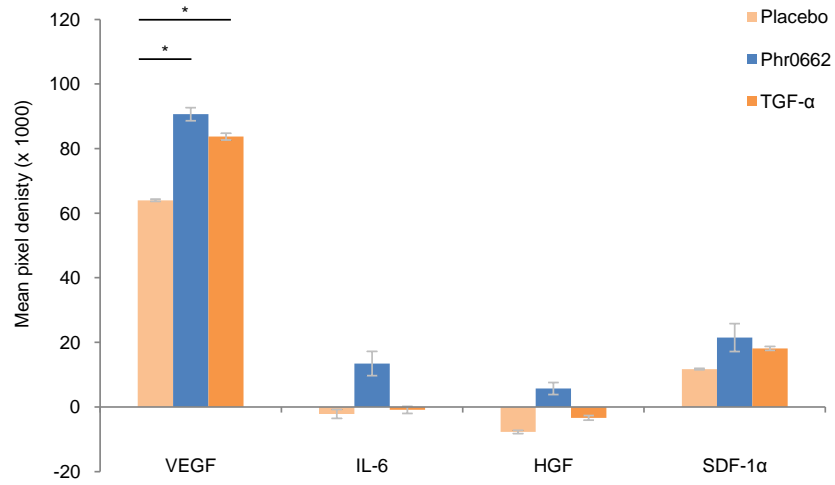
Figure 2: Neovascularisation and cell invasion after Phr0662 (100 nM) addition to HCT-8/E11 cells on CAM.

Placebo sample serves as negative control and TGF-α (0.1 μg/ml) as positive control. **(A)** Macroscopic images, observed 6 days after (pre-treated) tumor cell transfer to the eggs. **(B)** Average CAM Score (*i.e.* number of blood vessels in the 1 mm diameter ring around the 2 mm radius centre) in the presence of tumor cells (mean ± SEM, n = 5 (Phr0662), n = 7 (TGF-α) or n = 3 (placebo)). **(C)** Average CAM Score when no tumor cells are present (mean ± SEM, n = 8 (Phr0662 and placebo) or n = 6 (TGF-α)). **(D)** Histological H&E evaluation of the CAM, with orange arrowheads indicating invasion of the tumor cells into the mesoderm.
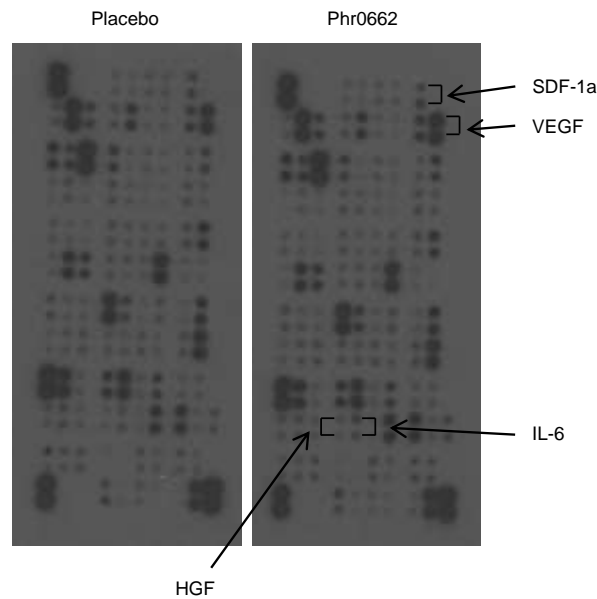
* p < 0.05 (Mann-Whitney U test)

Figure 3: Effect of quorum sensing peptide Phr0662 (100 nM) on cytokine expression in HCT-8/E11 colon cancer cells. Placebo sample serves as the negative control and TGF-α (0.1 μg/ml) as the positive control. **(A)** Mean (n = 2, ± SEM) pixel density of some pro-angiogenic cytokines, demonstrating increasing cytokine concentrations after Phr0662 addition (24 hours of peptide incubation); * $p < 0.05$ (independent samples t-test). **(B)** Human XL Cytokine Array visually demonstrating increased cytokine levels after peptide treatment of HCT-8/E11 cells, compared to placebo samples. The selected pro-angiogenic cytokines (in duplo) are indicated.
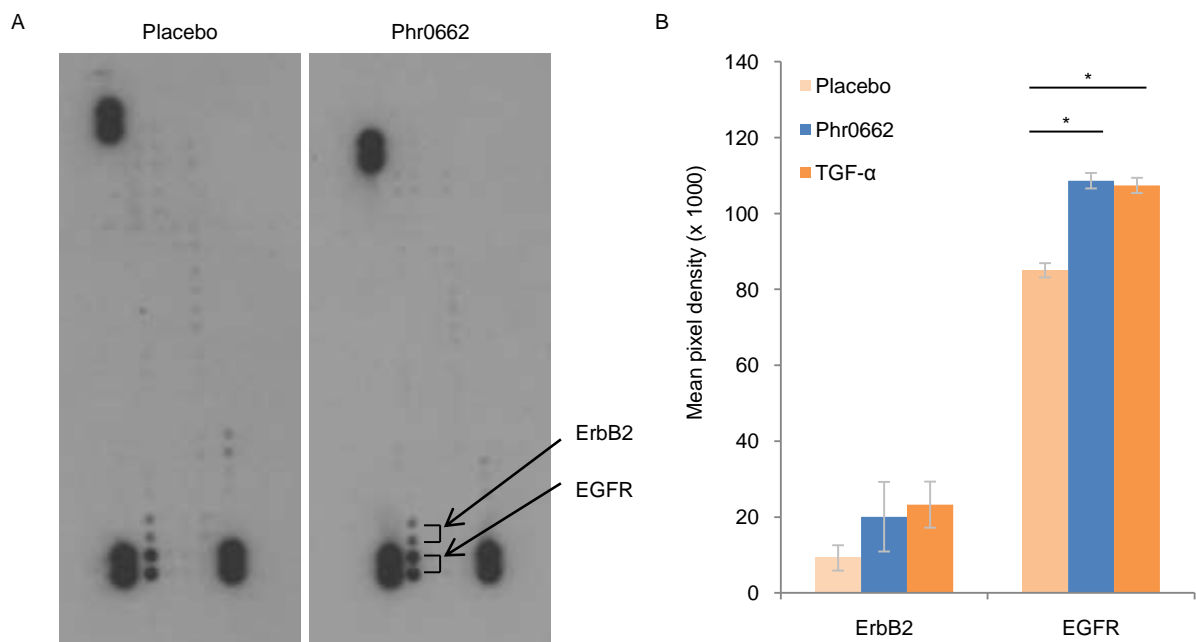
Figure 4: Effect of quorum sensing peptide Phr0662 (100 nM) on receptor tyrosine kinase activation in colon cancer cells. Placebo sample serves as the negative control and TGF-α (0.1 µg/ml) as the positive control. **(A)** Receptor tyrosine kinase (RTK) array demonstrating the tyrosine phosphorylation level of 42 RTK after Phr0662 treatment of HCT-8/E11. Each RTK is spotted in duplicate and the phosphorylated EGFR and ErbB2 are indicated. **(B)** Mean (n = 2, ± SEM) pixel density of ErbB2 and EGFR spots after Phr0662 addition (5 minutes of peptide incubation); * $p < 0.05$ (independent samples t-test). TGF-α serves as the cognate ligand for EGFR, demonstrating thus EGFR binding properties for the quorum sensing peptide.
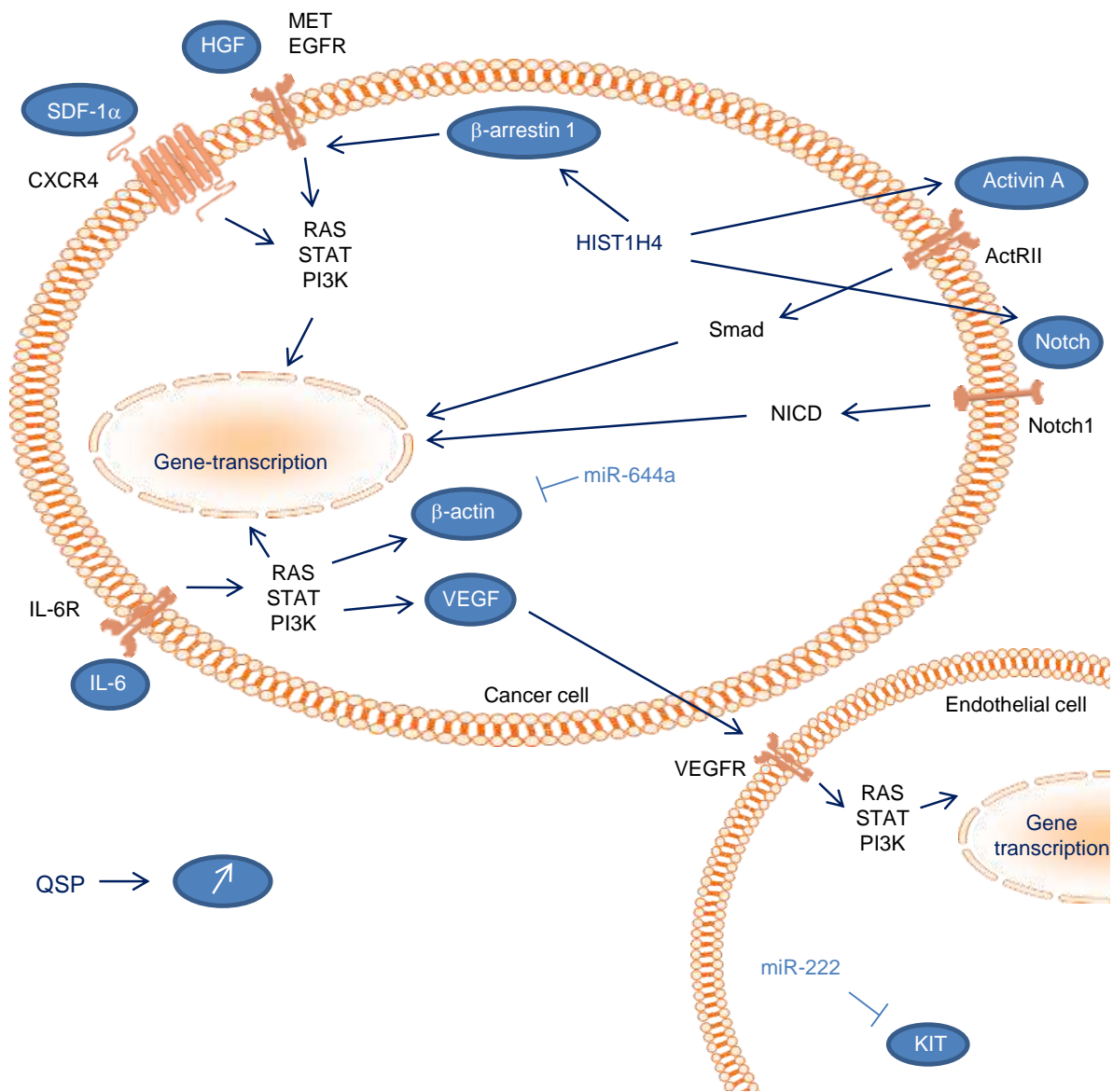
Figure 5: Possible pathway-map for quorum sensing peptides in tumor progression. Quorum sensing peptides promote cancer metastasis and tumor progression by activating different receptor pathways.

8. TABLES

Table 1: Selection of transcriptome alterations after quorum sensing peptide addition to HCT-8/E11 cells. Gene expression 24 hours post-treatment, compared to placebo samples. Mean fold change is calculated from duplicate samples (cut off: > 1.5 or < -1.5).

	Gene symbol	Fold change	ANOVA p-value	Description
Phr0662				
UP-REGULATED	SCARNA10	1.99	0.024	Small Cajal body-specific RNA 10
	RN5S348	1.69	0.272	RNA, 5S ribosomal 348
	MIR4521	1.60	0.335	MicroRNA 4521
	HIST1H4A-F/H-L	2.02	0.647	Histone cluster 1, H4d,…
	CXorf61	1.54	0.054	Chromosome X open reading frame 61
	SNORA26	1.63	0.095	Small nucleolar RNA, H/ACA box 26
DOWN-REGULATED	RPL36AP33	-1.75	0.576	Ribosomal protein L36a pseudogene 33
	RNU6-51	-1.99	0.565	RNA, U6 small nuclear 51
	MIR222	-1.60	0.593	MicroRNA 222
	SNORD121B	-1.88	0.242	Small nucleolar RNA, C/D box 121B
	SNORD85	-1.84	0.323	Small nucleolar RNA, C/D box 85
	ARL17A	-1.74	0.543	ADP-ribosylation factor-like 17A
	RNU7-47P	-1.72	0.463	RNA, U7 small nuclear 47 pseudogene
EntF-metabolite				
UP-REGULATED	RN5S62	1.79	0.534	RNA, 5S ribosomal 62
	OR52E6	1.58	0.479	Olfactory receptor, family 52, subfamily E, member 6
DOWN-REGULATED	RNU7-47P	-1.83	0.340	RNA, U7 small nuclear 47 pseudogene
	MIR597	-1.65	0.599	MicroRNA 597
	MIR548T	-1.58	0.597	MicroRNA 548t
	RNY4	-1.56	0.007	RNA, Ro-associated Y4
	MIR558	-1.68	0.090	MicroRNA 558
	LOC286437	-1.64	0.390	Uncharacterized LOC286437
	RNY4P2	-1.61	0.231	RNA, Ro-associated Y4 pseudogene 2
	MIR644A	-1.98	0.443	MicroRNA 644a
	MIR3975	-1.63	0.088	MicroRNA 3975
	MIR553	-1.95	0.328	MicroRNA 553
EDF-analogue				
UP-REGULATED	OR52E6	1.70	0.384	Olfactory receptor, family 52, subfamily E, member 6
DOWN-REGULATED	MIR222	-1.51	0.641	MicroRNA 222
	RNU7-19P	-1.68	0.008	RNA, U7 small nuclear 19 pseudogene

Table S1. Peptide information

Quorumpeps ID	Sequence
2	FNTIPSY
5	Ac-CGSLF, thiolacton linkage between C1 and F5
7	FNTWPSY
10	ADLPFEF
11	AGTKPQGKPASNLVECVFSLFKKCN
13	AIFILAS
14	AITLIFI
15	AKDEH
16	AKTVQ
17	ALILTLVS
18	ARNQT
19	NNWNN
22	CVGIW, thiolacton linkage between C1 and W5
24	CTFTLPGGGGVCTLTSECIC
25	CVFSLFKKCN
28	DIRHRINNSIWRDIFLKRK
30	DLRGVPNPWGWIFGR
31	DLRNIFLKIKFKKK
32	DMCNGYF, thiolacton linkage between C3 and F7
34	DRVGA
40	DSVCASYF, thiolacton linkage between C4 and F8
42	DWRFLNSIRDLIFPKRK
44	EKMIG
45	EMRISRIILDFLFLRKK
46	EMRKSNNNFFHFLRRI
47	EMRLPKILRDFIFPRKK
49	EQLSFTSIGILQLLTIGTRSCWFFYCRY
50	ERGMT
51	ERNNT
52	ERPVG
53	ESRLPKILLDFLFLRKK
54	ESRLPKIRFDFIFPRKK
55	ESRVSRIILDFLFQRKK
56	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP
58	DSRIRMGFDFSKLFGK
62	ESRISDILLDFLFQRKK
71	QNCPNIFGQWM, lacton linkage between S3 and M11
75	SINSQIGKATSNLVECVFSLFKKCN
76	SNLVECVFSLFKKCN
81	FNTIPKY
82	NTIPKY

Table S1. Peptide information (continued)

Quorumpeps ID	Sequence
84	FFNTCPSY
85	FNTCPSY
92	FHWWQTSPAHFS
93	FLVMFLSG
97	QNSPNIFGQWM, lacton linkage between S3 and M11
99	GKAEF
100	GKATSSISKCVFSFFKKC
101	GLWEDILYSLNIIKHNNTKGLHHPIQL
102	GLWEDLLYNINRYAHYIT
103	GNWNN
105	GSQKGVYASQRSFVPSWFRKIFRN
107	GVNACSSLF, thiolacton linkage between C5 and F9
111	GWWEDFLYRFNIIEQKNTKGFYQPIQL
121	ILSGAPCIPW
123	IRFVT
125	KSSAYSLQMGATAIKQVKKLFKKWGW
132	LFSLVLAG
133	LFVVTLVG
134	LPFEF
135	LPFEH
137	LVTLVFV
138	MAGNSSNFIHKIKQIFTHR
140	MKAEH
143	MPFEF
146	NEVPFEF
147	NGWNN
148	YSTCDFIM, thiolacton linkage between C4 and M8
151	NNGNN
152	NNNWNNN
153	NNWGN
154	NNWNG
155	NWN
156	FNTIP
157	FNTWP
160	QKGMY
162	QRGMI
164	SDLPFEH
165	SDMPFEF
166	SGSLSTFFLLFNRSFTQALGK
174	SGSLSTFFRLFNRSFTQALG
176	SGSLSTFFRLFNRSFTQALGK

Table S1. Peptide information (continued)

Quorumpeps ID	Sequence
177	SGSLSTFFRLFNRSFTQALGKIR
180	SGSLSTFFRLFNRSQTQALGK
184	SIFTLVA
186	SKDYN
188	SLSTFFRLFNFSFTQALG
191	SRKAT
192	SRNAT
193	SRNVT
206	SYPGWSW
207	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK
208	TNRNYGKPNKDIGTCIWSGFRHC
210	VAVLVLGA
212	VPFEF
214	WPFAHWPWQYPR
215	FNTWPKY
218	YNPCSNYL, thiolacton linkage between C4 and L8