Novel sites of CCK2R expression: Characterisation and functional significance of gastrin receptor expression in gastrointestinal myofibroblasts and human melanoma cancer cells

dual Ph.D. Thesis

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Abbreviations:

ASI (Acid secretion inhibitor), ATM (Adjacent tissue myofibroblast), BCC (Basal cell cancer), CAF (Cancer-associated fibroblasts), CAM (Cancer-associated myofibroblasts), CCK2R/CCKB (Cholecystokinin-2 / -B / gastrin receptor), CCK-8 / CeCo (Cell counting Kit-8), CNS (Central nervous system), CXCL12 (C-X-C motif chemokine ligand 12), DAB (3,3-diaminobenzidine), ECL (Enterochromaffin-like cell), ECM (Extracellular matrix), EdU (5-Ethynyl-2´-deoxyuridine), EGF (Epidermal growth factor), ELISA (Enzyme-linked immunoassay), ERK (Extracellular signal-regulated kinase), FGF (Fibroblast growth factor), FITC (Fluorescein isothiocyanate), FM (Full medium), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), GI (Gastrointestinal tract), GIST (Gastrointestinal stromal tumour), GLP (Glucagon-like peptide), GPCR (G-protein-coupled receptors), *H. pylori* / *Hp* (Helicobacter pylori), H2RB / H2RA (Histamine type 2 receptor antagonist), HB-EGF (Heparin-binding EGF-like growth factor), hG17 (Human heptadecapeptide gastrin), HGF (Hepatocyte growth factor), IGF (Insulin-like growth factor), JNK (c-JUN N-terminal kinase), KGF (Keratinocyte growth factor), MAPK (Mitogen-activate protein kinase), MM (Malignant melanoma), MMP (Matrix metalloprotease), MSC (Mesenchymal stem/stromal cell), NTM (Normal tissue myofibroblast), OD (Optical densitometry), PDGF (Platelet derived growth factor), PPI (Proton pump inhibitor), qPCR (Quantitative polymerase chain reaction), SF (Serum free), SIDLS (Stable Isotope Dynamic Labelling of Secretomes), SMA (Smooth muscle actin), TAM (Tumour-associated macrophage), TGF (Transforming growth factor), TIMP (Tissue inhibitors of matrix metalloproteases).

1 Introduction

There is growing recognition of the role of gastrin in gastrointestinal cancers including those of the oesophagus, stomach, pancreas and colon (Ferrand et al. 2006). The concept that tumours are wounds that do not heal is well recognised (Dvorak 1986, Desmouliere et al. 2004). In this context it is notable that expression of gastrin receptors (CCK2Rs) occurs during wound healing in the stomach. Schmassmann and Reubi (2000) used *in situ* hybridization to show increased CCK2R in rat stomach following cryo-ulceration (Schmassmann et al. 2000). Ashurst *et al.* (2008) then showed that after gastric cryo-ulceration in mice, CCK2R expression was co-localised with α-smooth muscle actin (α-SMA) which is a biomarker for myofibroblasts (Ashurst et al. 2008). However these data are derived from rodent models and the specific physiological mechanisms leading to *de novo* receptor expression and cell recruitment in a clinical context remain unknown.

The data therefore raise the possibility that the gastrin receptor CCK2R is also expressed in activated myofibroblasts, but even so the significance of this is poorly understood and there has been no direct study of CCK2R expression in human myofibroblasts. Because myofibroblasts are motile the data also suggest the hypothesis that gastrin regulates migration of these cells via CCK2R.

Melanoma arising from malignantly transformed melanocytes (Paek et al. 2008) is one of the most dangerous forms of skin cancer with a high metastatic potential (Ingraffea 2013). Data from Human Protein Atlas (HPA), the Cancer Genome Atlas, Genotype-Tissue Expression (GTEx) project and CAGE data from the FANTOM5 project reveal expression of CCK2R in melanoma. These findings raise the interesting question of whether there is a relationship between hypergastrinaemia and the progression of melanoma. Previous studies suggested that gastrin might have pleiotropic effects on melanoma cells, but the relevant pathways and receptors are far from fully described and the available data are inconclusive (Mathieu et al. 2005). The issue is important since patients with hypergastrinaemia due to decreased acid production as a consequence of long-term PPI therapy, *H.pylori* infection or atrophic corpus gastritis might therefore have a higher risk of developing distant metastasis of coincidental melanoma, compared to patients with normal circulating gastrin (Heidelbaugh et al. 2012, Smolka et al. 2012, Watari et al. 2014, Dacha et al. 2015, Haastrup et al. 2018).

2 Aims and objectives

The aim of this research was to identify novels sites of CCK2R expression and characterise their functional significance. As previously highlighted by a number of authors despite the broad knowledge we have of gastrin and the CCK2 receptor new aspects of their multiplex role in human physiology and pathophysiology are still emerging. Stromal myofibroblasts form a niche of cells which are important determinants of the tumour microenvironment and tissue regeneration. The recent concept that tumours can also be considered as chronic non healing wounds adds another link to this issue. There is a suggesting in the literature that myofibroblasts of the gut might express the gastrin receptor, however the role of this is far from clear. Hence we decided to screen the complete alimentary tract for myofibroblasts expressing the CCK2 receptor with experiments aimed at understanding the functionality of it.

Elevated plasma concentration of gastrin is a common complication of long term acid secretion inhibitor therapy. Open source protein databases provide evidence that melanoma cancer cells express the gastrin receptor. Taken together with the possibility that dermal stromal cells might also express CCK2R we decided to investigate a possible relation between gastrin and melanoma behaviour both *in vitro* and *in vivo,* including the prospective collection of histology and serum samples from patients with different stage melanomas.

Overview of objectives:

1. To characterise CCK2R expression in myofibroblasts derived from different parts of the GI tract. Investigate receptor functionality and the effect of gastrin on stromal cell growth and motility.

2. To evaluate CCK2R expression in biopsy samples from melanoma patients, to determine serum gastrin concentrations in these patients and examine possible correlating with pTNM stages, *H.pylori* infection and acid secretion inhibitor consumption.

3. To investigate the effect of gastrin and stromal cells on melanoma cell proliferation, adhesion, migration and invasion.

4. To analyse the secretome of gastrin-treated melanoma cells and to identify relevant pathways

3 Materials and methods

3.1 *Cell culture*

Unless otherwise stated experiments were performed on human melanoma cell lines G361 and SK-MEL-2, dermal fibroblasts, human mesenchymal stromal cells, human primary gastric CAMs or ATMs from colonic, pancreatic or oesophageal cancer, NTMs from healthy stomach and oesophagus, and myofibroblasts from chronic pancreatitis (Czepan et al. 2012, Holmberg et al. 2012, Kumar et al. 2014).

3.2 *Intracellular calcium*

Subconfluent cells were loaded with the Ca^{2+} fluorophore Fluo-4 AM as previously described (Homolya et al. 1993, Kao 1994). Myofibroblasts and melanoma cells were stimulated with hG17 (10 nmol/L) or ionomycin (1 µmol/L) as a positive control.

3.3 *Immunocytochemistry*

Myofibroblasts and melanoma cultures were stained for CCK2R using polyclonal IgG antibody visualized with FITC conjugated anti-rabbit secondary antibody or peroxidase (DAB) reaction as described before (Wroblewski et al. 2003, Kumar et al. 2014).

3.4 *Patient recruitment and database*

Whole blood was collected from patients referred to the Department of Dermatology, University of Szeged between February and May 2017 with the initial diagnosis of malignant melanoma or basal cell cancer. Paraffin embedded and formalin fixed patient samples for immunohistochemistry were retrieved from tissue archives of the Departments of Dermatology and Pathology, University of Szeged.

3.5 *Gastrin radioimmunoassay*

Serum gastrin was determined by radioimmunoassay as described earlier (Dodd et al. 2019).

3.6 *H.pylori detection*

H.pylori antibodies were detected in serum samples of patients using a commercially available ELISA kit (Biohit Health Care, UK).

3.7 *qPCR*

Myofibroblasts (0.25 - 1.5 x 10^6) and melanoma cells (5 x 10^5 / Petri dish of 5 cm) were incubated in full media for 72h. Cells were lysed prior to RNA extraction and were probed for sets of human IGF-1, IGF-2 and CCK2R using TaqMan primer/probe sequences.

3.8 *EdU proliferation assay*

Myofibroblasts and melanoma cells were treated with hG17 (0.1 – 1.0 – 10.0 nmol/L) for 24 h. Proliferating cells were identified using a Click-iT™ EdU imaging kit (Invitrogen, Paisley, UK) as described earlier (Varga et al. 2017).

3.9 *CeCo proliferation assay*

Cell Counting Kit-8 assay (Dojindo Laboratories, Munich, Germany) was used to detect proliferative activity of melanoma cells.

3.10 *Flow cytometry*

Myofibroblasts and melanoma cells (10^6 seeded in T75 flasks) were treated with 10nM G17 or FM overnight. Cells were sorted using FACS Canto II flow cytometer as described earlier (Varga et al. 2017).

3.11 *Spheroids*

Skmel-2 cells (3,000 per spheroid droplet) were seeded and incubated up to six days with or without myofibroblasts (10,000 per well in 24 well plates). Growth was calculated based on surface area expressed as fold-increase.

3.12 *Organotypic cultures*

Organotypic cultures were prepared by seeding Skmel-2 melanoma cells (1x 10^6) on top of a 1:1 Matrigel (Corning, NY, USA):collagen-I (Millipore, MA, USA) layer with or without myofibroblasts (0.5 x 10^6). Invasion of cancer cells was measured to the contact region of the melanoma cell layer with Matrigel.

3.13 *Proteomic analysis of conditioned media*

Putative gastrin targets in Skmel-2 and G361 melanoma secretomes were identified using Stable Isotope Dynamic Labelling of Secretomes (SIDLS) technique (Kristensen et al. 2012, Hammond et al. 2018). Filtered data were uploaded to Panther v.10. to predict the significantly enriched ($p<0.05$) protein classes, molecular functions and biological process.

3.14 *Western blots*

Media samples were concentrated with StrataClean resins (Agilent Technologies Ltd) and processed for Western blotting as previously described (Hemers et al. 2005) using antibodies to MMP-2, TIMP-3 (R&D Systems), TIMP-1, TIMP-2, and prosaposin (R&D Systems).

3.15 *ELISA*

Media and serum samples obtained from melanoma and basal cell cancer patients were analysed for TIMP-3 and MMP-2 using precoated colorimetric ELISA plates.

3.16 *Migration and invasion assays*

BD inserts or BD BioCoat™ Matrigel™ chambers (SLS, Nottingham, UK) were used for migration and invasion chemotactic assays respectively as described previously (Varro et al. 2004). Gastrin treatment was applied for 24 h at 10 nmol/L.

3.17 *Transfection of melanoma cells with siRNA for MMP-2*

Melanoma cells were transiently transfected using Amaxa™ Cell line Nucleofector™ kits V using the program T-19 for high transfection efficiency (Amaxa, Köln, Germany) as described earlier (Kumar et al. 2014).

3.18 *Immunohistochemistry*

Samples from MM, BCC patients and controls from gastric and bladder cancer patients were stained for CCK2R, MMP-2, TIMP-3 and Melan-A in an automatic and standardized manner with Leica BOND Max Autostainer.

3.19 *Statistics*

Results were calculated as mean ± standard error of means (SEM). Parametric tests including student t-test, Fisher exact test, Pearson's chi-squared test and ANOVA were performed on the data as appropriate. Where normal distribution failed Mann-Whitney and Kruskal-Wallis tests were applied with significance at $p<0.05$ using Systat Software Inc. v. 12.0 (London, UK) and IBM SPSS Statistics V26.0 (New York, USA) unless otherwise stated.

4 Results

4.1 *Myofibroblasts*

4.1.1 *Systematic screening of GI tract myofibroblasts revealed consistent expression of CCK2 receptors in a subset of cells*

We examined expression of CCK2R in a number of myofibroblast populations recovered from normal tissue, cancer or cancer-adjacent tissue from oesophagus, stomach, omentum, colon and pancreas. We furthermore examined myofibroblast cell lines derived from patients with GI disorders i.e. chronic pancreatitis, vipoma, GIST or pernicious anaemia. In all cases, only a subpopulation of cells were positive for CCK2R, ranging from 1 to 6%. Highest expression was seen in colon (5.96 ± 1.41%), followed by oesophagus (5.63 ± 0.81%) and stomach (3.87 ± 0.49%). Myofibroblasts derived from patients with pernicious anaemia, chronic pancreatitis, Vipoma or GIST showed lowest expression. In the case of stomach and oesophagus, there was a significant difference (5.96% vs 0.39% and 5.63% vs 1.75%; $p < 0.05$ respectively) between CAMs and ATMs or NTMs.

4.1.2 *CAMs showed increased CCK2R expression compared to their ATM counterparts in patients with advanced lymph node metastasis*

Investigating expression of CCK2R in a microarray dataset of CAMs and ATMs prepared from 13 patients with gastric cancer (Balabanova et al. 2014) we found that in patients with high lymph node involvement (graded pN2-4), the expression of CCK2R was higher in 5 of 6 CAMs compared with their matched ATMs, whereas in the subgroup with low lymph node involvement (pN0-1) 6 of 7 CAMs expressed lower CCK2R compared with their matched ATMs and the difference was statistically significant ($P < 0.05$, Fisher exact test). When grouping patients into ATM and corresponding CAM pairs CCK2R expression was significantly higher in the cancer associated myofibroblast group in the case of patients with multiple lymph node metastasis (SCAM4/ATM4, pN2; S-CAM1/ATM1, pN2) compared to patients with a lower lymph node involvement (S-CAM2/ATM2, pN0; S-CAM3/ATM3, N1) where the converse was true.

4.1.3 *Gastrin increases intracellular calcium in a subset of myofibroblasts*

In order to determine whether myofibroblasts are capable of mounting a functional response to gastrin we examined changes in cytosolic calcium on administration of hG17. Administration of hG17 (10 nmol/L) produced a prompt increase in intracellular calcium in a subset of gastric CAMs (5.3 ± 1.4%).

4.1.4 *CCK2R expression is associated with the cell cycle*

After synchronisation, cells were examined for co-localisation of EdU (proliferation marker incorporated into the DNA) and CCK2R. In the conditions of the experiment most cells were negative for both EdU and CCK2R (79.5 ± 4.0%). There were substantial numbers of EdU positive cells that were CCK2R negative (15.9 ± 4.0%). CCK2R positive cells accounted for 5% of the total population. Interestingly, over 80% of cells expressing CCK2R were found to be labelled with EdU. In order to resolve the possibility of a cell cycle dependent receptor expression we performed kinetic experiments. As expected, cells incubated in FM exhibited significantly higher labelling with EdU compared with cells in SF medium after 4 h (28.7 ± 0.3 vs 5.2 ± 0.6% cells incorporating EdU, respectively, $p < 0.001$). However, while the proportion of cells showing EdU incorporation increased with duration of incubation in FM the proportion of CCK2R labelled cells remained relatively constant at 3-10% of total. CCK2R expression was highest in the earlier time points and then declined after about 4h in EdU. Similarly, the subpopulation of CCK2R positive cells that were not labelled with EdU (<1% of total) remained relatively constant. The data are consistent with transient expression of CCK2R by cells in S-phase with a possible loss of expression or inactivation thereafter.

4.1.5 *Gastrin stimulates migration and invasion of EdU-labelled and unlabelled gastric myofibroblasts*

There was a clear stimulation of both migration and invasion ($p<0.05$; ANOVA) by 10 nmol/L hG17 that was inhibited by the CCK2R antagonist 100 nmol/L L740093 in gastric myofibroblasts using transwell Boyden chemotactic assays.

Moreover there was hG17-stimulated migration of EdU labelled cells that was inhibited by L740093 ($p < 0.05$; ANOVA). However, only about 40% of the migrating cells were labelled with EdU suggesting that cells expressing CCK2R were also able to activate other cells via a paracrine pathway. Both the number of EdU labelled and non-labelled myofibroblasts increased after gastrin treatment and this correlated with concentration of hG17. Nonetheless the proportion of proliferating cells compared with non-EdU labelled ones, expressed as a percentage ($\Delta_{\Sigma\text{-EdU}}\Delta hG17$= -7.57%) showed a modest decrease with higher gastrin concentrations. This most likely puts emphasis on the main, direct route especially at higher gastrin concentrations

IGF receptor tyrosine kinase inhibitor AG1024 suppressed hG17-stimulated migration, and to a lesser extent invasion. Additionally, in the presence of hG17 the relative abundance of IGF-2 transcripts was 2.1 ± 0.1 fold higher than control ($p < 0.05$); IGF-1 transcript abundance was virtually undetectabl. GLP-2 stimulated myofibroblasts were used as positive controls for IGF detection (Shawe-Taylor et al. 2017).

4.2 *Melanoma cells*

4.2.1 *CCK2R is expressed in a subset of human melanoma cells*

Immunohistochemistry was performed on skin samples from patients with malignant melanoma. Healthy skin areas from the same patient served for comparison. A low proportion (2-3%) of healthy melanocytes located in the stratum basale of the epidermis exhibited trace of CCK2R expression. There were also cases where aggressive tumour cells after dedifferentiation lacked Melan-A positivity, but retained CCK2R expression. Double labelling confirmed that melanoma cells expressed the receptor in all seven patients examined. Densitometry revealed higher densities in early stage compared with later stage melanomas (0.18 ± 0.03 vs 0.11 ± 0.02 OD units in <pT2a, n = 5, and >pT2b, n = 2 melanomas, respectively; t-test p=0.166). A similar pattern emerged from a comparison of melanocytes with melanoma cells from the same patients (pT3b; n = 2) (0.19 ± 0.02 vs 0.11 ± 0.02 OD units in melanocytes and melanoma cells, respectively; t-test p=0.139).

For a point of comparison, we examined expression of CCK2R in basal cell cancer (n = 3). Latter showed no detectable CCK2R expression compared with melanoma described above. Double labelling revealed that although there were abundant Melan-A positive melanocytes encapsulated inside the basal cell tumours, CCK2R expression did not change. Independently from tumour type, there were cells of fibroblastic origin (i.e. dermal fibroblasts, myofibroblasts) in the stroma, which showed CCK2R positivity.

In order to provide a more quantitative basis for the comparison, the presence of receptor expressing cancer cells was assessed by allocating samples to categories of 0%, 1-25% (designed 1+), 26-50% (designed 2+) or 51-100% (designed 3+) positive cells counted per field at 20x magnification. Receptor expressing cells were significantly more abundant in patients with malignant melanoma compared to the control basal cell carcinoma group (χ2-test, $p < 0.05$). CCK2R was detectable in 15 out of 18 melanoma patients, whereas it was only present in 2 out of 10 cases in patients with basal cell carcinoma. This was further confirmed by optical densitometry (Mann-Whitney U-test, $p < 0.05$).

4.2.2 *Serum gastrin concentration associated with the progression of melanoma*

Patients were divided into stage I and II groups defined by pT<2a and pT>2b respectively. This division follows the staging system defined by the 8th edition of American Joint Committee on Cancer used for distinguishing between melanoma patients from a prognostic aspect; stage I refers to patients with low-risk primary melanomas (pT1a, pT1b and pT2a), while stage II includes patients with primary tumours that are at higher risk of recurrence (T2b, T3a, T3b, T4a, and T4b) but without regional lymph node involvement or distant metastasis (Gershenwald et al. 2017). The percentage of patients with low serum gastrin was significantly higher in Stage I (Δ%=43.1 of total cohort). This difference was reduced having almost equal number of patients with low and high gastrin concentrations in Stage II (Δ%=6.8). The data indicated that MM patients in the stage II group had significantly higher gastrin concentrations (OR 8.5, $p < 0.0005$; Fisher exact test $p < 0.0003$), than in stage I (40 ± 6.8 pM *vs* 24 ± 4.4 pM in Stages II and I, respectively).

4.2.3 *Expression of CCK2R in human derived melanoma cell lines*

In contrast to previous research in this area (Mathieu et al. 2005) we were able to detect CCK2R expression using immunocytochemistry in two melanoma cell lines selected for the purpose (Skmel-2; G361). Immunofluorescence showed punctate cytoplasmic staining of the receptor. Peroxidase reaction revealed CCK2R expression in 21 ± 5% of cells. Transcripts of CCK2R were then detected by qPCR in both cell lines. Cycle threshold values for Skmel-2 and G361 were 35.5 ± 0.3 and 33.8 ± 0.4 respectively. Using GAPDH as reference, ΔCt values for Skmel-2 and G361 were 15.9 ± 0.2 and 15.2 ± 0.2 respectively.

4.2.4 *Gastrin stimulates migration and invasion of melanoma cells*

Gastrin significantly increased migration of melanoma cells (both Skmel-2 and G361) in Boyden chamber transwell chemotaxis assays (One-way ANOVA, $p < 0.01$). This effect was inhibited by the CCK2 receptor antagonist L740093 at 100 nmol/L which has previously shown to be an effective concentration *in vitro* (One-way ANOVA, $p < 0.01$). Similarly, gastrin stimulated invasion of Skmel-2 and G261 melanoma cells and the response was reduced by L740093 (One-way ANOVA, $p < 0.01$).

In view of the strong indication that gastrin stimulated melanoma cell invasion we then turned to a different model of invasion in which Skmel-2 cells were seeded on the top of a layer of a mixture of type I collagen and Matrigel. The incorporation of dispersed gastric CAMs stimulates melanoma cell invasion from the cancer layer into the deeper structures. When organoids were treated with gastrin (10 nM hG17), the depth of invasion was significantly increased (One-way ANOVA; SF vs. Myo: $p < 0.001$ and Myo vs. Myo + hG17: $p = 0.008$). This effect was not seen when organoids were kept in SF media untreated.

4.2.5 *Dermal fibroblasts stimulate melanoma growth in cancer spheroid models*

Similar to the findings with gastric CAMs, incubation of melanoma cell spheroids in a Matrigel-collagen bed containing dermal stromal cells resulted in a rapid growth reaching a 1.3 ± 0.1-fold change of mean surface area over a 6-day period compared to controls (Kruskal-Wallis test; fib. vs. no fib.: $p=0.01$). When spheroids were treated with 10nM G17 for the duration of the incubation, there were no effects on spheroid growth either with fibroblasts (Δ_{6days}F.Ch: 1.3 ± 0.1 *vs* 1.3 ± 0.2; treated and controls respectively) or without (Δ_{6days}F.Ch: 1.1 ± 0.1 *vs* 1.0 ± 0.1). The effect of dermal fibroblasts on melanoma growth was seen independently of gastrin treatment (Kruskal-Wallis test; fibs. + hG17 vs. fibs. + no G17 $p = 1.0$).

Data from spheroids were also assessed for single cell migration from the spheroid core. Although overall growth of cancer foci was not affected by gastrin, individual cells most likely expressing the receptor were able to migrate furthest when melanoma spheroids surrounded by dermal fibroblasts were treated with gastrin. This burst however in the number of migrating cells was not observed when spheroids were treated with gastrin in the absence of fibroblasts (One-way ANOVA, gastrin vs. gastrin + fibs: $p < 0.001$), also there was no effect of fibroblasts on cancer cell migration without gastrin (One-way ANOVA, fibs vs gastrin + fibs.: $p < 0.001$).

4.2.6 *Gastrin increases invasion of melanoma cancer cells in the presence of dermal fibroblasts and myofibroblasts*

Using melanoma cell organoids, the cancer-stromal border resembling the epidermal-dermal junction remained intact in control samples during the whole observation period (2 weeks) despite the lack of an artificial basement membrane. When melanoma cells were layered on type I collagen matrix with fibroblasts dispersed in it, groups of cancer cells in the form of pedicles or detached nodules started to penetrate deeper structures (One-way ANOVA, fibs. vs. acellular collagen matrix: $p < 0.001$). Invasion of tumour cells was further increased when organoids with fibroblasts (One-way ANOVA, fibroblasts + G17 *vs* acellular collagen matrix: $p < 0.001$; fibroblasts vs. fibroblasts + G17, $p = 0.002$) were treated with gastrin. This stimulatory effect of gastrin was, however, not seen in the absence of stromal cells in the collagen matrix.

4.2.7 *Secretome and associated pathway analysis of gastrin treated-melanoma cell cultures reveals TIMP-3 inhibition and increased secretion of MMP-2*

Cultured melanoma cells were treated with gastrin and secretomes were analysed using methods designed to identify classically secreted proteins that were up or downregulated as a result of gastrin treatment. Proteins were identified based on at least one unique tryptic peptide. Around 10% of total hits (134 and 187 out of total 1266 and 1507 hits in Skmel-2 and G361 respectively) incorporated Lys in a ratio of H to L isotopes ranging from 0.0012-2.193 in Skmel-2 and 0.0011-1.785 in G361 cells, out of which approximately 50% (65 and 93 in Skmel-2 and G361 respectively) were identified as classical secreted ones based on a ubiquitous protein sorting signal sequence with a threshold of SignalP D-score > 0.45 as suggested by Petersen et al (Petersen et al. 2011). Incorporation of heavy lysine (treated samples) expressed as a ratio to light lysine (control samples) revealed increased MMP-2 secretion in Skmel-2 melanoma cells (H to L ratio 1.5). Interestingly the converse was true for G361 melanoma cells, where MMP-2 expression was not influenced, however there was a significant decrease in metalloproteinase inhibitor secretion, namely of TIMP-3 and to a lesser extent TIMP-1 and 2 (0.125; 0.87; 0.7 respectively). Since MMP-2 and the TIMPs are complementary to each other in determining ECM remodelling, this highlights a possible mechanism by which melanoma cells gain increased invading capacity as a result of CCK2R activation.

4.2.8 *Chemotactic assays with MMP-2 siRNA transfected melanoma cultures reveal absence of migration and invasion after gastrin stimulation*

To investigate the functional significance of proteomic data, we used siRNA to knock down MMP-2 expression in Skmel-2 melanoma cells. Melanoma monolayers on Boyden inserts and Matrigel coated bio-membranes were treated with gastrin for 24h. The stimulatory effect of gastrin on cell migration was significantly impaired in the absence of MMP-2 (control treated *vs*. MMP-2 knock down: one-way ANOVA; $p < 0.05$). Nonetheless even with significantly reduced MMP-2 production gastrin still managed to stimulate migration compared to untreated controls (control *vs* knock down; one-way ANOVA; $p < 0.05$). This effect is most likely mediated through a number of paracrine signalling cascades similar to what has already been described with epithelial cells or gastric myofibroblasts (namely IGF system, IL-8 or prostaglandins) (Noble et al. 2003, Hemers et al. 2005, Varga et al. 2017). The invading capability of melanoma cells was more severely impaired by reduced MMP-2, but the

stimulatory effect of gastrin was still present compared to controls (gastrin treated *vs* MMP-2 knock down; One-Way ANOVA; $p < 0.05$, MMP-2 knock down control vs. treated; One-Way ANOVA; $p < 0.05$)

In G361 melanoma cells, where secretome analysis revealed reduced TIMP-3 secretion as a result of gastrin treatment substitution of this metalloproteinase inhibitor successfully reduced the number of migrating cells compared to controls (TIMP-3 + G17 vs. G17; One-Way ANOVA; $p < 0.05$). This inhibitory effect was more robust when melanoma cells had to digest themselves through a collagen matrix with invasion almost reduced to basal levels (TIMP-3 + G17 vs G17; One-Way ANOVA; $p < 0.05$).

4.2.9 *Detection of MMP-2 and TIMP-3 in serum samples of melanoma and basal cell carcinoma patients*

Investigating MMP-2 serum concentrations in skin cancer showed no difference between patients with basal cell cancer or melanoma (227 ± 8 vs. 240 ± 17 in MM and BCC respectively). There was also no correlation with tumour thickness and overall disease progression (230 ± 11 vs. 226 ± 12 in Stage I and II respectively). MMP-2 concentrations were in the range between 200-250 ng/ml which is the reported range for control, healthy individuals. Similar observations were made with TIMP-3. There was no difference between the two patient cohorts (14 ± 1 vs. 17 ± 4 in MM and BCC respectively). Advanced melanoma stage did not influence serum TIMP-3 concentration (15 ± 2 vs. 14 ± 2 in Stage I and II respectively).

We performed immunohistochemistry for MMP-2 and TIMP-3 on randomly selected histology samples from melanoma and basal cell cancer patients. Both in the case of MM and BCCs there was a distinct positive staining of MMP-2 mainly localised to the tumour stroma. Although there are studies which report TIMP-3 expression to be decreased with melanoma progression, we were not able to detect TIMP-3 either in MM or in BCC (Das et al. 2016).

5 Discussion

5.1 *CCK2R expression by myofibroblasts*

CCK2Rs were first described almost 40 years ago initially isolated from the brain (Saito et al. 1980). The main sites of expression in the gut include ECL, parietal and smooth muscle cells (Dockray 2004). Wound healing rodent models revealed *de novo* CCK2R expression in the mucosa and submucosa of repairing cryoulcers in the stomach. Many of these cells were found to be myofibroblasts based on coexpression of vimentin and smooth muscle α-actin (Schmassmann et al. 2000, Ashurst et al. 2008). Systematic screening of the GI tract revealed CCK2Rs expression in 1-6% of myofibroblasts. Expression was related to the proliferative activity of cells, since myofibroblasts from the tumour stoma (CAMs) exhibited higher levels compared to tumour adjacent and normal tissue derived ones (NTMs) (Holmberg et al. 2012). EdU labelling provided direct evidence of a cell cycle, specifically S phase associated transient receptor expression.

CCK2Rs belong to the G-protein coupled seven-transmembrane domain receptor family (Dufresne et al. 2006). Calcium detection after gastrin stimulation revealed a functional receptor present on myofibroblast. GPCR agonists are known to have a mitogenic effect through the release of different growth factors and cytokines (i.e. IGF, EGF etc.) (Varro et al. 2002). The trophic and modulatory effect of gastrin on cell migration and invasion via multiple paracrine pathways and direct CCK2R activation is well described with gastric glandular epithelial cells (Noble et al. 2003, Kumar et al. 2015).

There is little evidence, however, available on the role of CCKRs expressed by myofibroblasts. Although gastrin did not significantly influence overall cell proliferation it did indeed increase the number of mitotic cells (with spindle structures and fragmented nuclei) suggesting an acceleratory effect on the cell cycle similar to that observed with chemokine receptors CXCR3 and GPR19 (Romagnani et al. 2001, Kastner et al. 2012). Furthermore, gastrin was found to stimulate migration and invasion of gastric myofibroblasts through CCK2Rs in chemotactic assays. Interestingly gastrin also stimulated migration of myofibroblasts independently of CCK2R and increased transcript abundance of IGF-2. These data suggest that gastrin not only has a direct effect on myofibroblasts but it is also capable of liberating paracrine acting factors i.e. IGF-2 and others, which need to be further characterised. Overall it looks like CCK2Rs expressed by myofibroblasts are mainly involved in tissue organisation and remodelling conducted by complex epithelial-mesenchymal interactions.

It is well known that myofibroblasts contribute to epithelial restitution and tissue regeneration through paracrine acting growth factors (i.e. HGFs, KGFs and CXCL12) of which their receptors are expressed by epithelial cells (Powell et al. 1999, Smith et al. 2005). Contrary to that, myofibroblast activation through epithelial derived TGF-β has also been described where increased αSMA and MMP expression related to changes in contractibility and matrix restoration respectively were observed in wound healing (Powell et al. 1999, Pender et al. 2004). Another site where epithelial-mesenchymal interaction is important became the centre of attention when the concept of the tumour microenvironment was introduced and neoplasias were no longer considered as a group of malignantly transformed cells that acquired limitless replicative capacity but rather lesions that influence and alter their microenvironment to achieve self-sufficiency and evade apoptosis (Hanahan et al. 2000).

Research in this area indicates that myofibroblast activation and recruitment plays an important role in cancer progression. As to whether these CAMs originate from transdifferentiated fibroblasts, IGF I-II/EGF/PDGF induced proliferation or circulating mesenchymal stem cells is still under debate (Elenbaas et al. 2001, Bhowmick et al. 2004, Brittan et al. 2004).

The role of CCK2Rs in epithelial-mesenchymal interaction in the gut is still not yet fully understood, however as stated before it is likely to be involved in defining the position of myofibroblasts once they exit S-phase of the cells cycle thereby contributing to tissue remodelling both in regenerative processes and also by allowing the assembly of a tumour microenvironment. Taken together this provides a novel dimension to understanding how gastrin might control gastric mucosal architecture both in health and disease.

5.2 *CCK2R expression by melanoma cells*

There is growing interest in the role of CCK2Rs in tissue regeneration and wound healing. As discussed earlier (in section 5.1) myofibroblasts can now be also listed among the known targets and key players of gastrin regulated epithelial restitution (Varga et al. 2017). In addition to these, there is ongoing intense research investigating CCK2Rs in cancer and precancerous conditions. Most of this work has been focused on gastrointestinal tumours, where the receptor was found to be involved in determining the tumour microenvironment (Hanahan et al. 2011, Quail et al. 2013). Data from the Human Protein Atlas provided preliminary evidence of a possible CCK2R expression by human melanoma cells . Given our previous observation with gastric and dermal myofibroblasts we decided to further investigate this relation.

Immunostaining of melanoma samples showed heterogeneous but nonetheless consistent CCK2R expression. A limitation of this study was that melanoma specimens were not assessed for alternative splice variants of the CCK2R, which would be worth studying in the future (Hellmich et al. 2000, Körner et al. 2010). Nonetheless dermal myofibroblasts, fibroblasts, melanoma cells and melanocytes to a lesser extent exhibited CCK2R expression. The latter raises the possibility that outside the CNS, there is another neural-derived cell population which expresses the gastrin receptor.

When fasting serum gastrin concentrations of melanoma patients were correlated with tumour thickness, there was a clear tendency indicating higher gastrin concentrations in advanced melanoma stages. *H.pylori* infection (which is a known risk of gastric cancer development) and long term acid inhibitor consumption were identified as main causes of moderate hypergastrinaemia observed in the investigated cohort. The percentage of patients with chronic hypergastrinaemia due to the aforementioned reasons were in the same range as reported by the literature (Krashias et al. 2013, Helgadottir et al. 2014). These data highlight the fact that overutilization of acid secretion inhibitors and asymptomatic *H.pylori* carriers represent a patient group with increased risk for melanoma progression. To have a better understanding how gastrin might control melanoma behaviour we used primary melanoma cultures (SKmel-2 and G361).

In vitro studies confirmed a functional CCK2R expression. The effect of gastrin on cancer progression has been in focus of gastrin related research in the last decade. CCK2Rs are reported to be expressed in a number of malignant conditions (i.e. pancreatic adenocarcinoma, medullary thyroid cancer, astrocytomas, etc.) (Goetze et al. 2000, Koh et al. 2004, Roy et al. 2016) however there is lack of data with skin tumours. The effect of gastrin on cancer progression is mainly associated with proliferation, adhesion and apoptosis through stimulation of Ras/Raf/MEK/ERK, JNK and p38-MAPK signalling pathways (Dehez et al. 2001, Dufresne et al. 2006).

A key step in melanoma progression is systemic dissemination of melanoma cells through invading lymphovascular structures in the vertical growth phase. CCK2R activation was shown to result in internalisation of β-catenins allowing epithelial cell detachment in non-tumorigenic models (Bierkamp et al. 2002). The involvement of integrins (namely β_3 integrin subunit of the $\alpha_v\beta_3$ vitronectin receptor) have also been identified to play role in vertical growth of melanoma (Sturm et al. 2002), which raises the possibility that gastrin might influence expression of adhesion molecules indirectly.

Although most available data indicates that gastrin is a direct growth factor of the gastric mucosa increasing evidence suggests that it also stimulates proliferation of cells absent the receptor via liberation of paracrine acting cytokines (Dockray et al. 2001, Varro et al. 2002). Melanoma cells did not respond to gastrin stimulation either in monocultures or spheroid like tumour aggregates embedded in a ECM mimicking collagen gel. Therefore, it seems unlikely that gastrin drives the growth of melanomas directly.

Interestingly however, when co-cultured with stromal cells (dermal myofibroblasts and MSCs) rapid tumour expansion was visible. This observation furthermore highlights the importance of the cancer milieu and cross communication between epithelial and mesenchymal structures. Cancer associated fibroblasts, myofibroblasts and macrophages (CAFs, CAMs and TAMs respectively) have been identified recently as key players sustaining a pro-tumorigenic environment (Joyce et al. 2009, Biswas et al. 2010). Association between CAMs and lymph node involvement in gastric cancer has also been described. Increased tumour mass in these cases suggest a paracrine activation from the tumour stroma through factors such as FGF, HB-EGF, TGF-b and IGFII although the mediators and pathways involved are far not yet fully characterised.

On the other hand, there was a clear stimulatory effect of gastrin on cell migration and invasion in chemotactic assays using Boyden chambers and *in vitro* organoids; the latter is a frequently applied model in melanoma research (Hill et al. 2015). Altogether these data suggest that gastrin stimulates invasion and migration of melanoma cells both directly and indirectly through CCK2R expressed by cancer and stromal cells with several putative paracrine factors involved.

To identify proteins responsible for gastrin mediated rapid ECM degradation, which leads to an increased metastasising capacity of melanoma cells, proteomic analysis was performed on conditioned media to define secretome. Gastrin relevant protein hits revealed upregulation of matrix metalloproteinase 2 and downregulation of tissue inhibitor of metalloprotease 3 expression. With the help of bioinformatics algorithms (PANTHER) pathway analysis confirmed adhesion as main biological function of classically secreted proteins related to gastrin stimulation, which was further validated by adhesion assays. MMPs have been broadly investigated in cancer as key elements responsible for ECM remodelling thereby allowing local tumour expansion and intravasation of cancer cells (Hojilla et al. 2008, Hua et al. 2011). Furthermore, overexpression of MMP-2 (which is an important gelatinases responsible for the degradation of basement membranes) was identified in several aggressive melanoma cell lines (Hofmann et al. 1999, Hofmann et al. 2000). Some authors report a relation between MMPs and disease specific survival while others showed correlation with lymph node metastasis suggesting a prognostic value (Vaisanen et al. 2011, Candrea et al. 2014). We found no association between serum MMP-2 concertation and melanoma progression, even though

immunostaining revealed an extensive presence of active MMP-2 in nearly all biopsy samples. Although we would be inclined not to propose MMP-2 as a serum biomarker, the local expression pattern without doubt adds useful information on prognosis and in particular the aggressiveness of a melanoma. Inhibition of TIMP-3 via epigenetic changes or posttranslational modifications is also reported in a number of malignant conditions including pigmented skin lesions however we did not detect changes of TIMP-3 on a tissue level (Hu et al. 2006, Martin del Campo et al. 2015, Das et al. 2016).

Knock down of MMP-2 expression with siRNA and substitution of TIMP-3 resulted in reduced invasion and migration of melanoma cells. Overall proteomic and functional data suggests that gastrin might influence melanoma progression through interfering in the protease / inhibitor axis stimulating ECM degradation and increasing dissemination of cancer cells.

In conclusion melanoma can be added to the group of tumours known to express the CCK2 receptor. Gastrin can now be proposed to promote melanoma progression and metastasis through liberation of proteases and growth factors directly and indirectly through stromal cells. Patients with hypergastrinaemia due to different reasons should therefore be considered as a high risk group for melanoma progression.

6 Summary and key results

This research was centred towards investigating the role of CCK2 receptors expressed by gastrointestinal myofibroblasts and human melanoma cells. The main findings of the thesis include: a) CCK2Rs are exhibited by a proportion of myofibroblasts ranging from 1 to 6%; b) cancer associated myofibroblasts have a higher prevalence of CCK2R expression; c) increased cytosolic calcium confirmed a functional receptor, which is temporarily present on the cells surface in S phase of the cell cycle; d) CCK2Rs on myofibroblasts are likely to be involved in gastrin regulated migration and cell invasion rather than proliferation; e) immunostaining of human melanoma samples revealed CCK2 expression of melanoma cells and melanocytes to a lesser extent; f) elevated serum gastrin concentration strongly correlates with advanced melanoma stage; g) primary cultures of melanoma cells (Skmel-2, G361), dermal fibroblasts and TGF-β transformed myofibroblasts also exhibit CCK2 receptors; h) gastrin does not affect melanoma cell proliferation, but increases invasion and migration *in vitro*; i) stromal cells (gastric CAMs, MSCs, dermal fibroblasts) stimulate cancer growth independently of gastrin in spheroid model; j) gastrin increases melanoma cell invasion in the presence of stromal cells in organotypic model; k) proteomic analysis of the gastrin stimulated melanoma secretome revealed upregulation of MMP-2 and downregulation of TIMP-3; l) inhibition of MMP-2 and substitution of TIMP-3 reduced invasion and migration of melanoma cells *in vitro*; m) alterations in serum concertation of MMP-2 and TIMP-3 showed no relation with disease progression, however MMP-2 remained detectable in all melanoma samples.

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8 List of publications

Thesis is based on the following publications:

Varga A, Kumar JD, Simpson AWM, Dodd S, Hegyi P, Dockray GJ, Varro A. Cell cycle dependent expression of the CCK2 receptor by gastrointestinal myofibroblasts: putative role in determining cell migration. Physiol Rep. 2017 Oct;5(19). pii: e13394.
Doi: 10.14814/phy2.13394; SJR **IF 0.948**

Shawe-Taylor M, Kumar JD, Holden W, Dodd S, **Varga A**, Giger O, Varro A, Dockray GJ. Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation, migration and invasion of both myofibroblasts and cancer cells via the IGF pathway. Peptides. 2017 May; 91:49-57.
Doi:10.1016/j.peptides.2017.03.008; **IF 2.851**

Other papers related to the subject of the dissertation:

Garalla HM, Lertkowit N, Tiszlavicz L, Reisz Z, Holmberg C, Beynon R, Simpson D, **Varga A**, Kumar JD, Dodd S, Pritchard DM, Moore AR, Rosztóczy AI, Wittman T, Simpson A, Dockray GJ, Varro A. Matrix metalloproteinase (MMP)-7 in Barrett's esophagus and esophageal adenocarcinoma: expression, metabolism, and functional significance. Physiol Rep. 2018 May;6(10):e13683.
Doi: 10.14814/phy2.13683; SJR **IF 0.963**

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