The gene expression profiles of canine mammary cancer cells grown with carcinoma-associated fibroblasts (CAFs) as a co-culture \textit{in vitro}\\

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Abstract

\textbf{Background:} It is supposed that fibroblasts present in tumour microenvironment increase cancer invasiveness and its ability to metastasize but the mechanisms have not been clearly defined yet. Thus, the current study was designed to assess changes in gene expression in five various cancer cell lines grown as a co-culture with the carcinoma-associated fibroblasts (CAFs) \textit{in vitro}.

\textbf{Results:} A carcinoma-associated fibroblast cell line was isolated from a canine mammary cancer. Then, a co-culture of cancer cells with the CAFs was established and maintained for 72 hrs. Having sorted the cells, a global gene expression in cancer cells using DNA microarrays was examined. The analysis revealed an up-regulation of 100 genes and a down-regulation of 106 genes in the cancer cells grown as a co-culture with the CAFs in comparison to control conditions. The PANTHER binomial statistics tool was applied to determine statistically over-manifested pathways ($p < 0.05$). Bulk of the up-regulated genes are involved in the adhesion, the angiogenesis, the epithelial-mesenchymal transition (EMT) and generally take part in the developmental processes. These results were further confirmed using real-time qPCR. Moreover, a wound-healing assay and growth characteristics on Matrigel matrix showed that CAFs increase cancer cell migration and matrix invasion.

\textbf{Conclusion:} The results of the current study showed that the co-culturing of cancer cells and the CAFs caused significant changes to the cancer gene expression. The presence of the CAFs in a microenvironment of cancer cells promotes adhesion, angiogenesis and EMT.

Background

Since canine mammary tumours in bulk are of epithelial origin this kind of cells is subjected to many studies. Over the last few years it has also been pin-pointed that concomitant changes occur within stromal cells, which contribute to the tumour microenvironment as well [1,2]. Tumour microenvironment embraces inflammatory, fibroblastic, endothelial cells, adipocytes and other. Changes within these stromal cells have been postulated to increase the tumorigenic phenotype of the epithelial cell, promote malignant transformation, induce epithelial-mesenchymal transition (EMT) and promote tumour spreading and metastasis [3]. It is worth noting however, that in almost all the tumours, the main cell type of cancer stromal compartment is fibroblast. These cells are usually atypical and are termed carcinoma-associated fibroblasts (CAFs). We assume there is a cross-talk between the tumour cells and the CAFs, which promotes migratory, and invasive properties of cancer cells [3] though their exact role within cancer microenvironment has not been fully defined yet. Thus, the study was conducted to assess the changes in gene expression in cancer cells grown as a co-culture with the CAFs \textit{in vitro}. As far as we know the study presented hereby is a pioneering microarray experiment in this field. Despite that our study involved five various cell lines, only one CAFs cell line was used, thus the results may be limited to this particular CAF model. Further studies in this field are required.
The analysis revealed an up-regulation within a span of 100 genes and a down-regulation within 106 genes in cancer cells grown as a co-culture with the CAFs, comparing against set control conditions. In this manuscript we focused mainly on the gene sets involved in adhesion, developmental process and neurotransmissions. The results of our study can be extrapolated on human research because canine mammary tumours are being considered a spontaneous animal model of human breast cancer [4]. There are many similarities between human and canine mammary cancers: in both species they represent a heterogeneous group in terms of morphology and biological behaviour [5], in both similar cancer-related pathways are activated [6-8] as much as both species live under similar environmental conditions.

**Methods**

**Cell lines**

The cell lines used for this study have previously been given an account of [9-12]. Two canine mammary adenocarcinoma cell lines (CMT-W1, CMT-W2), an anaplastic cancer cell line (P114), a simple carcinoma cell line (CMT-U27) and a spindle-cell mammary tumour cell line (CMT-U309) were examined. The CMT-W1 and the CMT-W2 cell lines had kindly been donated by Prof. Dr. Maciej Ugorski and Dr. Joanna Polanska from Wrocław University of Environmental and Life Sciences (Poland). The CMT-U27 cell line had kindly been donated by Dr. Eva Hellmen from Swedish University of Agricultural Sciences (Sweden) and the P114 cell line had kindly been donated by Dr. Gerard Rutteman from Utrecht University (The Netherlands).

The cells were cultured under optimal conditions: a medium (RPMI-1640) enriched with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin (50 iU mL-1), and fungizone (2.5 mg mL-1) (reagents obtained from Sigma Aldrich, USA), a medium that encourages cell proliferation and routinely sub-cultured every other day. The medium RPMI 1640 medium had been used to maintain the cancer cell lines, a medium that encourages preferential fibroblastic outgrowth.

**Tumour sample**

A mammary tumour was surgically removed during mastectomy on a 12 years old mixed breeds female. The tumour then, was divided into equal halves, one of them was fixed in 10% neutral buffered formalin and routinely embedded in paraffin to perform histological assay. The other, was used to isolate and establish a carcinoma-associated fibroblast cell line.

**Carcinoma-associated fibroblasts isolation**

The cells isolation from cancer tissue has been described in our previous manuscript [13]. The tumor sample was collected into the medium RPMI 1640 (Sigma Aldrich, USA) containing flask immediately after mastectomy. The RPMI 1640 medium had been used to maintain the same culturing conditions for mono- and co-culture. The tumour sample was then sliced and cultured overnight in collagenase containing medium RPMI 1640 according to the Limon et al. [14] protocol (modified by Dr Eva Hellmen, Swedish University of Agricultural Sciences, Sweden). The following day, the medium was centrifuged and pellet was suspended in a fresh culture medium supplemented in FGF (10 nM/ml, obtained from Sigma Aldrich, USA), a medium that encourages preferential fibroblastic outgrowth.

**Histopathological examination**

The tissue sample embedded in paraffin block was cut into five μm sections and baked in 37°C overnight. After dewaxing in xylene and rehydration in ethanol, for antigen retrieval, the slides were placed in 0.02 M citrate buffer, pH 6.0 and boiled in the decloaking chamber. The tumor type was classified based on the World Health Organization (WHO) Histological Classification and Mammary Tumors of the Dog and Cat classification [15,16]. The mammary carcinoma grading was assessed in respect to tubule formation, degree of differentiation and mitotic index.

The carcinoma-associated fibroblasts and the canine mammary cancer cells were cultured on Lab-Tek (Nunc Inc., USA) 4-chamber culture slides and were then fixed with ethanol after the 24 hrs.

The immunohistochemical examination of expression of Ki67, cytokeratin, vimentin, smooth muscle actin, s100 protein, p63 protein was performed on the tissue sample as well as on carcinoma-associated fibroblasts to confirm the origin of cell culture. The MUC1 expression was analyzed in the canine mammary cancer cell lines.

The samples were incubated in the Peroxidase Blocking Reagent (Dako, Denmark) for 10 min at room temperature prior to the antibody incubation. After 30 min incubation in 5% bovine serum albumin (Sigma Aldrich, Germany), the following primary antibodies were used (diluted in 1% bovine serum): mouse monoclonal anti-Ki67 (Clone MIB-1) at the concentration 1:75; monoclonal mouse anti-human cytokeratin (Clone MNF116) at the concentration 1:50; monoclonal mouse anti-human vimentin (Clone Vim 3B4) at the concentration 1:50; monoclonal mouse anti-human actin (Clone HHF35) at the concentration 1:50; polyclonal rabbit anti-S100 (ready to use solution) all obtained from Dako (Denmark); monoclonal mouse anti-p63 protein (Santa Cruz Biotechnology, USA) and monoclonal mouse anti-MUC1 (Abcam, United Kindgdom) at the concentration 1:10. According to the manufacturer’s instructions the slides were incubated with antibodies at +4°C overnight.
or 1 hr at room temperature. For the staining the anti-
mouse or anti-rabbit EnVision kits (Labelled Polymers
consist of secondary anti-rabbit antibodies conjugated
with the HRP enzyme complex obtained from Dako) were
used. To develop the coloured product, the 3,3’-
Diaminobenzidine (DAB) substrate was used (Dako).
Finally, the haematoxylin was used for nuclei
counterstaining.

For each immunohistochemical analysis as the nega-
tive control, the staining without the use of primary
antibodies was done. The pictures were taken using
Olympus microscopy BX60 (Olympus, Germany).

Co-culture and sorting
The CAFs (10^5 cells) were grown on 75 cm^2 culture
flasks and the cancer cells (CMT-W1, CMT-W2, CMT-
U27, CMT-U309, P114) were layered (5 × 10^5 cells) on
the top of the CAFs (fibroblasts and cancer cells at 1:5
ratio [17]). An Orange CellTracker fluorescent dye
CMTMR (Invitrogen, USA) was used to stain the CAFs’
population before the cancer cell population was added.
Initially, optimal staining conditions were determined by
incubating CAFs in various concentrations of CMTMR
(5-25 μM dye, according to the manufacturer’s instruc-
tions) and checking the fluorescence signal after 72 hrs
using FACS. The lowest concentration that gives posi-
tive results has been used in further experiments (5
μM). Staining was accomplished by incubation in
serum/anti-biotics-free RPMI medium containing 5 μM
CMTMR (10 mM stock in DMSO; Sigma Aldrich, USA)
for 45 min at 37°C. Subsequently, the medium was aspi-
rated, and the CAFs were washed with PBS twice and
incubated with complete RPMI for 1 hr and then again
washed to remove any remnant non-metabolized
CMTMR. The cancer cells were placed on the
CMTMR-stained CAFs.

The co-culture was maintained for 72 hrs. Then, the
cells were harvested by trypsynization, analyzed and
sorted using FACS Aria II high speed cell sorter with
Diva 5.0 software (Becton Dickinson, USA). Based on
the FSC and SSC cytogram, live cells were gated to
exclude all dead cells, cell debris and cell clumps.
Within the gated cell populations, fluorescing cells were
identified as CMTMR-labelled carcinoma-associated
fibroblasts and non-fluorescent as cancer cells. Ex cita-
tion wavelength used was 488 nm, whereas emission
wavelength used was 578 nm. Cancer cells were sorted
into RPMI 1640 medium in 15 ml polypropylene tubes
(BD Biosciences).

Confocal microscopy
The CAFs grown as a mono-culture were stained using
Orange CellTracker fluorescent dye CMTMR, as
described above. The cells grown on plastic were fixed
in 70% ethanol (10 min), washed in PBS three times and
the coverslips were mounted on microscope slides using
ICN mounting medium. The cell imaging was per-
formed on confocal laser scanning microscope FV-500
system (Olympus Optical Co, Germany) after 1 hr, then
after 72 hrs after the staining. The excitation/emission
were: HeNe 543 nm laser with 610 nm filter for
CMTMR staining. The cells were examined using the
Fluoview program (Olympus Optical Co., Germany).
The pictures have been analyzed using a computer-
assisted image analyzer (Olympus Microimage™ Image
Analysis, software version 4.0 for Windows, USA).

Wound-healing assay
To assess the migration ability of cancer cells grown as
a co-culture with CAFs, we applied a wound-healing
test. The cancer cells (grown as the co-culture with
CAFs at the 5:1 ratio, and normal control cells) were
separately seeded in multi-well plates and then, (after 72
hrs when the cells were confluent) using a pipette tip
(100 ul) a straight scratch had been made, simulating a
wound. The images were captured at the beginning and
at regular intervals (after 2, 4 and 6 hours) during cell
migration to close the wound. The images then were
compared to quantify the cells’ migration rate. This
method is particularly suitable for studies of cell-cell
interaction on cell migration [18]. The pictures have
been analyzed using a computer-assisted image analyzer
(Olympus Microimage™ Image Analysis, software ver-
version 4.0 for Windows, USA).

3D culture
Cancer cells were treated with trypsin and resuspended
in culture medium. 35 mm culture plates (Corning Inc.)
were coated with 100 μl of growth factor reduced Matri-
gel (BD Biosciences) and left to solidify for 30 min. at
37°C. The control cells were then plated at a concen-
tration of 10^4 cells/ml. Co-cultured cells were plated at the
same concentration (cancer cells and CAFs at 5:1 ratio).
The growth of cells on Matrigel was observed everyday
under phase-contrast microscope (Olympus).

Microarray analysis
The sorted cancer cells grown as a co-culture were cen-
trifuged (2,500 rpm for 5 min), whereas cancer cells
grown as mono-cultures were washed with PBS and har-
vested by trypsynization and centrifuged (2,500 rpm for
5 min). The total RNA from the samples was isolated
using a Total RNA kit (A&A Biotechnology, Poland)
according to the manufacturer’s protocol. The isolated
RNA samples were dissolved in RNase-free water. The
quantity of the isolated RNA was measured using Nano-
Drop (NanoDrop Technologies, USA). The samples with
adequate amounts of RNA were treated with DNase1 to
eliminate DNA contamination. The samples were subsequently purified using RNeasy MiniElute Cleanup Kit (Qiagen, Germany). Finally the RNA samples were analyzed on a BioAnalyzer (Agilent, USA) to measure the final RNA quality and integrity.

The total RNA (10 μg) of each cell line was reverse-transcribed using SuperScript Plus Direct cDNA Labeling System, (Invitrogen, USA) according to the manufacturer's protocol for each microarray slide. Single-strand cDNAs were stained with Alexa 647 and Alexa 555 (Invitrogen). Dog-specific oligonucleotide microarray slides Canis familiaris V1.0.1 AROS (Operon, USA) with 25,383 probes were used for the hybridization. Hybridization was performed using automatic hybridization station HybArray12 (PerkinElmer, USA). Two replicates were made (dye-swap).

The slides were analyzed using microarray scanner ScanArray HT and ScanExpress software (PerkinElmer, USA).

Real-time qPCR

The mRNA sequences of the key genes were obtained from NCBI database. Primers were designed using PRIMER3 software (free on-line access) and checked using Oligo Calculator (free on-line access) and Primer-Blast (NCBI database). Primers’ sequences are listed in Table 1. HPRT and RPS19 genes were used as non-regulated reference genes for normalization of target gene expression [19,20]. Quantitative RT-PCR was performed using fluorogenic Lightcycler Fast Strand DNA Sybr Green (Roche) and the Light Cycler (Roche). The results were analyzed using comparative Ct method [21]. Relative transcript abundance of the gene equals ΔCt values (ΔCt = Ctreference - Ct(target)). Relative changes in transcript are expressed as ΔΔCt values (ΔΔCt = ΔCt(target) - ΔCt(control)). The experiment was conducted three times.

Statistical analysis

In the analysis of differential gene expression, background-corrected value of signal in each microarray channel was used. Prior to the analysis, non-specific filtering was performed, i.e. genes with small level of expression were removed (we set an arbitrary threshold according to which at least half of the samples’ expression was to be at least 100). This reduced the number of genes down to 24 842. Then the log2 ratio of the sample vs control channels was calculated and the signal was loes normalized. Quality control, including MA analysis, and signal normalization were done with the Bioconductor software. The analysis of differential expression was performed using linear methods for microarrays (limma package in Bioconductor software) [22]. The method tests the null hypothesis of no differential expression between the sample and control groups using the moderated t-statistic [22], which has similar interpretation as the ordinary t-test statistic. We identified 206 genes with the p-value below 0.05 and fold change > 2.0.

The microarray data discussed in this publication has been deposited in NCBI’s Gene Expression Omnibus and is freely accessible through GEO Series accession number GSE29601.

The gene function was identified using the NCBI database and PANTHER pathway analysis software [23]. The pathway analyses were conducted using binominal statistic test (PANTHER) with the cut-off value p < 0.05.

The statistical analysis of optical density, wound healing assay and Real-time qPCR was conducted using Prism version 5.00 software (GraphPad Software, USA). The one-way ANOVA, and ANOVA + Tukey HSD (Honestly Significant Difference) post-hoc test as well as t-test were applied. The p-value < 0.05 was regarded as significant whereas p-value < 0.01 and p-value < 0.001 as highly significant.

Results

Tumor sample and CAFs examination

The histopathological and immunohistochemical assessment of the tissue sample, from which the carcinoma-associated fibroblasts were isolated, showed that the tumour type was a complex carcinoma of the 1st grade malignancy (Figure 1a). The histopathological and immunohistochemical analysis of the isolated cells

| Gene symbol | Forward primer | Reverse primer | Optimum annealing temp. (°C) | Optimum annealing time (sec) |
|-------------|----------------|----------------|------------------------------|-----------------------------|
| DSP         | CAGACTCACCAGAAGGAAA | CTGCTGTGAAGTCTGGGAGT | 61 | 7 |
| MAG         | TGCCATCGTGCATCTCATTA | CAGTCGCCTCACTCCTCAT | 60 | 6 |
| PCDH19      | CTTTACATGTCGACTCAG | GTTGGTGAGGGAGGATTGCC | 61 | 6 |
| HPRT        | AGCTTGTGGGGAAAAGGAC | TTATAGTCAAGGGCATATCC | 59 | 6 |
| RPS19       | CTTCCTCAAAAAGTCTGGG | GTTTCATCGTAGGGAGCAAG | 61 | 10 |

Primers sequences used in this study and their annealing optimal temperature and time. The mRNA sequences of key genes were obtained from NCBI database. Primers were designed using PRIMER3 software (free on-line access) and checked using Oligo Calculator (free on-line access) and Primer-Blast (NCBI database). HPRT and RPS19 genes were used as non-regulated reference genes for normalization of target gene expression [19,20].
(Figure 1b) revealed that the cell line did not express cytokeratin, S100 protein, p63 protein and actin, whereas a strong vimentin expression was detected (at the level of 3 in 0-3 scale) (Figure 1c). The analysis confirmed that the isolated cell line is an atypical colony of fibroblasts which are termed the CAFs.

**Sorting of the co-cultured cells**

The Flow-Cytometry easily distinguished the CMTMR-stained cells from the unstained ones (Figure 2a) and allowed the proper further sorting of each population (Figure 2b, c). The co-culture was maintained for 72 hrs. According to the manufacturer’s instruction, CMTMR probes remain vividly fluorescent for at least 72 hrs after incubation in fresh medium at 37°C and through at least four cell divisions. The confocal observations confirmed that after 72 hrs of the staining with the CMTMR all the cells showed red-staining cytoplasm pattern (Figure 2c, d, e). No detrimental effects on proliferation and plating efficiency was observed. Our analysis of optical density of the red dye fluorescence with Nomarski Interferenced Contrast showed that all of the stained CAFs maintained their staining pattern after the 72 hrs. Thus, the artificial sorting of unstained CAFs as cancer cells is not very probable. Our FACS sorting isolated a 97-99% pure population on postsort (assessed by BC FACS Diva 5.0 software) what was checked by FACS (Figure 2b, c) and fluorescence microscopy (data not shown). Previously published study of the human fibroblasts and epithelial cells sorting based on the cell tracker staining showed similar results [25].

**Migration assay and growth characteristics on Matrigel matrix**

The wound healing assay showed that in all the cancer cell lines the co-culturing with CAFs increased their migratory abilities (Figure 3). CMT-U27 cells grown with CAFs almost completely closed the wound (99%) in 6 hours, whereas CMT-U27 control cells after 6 hrs closed 68% of the wound. Similarly, CMT-U309 and P114 cells (grown with CAFs) after 6 hrs completely closed the wound (100%), whereas control cells closed only 55% and 50% (respectively) of the wound. CMT-W1 cells grown with CAFs completely closed the wound after 4 hrs, whereas CMT-W1 control cells after 6 hrs (after 4 hrs 64% of the wound was closed). CMT-W2 cells grown with CAFs closed 93% of the wound after 6 hrs, whereas control cells closed only 52% of the wound.

To assess the ability of the cell lines to matrix invasion, we have assessed their growth characteristics on Matrigel matrix (Figure 4). After 72 hrs of culturing (similarly as in all experiments) on Matrigel CMT-U27, CMT-U309 and P114 cell lines formed colonies, whereas CMT-W1 and CMT-W2 cell lines formed branching structures (Figure 4a) what indicated their invasive phenotype. However, all the cell lines grown as a co-culture with CAFs after 72 hrs were dispersed (Figure 4b) what indicated increase of their matrix invasion ability by CAFs.

**Gene expression analysis**

The gene expression analysis showed similar rate of gene expression in each of the dye-swap experiment. This result indicates that all microarray samples were successfully labeled, hybridized, and scanned. The discriminating analysis (with p value cut-off < 0.05; fold change > 2.0) revealed 106 up-regulated (Table 2) and 100 down-regulated (Table 3) genes in cancer cells grown as a co-culture with the CAFs in each of the
These up/down-regulated genes were common for each cell line examined individually, compared to the same cell line grown as a mono-culture. Thus, these genes were activated/inactivated in all of the cell lines under co-culture conditions with the CAFs.

The PANTHER binomial statistics tool to compare classifications of multiple clusters of lists to a reference list of Canis familiaris genes allowed us to statistically determine over-manifestation of PANTHER biological process and pathways classification categories. The PANTHER biological process analysis revealed that most of the up-regulated genes in cancer cells grown as a co-culture with the CAFs were involved in: cell surface receptor linked signal transduction ($p = 9.05 \text{E}-03$), lysosomal transport ($p = 9.2 \text{E}-03$), developmental process ($p = 3.64 \text{E}-02$), antigen processing and presentation ($p = 4.22 \text{E}-02$), signal transduction ($p = 4.35 \text{E}-02$), cell communication ($p = 4.55 \text{E}-02$), nervous system development ($p = 4.97 \text{E}-02$).

Because our concern involved interactions between cancer cells and carcinoma associated fibroblasts which could predispose cancer to metastasis, we specifically focused on the up-regulated genes involved in cell adhesion and cellular morphogenesis: QRICH2, CHAD,
Interestingly, the gene expression analysis also revealed an up-regulation of 24 genes involved in developmental processes such as: a mammary gland development, a mesoderm development, an ectoderm development, a skeletal system development, a nervous system development, an embryonic development, a heart development, and a muscle-organ development. The pathway analysis revealed that significantly over-manifested were: the salvage pyrimidine ribonucleotides ($p = 0.87E-03$), the oxytocin receptor mediated signaling pathway ($p = 4.76E-03$), the thyrotropin-releasing hormone receptor signaling ($p = 5.21E-03$), the 5HT2 type receptor mediated signaling pathway ($p = 6.99E-03$), the metabotropic glutamate receptor III ($p = 8.14E-03$), the metabotropic glutamate receptor group II ($p = 3.33E-02$), the metabotropic glutamate receptor I ($p = 1.75E-02$), the Beta 2 and Beta1 adrenergic receptor signaling ($p = 2.54E-02$) and the histamine H1 receptor mediated signaling ($p = 2.87E-02$).

Among the down-regulated genes no significant pathways or biological processes and their over-manifestation were found (Table 3) comparing to a reference list of *Canis familiaris* genes.

The results were confirmed at mRNA level using real-time qPCR analysis

For the purposes of the microarray data validation, we have randomly selected 3 of all the genes that may play
the most important role in the cancer cells-CAFs interactions: PCDH19, DSP and MAG. Real-time qPCR results showed similar trends in gene expression changes as were observed in microarray studies (Figure 5).

MUC1 expression detection in cancer cell lines
Because the MAG gene up-regulation was found in cancer cells grown as a co-culture with the CAFs, the expression of MUC1 (which binds MAG) was examined immunohistochemically (Figure 6). The MUC1 expression was confirmed in all of the examined cell lines.

Discussion
Canine mammary cancers in bulk arise from epithelial cells. Several genetic alterations have been detected, that may predispose these cells to the malignant transformation [10-12]. However, researches over the last few years suggested that concomitant changes also occur in stromal cells that form the tumour microenvironment [1,2]. The hypothesis of stromal cells involvement in tumorigenesis is based on a study of embryological development where interactions between various cells are necessary for programming and maintaining epithelial structure and function. The embryonic epithelial and stromal cells of mesenchymal origin engage in a molecular dialogue that ensures the proper organ development and function [3].

The study showed in cancer cells after a co-culture with CAFs an up-regulation of 23 genes (Table 2) involved in developmental processes (a nervous system development, an embryonic development, a mesoderm and ectoderm development).

The involvement of fibroblasts in the malignant transformation of epithelial cells has previously been documented [3,26-28]. Moreover, the histology and growth characteristics of CAFs were found different from those of the fibroblasts associated with normal epithelial cells [3]. Mishra et al. [29] have proposed bidirectional cross-talk between the CAFs and the cancer cells which release proteins that increase the fibroblasts ability to secrete a variety of tumour-promoting factors, which then act back on the malignant cells to change their gene expression and promote their proliferative, migratory, and invasive properties. On the other hand, other studies showed that only direct contact of fibroblasts with cancer cells is able to cause changes in their gene expression and biology [30-32].

So far several papers have been published about gene expression in tumour microenvironment. Most of them describe gene expression in fibroblasts, but not in cancer cells, however there are some papers available about the changes in gene expression in cancer cells [33-36]. These reports indicated up-regulation of genes involved in angiogenesis, EMT and migration in cancer cells grown with fibroblasts. Surprisingly, some of the genes identified, even though functionally identical turned out to be of different names. The studies have been conducted using various cancer models (various species) and various cell lines, so the differences are possible.
| No | Fold change | Gene ID  | Gene name                     | Molecular function                                                                 | Biological process                                                                 |
|----|-------------|---------|-------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| 1  | 4.18        | TRIM6   | Tripartite motif-containing protein 6 | ubiquitin-protein ligase activity; structural constituent of cytoskeleton; RNA binding; cytoskeletal protein binding | spermatogenesis, neurotransmitter secretion; intracellular protein transport; exocytosis; cell cycle; signal transduction; synaptic transmission; carbohydrate metabolic process; protein metabolic process; cell-cell signaling; dorsal/ventral axis specification; mesoderm development; mammary gland development |
| 2  | 3.76        | PPP1R12A| Protein phosphatase 1 regulatory subunit 12A | protein binding; phosphatase regulator activity                                    | protein metabolic process                                                            |
| 3  | 3.73        | TCHHL1  | Trichohyalin-like protein 1     |                                                                                     |                                                                                      |
| 4  | 3.24        | QRIC2   | Glutamine-rich protein 2        | receptor activity                                                                   | fertilization; cell adhesion                                                        |
| 5  | 3.14        | TMEM82  | Transmembrane protein 82        |                                                                                     |                                                                                      |
| 6  | 3.14        | ZNF212  | Zinc finger protein 212         | DNA binding, transcription factor activity                                         | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process                |
| 7  | 3.13        | PYROXD1 | Pyridine nucleotide-disulphide oxidoreductase domain-containing protein 1     | oxidoreductase activity                                                            | immune system process; respiratory electron transport chain; apoptosis; ferredoxin metabolic process; oxygen and reactive oxygen species metabolic process |
| 8  | 3.10        | PDESA   | cGMP-specific 3',5'-cyclic phosphodiesterase                                 | hydrolase activity, acting on ester bonds                                           | visual perception; sensory perception; signal transduction; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; signal transduction |
| 9  | 3.02        | CHAD    | Chondroadherin                  | receptor activity                                                                   | immune system process; cell surface receptor linked signal transduction; cell-cell adhesion, mesoderm development; skeletal system development |
| 10 | 3.00        | QSER1   | Glutamine and serine-rich protein 1                                         |                                                                                     |                                                                                      |
| 11 | 2.96        | PRRX    | Serine/threonine-protein kinase   | kinase activity                                                                     | muscle contraction; neurological system process; mitosis; intracellular signaling cascade; protein metabolic process; signal transduction; |
| 12 | 2.90        | ZFAND5  | AN1-type zinc finger protein 5   | nucleic acid binding                                                                | sensory perception; respiratory electron transport chain                               |
| 13 | 2.86        | C2CD3   | C2 domain-containing protein 3   |                                                                                     |                                                                                      |
| 14 | 2.82        | CEP110  | Centrinol, Centrosomal protein of 110 kDa                                    |                                                                                     |                                                                                      |
| 15 | 2.80        | FOXQ1   | Forkhead box protein Q1         | DNA binding, transcription factor activity                                         | visual perception; sensory perception; cell cycle; cell surface receptor linked signal transduction; carbohydrate metabolic process; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; cellular component morphogenesis; segment specification; anterior/posterior axis specification; ectoderm development; mesoderm development; embryonic development; nervous system development |
| 16 | 2.80        | NUP210L | Nuclear pore membrane glycoprotein 210-like                                 | ATPase activity, coupled to transmembrane movement of substances, ligase activity; carbohydrate transmembrane transporter activity; cation transmembrane transporter activity | intracellular protein transport; nuclear transport                                   |
| 17 | 2.79        | SLC22A11| Solute carrier family 22 member 11                                         |                                                                                     | cation transport; anion transport; extracellular transport; carbohydrate transport; carbohydrate metabolic process |
Table 2 Up-regulated genes in cancer cells grown as a co-culture with CAFs (Continued)

| No. | Log2FoldChange | Gene Name | Description | Functions |
|-----|----------------|-----------|-------------|-----------|
| 18  | 2.78           | HERC2     | Probable E3 ubiquitin-protein ligase HERC2 | ubiquitin-protein ligase activity; protein metabolic process; ectoderm development; mesoderm development; skeletal system development; nervous system development |
| 19  | 2.76           | PCSK6     | Proprotein convertase subtilisin/kexin type 6 | peptidase activity; cell surface receptor linked signal transduction; cell-matrix adhesion; protein metabolic process; signal transduction; mesoderm development |
| 20  | 2.76           | UCK2      | Uridine-cytidine kinase 2 | kinase activity; transferase activity, transferring glycosyl groups; carbohydrate metabolic process; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 21  | 2.76           | WFDC5     | WAP four-disulfide core domain protein 5 | protein binding; peptidase inhibitor activity; protein metabolic process |
| 22  | 2.75           | RPS10     | 40S ribosomal protein S10 | structural constituent of ribosome; nucleic acid binding; protein metabolic process |
| 23  | 2.75           | SLC7A14   | Probable cationic amino acid transporter | amino acid transmembrane transporter activity; transmembrane transporter activity; amino acid transport; cellular amino acid and derivative metabolic process |
| 24  | 2.75           | UBQLN2    | Ubiquilin-2/UBQLN2 | protein metabolic process |
| 25  | 2.70           | CLEC7A    | C-type lectin domain family 7 member A | receptor activity; receptor binding; B cell mediated immunity; natural killer cell activation; intracellular protein transport; endocytosis; signal transduction; cell-cell adhesion; signal transduction; cellular defense response |
| 26  | 2.70           | SH3BP1    | SH3 domain-binding protein 1 | protein binding; small GTPase regulator activity; cell surface receptor linked signal transduction; signal transduction; cellular component morphogenesis |
| 27  | 2.68           | HMGB2     | High mobility group protein B2 | DNA binding; chromatin binding; receptor binding; transcription factor activity; intracellular signaling cascade; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; signal transduction; organelle organization; establishment or maintenance of chromatin architecture |
| 28  | 2.68           | TM5B10    | Thymosin beta-10 | structural constituent of cytoskeleton; cellular component morphogenesis; ectoderm development |
| 29  | 2.67           | NEFM      | Neurofilament medium polypeptide | structural constituent of cytoskeleton; cellular component morphogenesis; ectoderm development |
| 30  | 2.67           | ZNF274    | Zinc finger protein 274 | DNA binding; transcription factor activity; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 31  | 2.66           | PCDH19    | Protocadherin-19 | G-protein coupled receptor activity; calcium ion binding; cell surface receptor linked signal transduction; cell-cell adhesion; cell motion; signal transduction; cellular component morphogenesis; ectoderm development; mesoderm development; embryonic development; nervous system development; heart development; muscle organ development |
| 32  | 2.61           | KCMF1     | E3 ubiquitin-protein ligase KCMF1 | protein metabolic process; ectoderm development; mesoderm development; skeletal system development; nervous system development |
| 33  | 2.60           | PIP4K2B   | Phosphatidylinositol-5-phosphate 4-kinase type-2 beta | kinase activity; cell surface receptor linked signal transduction; lipid metabolic process; signal transduction |
| 34  | 2.57           | GNAT3     | Guanine nucleotide-binding protein G(t) subunit alpha-3 | GTPase activity; protein binding; cell surface receptor linked signal transduction; signal transduction |
| 35  | 2.56           | LPCAT1    | 1-acylglycerophosphocholine O-acyltransferase 1 | acyltransferase activity; calcium ion binding; calmodulin binding; calcium-dependent phospholipid binding; metabolic process |
| 36  | 2.56           | XPR1      | Xenotropic and polytropic retrovirus receptor 1 | G-protein coupled receptor activity; cell surface receptor linked signal transduction; signal transduction; embryonic development |
Table 2 Up-regulated genes in cancer cells grown as a co-culture with CAFs (Continued)

| Gene ID | log2 Fold Change | Gene Symbol  | Description                                                                                     |
|--------|------------------|--------------|-------------------------------------------------------------------------------------------------|
| 37     | 2.54             | LPHN2        | Latrophilin-2; G-protein coupled receptor activity; immune system process; neurotransmitter secretion; intracellular protein transport; cell adhesion; cell-cell signaling; mesoderm development; angiogenesis; heart development |
| 38     | 2.54             | MPHOSPH6     | M-phase phosphoprotein 6; cell cycle                                                            |
| 39     | 2.52             | NDUFB10      | NADH dehydrogenase ubiquinone 1 beta subcomplex subunit 10; oxidoreductase activity; oxidative phosphorylation; respiratory electron transport chain |
| 40     | 2.51             | DSP          | Desmoplakin; structural constituent of cytoskeleton; cytoskeletal protein binding; cell adhesion; cell-cell signaling; mesoderm development; angiogenesis; heart development |
| 41     | 2.51             | EXOC3L2      | Exocyst complex component 3-like protein 2; spermatogenesis; immune response; macrophage activation; intracellular protein transport; cell adhesion; mesoderm development; angiogenesis; hemopoiesis; response to stimulus |
| 42     | 2.50             | UBXN1        | UBX domain-containing protein 1; intracellular protein transport; cellular component morphogenesis |
| 43     | 2.49             | MYOT         | Myotilin; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; organelle organization; establishment or maintenance of chromatin architecture |
| 44     | 2.49             | SHPRH        | E3 ubiquitin-protein ligase; DNA helicase activity; nucleic acid binding; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 45     | 2.49             | SMYD1        | SET and MYND domain-containing protein 1; DNA binding; transcription factor activity; transcription cofactor activity; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 46     | 2.48             | KTN1         | Kinectin; structural constituent of cytoskeleton; intracellular protein transport; cellular component morphogenesis |
| 47     | 2.48             | PJA2         | E3 ubiquitin-protein ligase Praja2; DNA binding; transcription factor activity; immune system process; mitosis; cell surface receptor linked signal transduction; intracellular signaling cascade; carbohydrate metabolic process; protein metabolic process; cell motion; mitosis; signal transduction; segment specification; ectoderm development; mesoderm development; embryonic development; nervous system development; response to stress |
| 48     | 2.48             | SRPK1        | Serine/threonine-protein kinase SRPK1; kinase activity; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 49     | 2.47             | ANKS3        | Ankyrin repeat and SAM domain-containing protein 3; immune system process; mitosis; cell surface receptor linked signal transduction; intracellular signaling cascade; carbohydrate metabolic process; protein metabolic process; cell motion; mitosis; signal transduction; segment specification; ectoderm development; mesoderm development; embryonic development; nervous system development; response to stress |
| 50     | 2.47             | C2orf26      | Uncharacterized protein C2orf26; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 51     | 2.47             | TREML2       | Trem-like transcript 2; protein; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 52     | 2.46             | TAB2         | Mitogen-activated protein kinase kinase kinase 7-interacting protein 2; DNA binding; transcription factor activity; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 53     | 2.45             | C1orf6       | Membralin; structural molecule activity                                                         |
| 54     | 2.45             | EPB41L2      | Band 4.1-like protein 2; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process    |
| 55     | 2.44             | CCDC71       | Coiled-coil domain-containing protein 71; mesoderm development; skeletal system development        |
| 56     | 2.44             | FGD1         | FYVE, RhoGEF and PH domain-containing protein 1; protein binding; small GTPase regulator activity; guanyl-nucleotide exchange factor activity; mesoderm development; skeletal system development |
| No. | Log2 Ratio | Gene Symbol | Gene Name |
|-----|------------|-------------|-----------|
| 57  | 2.44       | VCAM1       | Vascular cell adhesion protein 1 |
| 58  | 2.42       | ANKRA2      | Ankyrin repeat family A protein 2 |
| 59  | 2.42       | KCNK17      | Potassium channel subfamily K member 17 |
| 60  | 2.42       | TXNDC15     | Thioredoxin domain-containing protein 15 |
| 61  | 2.41       | TBPL2       | TATA box-binding protein-like protein 2 |
| 62  | 2.40       | ALDH3A2     | Fatty aldehyde dehydrogenase |
| 63  | 2.40       | LAMP3       | Lysozyme-associated membrane glycoprotein 3 |
| 64  | 2.40       | PNLP        | Pancreatic triacylglycerol lipase |
| 65  | 2.37       | C17orf28    | Transmembrane protein C17orf28 |
| 66  | 2.36       | GPR137      | Integral membrane protein GPR137 |
| 67  | 2.35       | WARS        | Tryptophanyl-tRNA synthetase, cytoplasmic |
| 68  | 2.34       | C11orf35    | Uncharacterized protein C11orf35 |
| 69  | 2.34       | SCML2       | Sex comb on midleg-like protein 2 |
| 70  | 2.33       | GNL1        | Guanine nucleotide-binding protein-like 1 |
| 71  | 2.33       | MORN1       | MORN repeat-containing protein 1 |
| 72  | 2.33       | TMEM149     | Transmembrane protein 149 |
| 73  | 2.32       | AK4         | Adenylate kinase isoenzyme 4, mitochondrial |
| 74  | 2.32       | TOMM34      | Mitochondrial import receptor subunit TOMM34 |
| 75  | 2.32       | ZNHIT6      | Zinc finger HIT domain-containing protein 6 |
| 76  | 2.31       | BTG1        | Protein BTG1 |
| 77  | 2.30       | GRAMD1A     | GRAM domain-containing protein 1A |

Table 2: Up-regulated genes in cancer cells grown as a co-culture with CAFs (Continued)
|   |   |   |   |   |
|---|---|---|---|---|
| 78 | 2.30 | GRIK5 | Glutamate receptor, ionotropic kainate 5 | glutamate receptor activity; ligand-gated ion channel activity | neurological system process; cation transport; cell surface receptor linked signal transduction; synaptic transmission; signal transduction; cell-cell signaling |
| 79 | 2.30 | TDRKH | Tudor and KH domain-containing protein | hydrolase activity, acting on ester bonds; nucleic acid binding | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 80 | 2.30 | TGIF1 | Homeobox protein TGIF1 | DNA-directed RNA polymerase activity; DNA binding; transcription factor activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; ectoderm development; nervous system development |
| 81 | 2.29 | FAM81B | Protein FAM81B | structural constituent of ribosome; nucleic acid binding | protein metabolic process |
| 82 | 2.28 | ANKRD46 | Ankyrin repeat domain-containing protein 46 | GTPase activity; structural constituent of cytoskeleton; protein binding | mitosis; cytokinesis |
| 83 | 2.27 | INSM1 | Insulinoma-associated protein 1 | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 84 | 2.27 | PBX1 | Pre-B-cell leukemia transcription factor 1 | DNA-directed RNA polymerase activity; DNA binding; transcription factor activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; ectoderm development; mesoderm development; nervous system development; hemopoiesis |
| 85 | 2.27 | TPRG1 | Tumor protein p63-regulated gene 1 protein | GTPase activity; structural constituent of cytoskeleton; protein binding | mitosis; chromosome segregation |
| 86 | 2.25 | FAM159B | Protein FAM159B | cation transmembrane transporter activity; voltage-gated sodium channel activity; cation channel activity | neuron action potential propagation; cation transport |
| 87 | 2.24 | AKAP8 | A-kinase anchor protein 8 | receptor activity; structural constituent of myelin sheath; receptor binding | 8 cell mediated immunity; cell surface receptor linked signal transduction; cell-cell adhesion; signal transduction; ectoderm development; nervous system development; response to stimulus |
| 88 | 2.24 | MAG | Myelin-associated glycoprotein | cation transmembrane transporter activity; voltage-gated sodium channel activity; cation channel activity | neurotransmitter secretion; intracellular protein transport; exocytosis; endocytosis; cell cycle; cell surface receptor linked signal transduction; intracellular signaling cascade; signal transduction |
| 89 | 2.21 | RASA3 | Ras GTPase-activating protein 3 | protein binding; small GTPase regulator activity | signal transduction |
| 90 | 2.21 | TMEM59L | Transmembrane protein 59-like | GTPase activity; protein binding | muscle contraction; neurotransmitter secretion; synaptic transmission; cell-cell signaling |
| 91 | 2.21 | CACNB3 | Voltage-dependent L-type calcium channel subunit beta-3 | GTPase activity; protein binding | muscle contraction; neurotransmitter secretion; synaptic transmission; cell-cell signaling |
| 92 | 2.21 | PLCD3 | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-3 | hydrolase activity, acting on ester bonds; calcium ion binding | cell surface receptor linked signal transduction; lipid metabolic process; signal transduction |
| 93 | 2.21 | PLCH1 | 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase eta-1 | hydrolase activity, acting on ester bonds; calcium ion binding; receptor binding; small GTPase regulator activity; guanyl-nucleotide exchange factor activity | cell surface receptor linked signal transduction; intracellular signaling cascade; lipid metabolic process; signal transduction |
| 94 | 2.21 | TULP1 | Tubby-related protein 1 | | visual perception; sensory perception; ectoderm development; nervous system development |
The results of the study hereby revealed increased expression of 13 genes involved in cell adhesion (Table 2) among cancer cells co-cultured with the CAFs. As much as 10 of them are involved in developmental processes as well. These genes seem to be particularly significant because the cell adhesion is responsible for tumour progression and metastasis, detachment from the primary tumour and spreading to the circulatory system. Moreover, the up-regulated genes responsible for adhesion are by rule involved in angiogenesis and lymphangiogenesis. For example, the vascular cell adhesion molecule-1 (VCAM-1) up-regulated in cancer cells grown as a co-culture with CAFs may be involved in the down regulation of keratin 20 (Table 3) in cancer cells following the co-culture with the CAFs [42]. It also increases expression of several junction proteins promoting cancer cells to gain the stem-cell-like properties and ensuring resistance to apoptosis [42-44]. Moreover, desmosomes may also be important in the epithelium-mesenchymal transition (EMT). The epithelium-mesenchymal transition is an indispensable mechanism for morphogenesis during embryonic development, and is implicated in conversion of early-stage tumours into invasive cancers. During EMT, epithelial cells undergo changes in morphology and acquire the migratory and invasive characteristics of mesenchymal cells [30]. EMT is also promoted by the FOXQ1, another up-regulated gene in cancer cells grown under co-culture conditions with the CAFs [42]. It also increases expression of several junction proteins promoting cancer cells to gain the stem-cell-like properties and ensuring resistance to apoptosis [42-44]. Moreover, the down regulation of keratin 20 (Table 3) in cancer cells following the co-culture with the CAFs may indicate the EMT induction [45,46].

Interestingly, another up-regulated gene in cancer cells grown with the CAFs, which contributes to cancer invasion is myelin-associated glycoprotein (MAG) that binds to the oncogenic glycoprotein MUC1 [47]. Swanson et al. [47] described an interaction between the MUC1 and the MAG in cancers that invade perineurally, including prostate, salivary, and breast carcinomas. Furthermore, breast cancers may metastasize to the brain where the MAG is abundantly expressed. Interactions between the MUC1 and the MAG have not fully been defined yet. We confirmed the MUC1 expression in all of the examined cell lines (Figure 6). Thus, based on our own

| Gene ID | Name | Molecular function and biological process | Table 2: Up-regulated genes in cancer cells grown as a co-culture with CAFs (Continued) |
|---------|------|-------------------------------------------|----------------------------------------------------------------------------------------|
| 101     | EFCA86 | EF-hand calcium-binding domain-containing protein 6 | calcium ion binding; receptor binding; calmodulin binding; enzyme regulator activity; cation transport; cell cycle; signal transduction |
| 102     | PTPN6 | Tyrosine-protein phosphatase non-receptor type 6 | hydrolase activity, acting on ester bonds; phosphatase activity, receptor activity; immune system process; intracellular protein transport; mitosis; cell surface receptor linked signal transduction; intracellular signaling cascade; cell-matrix adhesion; cell-cell adhesion; protein metabolic process; cytokinesis; cell motion; signal transduction; nervous system development; cellular glucose homeostasis |
| 103     | GPR155 | Integral membrane protein GPR155 | receptor activity; neurological system process; cell surface receptor linked signal transduction; cell-cell signaling; signal transduction; ectoderm development; nervous system development |
| 104     | PSPN | Persephin | receptor binding; transmembrane transporter activity; protein binding; kinase activator activity; kinase regulator activity |
| 105     | STAM2 | Signal transducing adapter molecule 2 | transmembrane transporter activity; protein binding; kinase activator activity; lysosomal transport; intracellular protein transport; endocytosis; intracellular signaling cascade; signal transduction |
| 106     | AGXT2L2 | Alanine-glyoxylate aminotransferase 2-like 2 | transaminase activity; visual perception; sensory perception; vitamin biosynthetic process; cellular amino acid and derivative metabolic process |
| No. | Fold change | Gene ID | Gene name | Molecular function | Biological process |
|-----|-------------|---------|-----------|--------------------|-------------------|
| 1   | 2.17        | EPS8L1  | Epidermal growth factor receptor kinase substrate 8-like protein 1 |  | intracellular signaling cascade; cell motion; signal transduction |
| 2   | 2.17        | OR4X1   | Olfactory receptor 4X1 |  |  |
| 3   | 2.18        | C2orf61 | Uncharacterized protein C2orf61 |  |  |
| 4   | 2.18        | DSN1    | Kinetochore-associated protein DSN1 homolog |  |  |
| 5   | 2.18        | PFAS    | Phosphoribosylformylglycinamidine synthase | ligase activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 6   | 2.18        | SLCSA2  | Sodium/glucose cotransporter 2 | | cation transport; extracellular transport; amino acid transport; carbohydrate metabolic process; cellular amino acid and derivative metabolic process |
| 7   | 2.18        | TMEM138 | Transmembrane protein 138 |  |  |
| 8   | 2.19        | FOLR1   | Folate receptor alpha | receptor activity; transmembrane transporter activity | vitamin transport |
| 9   | 2.19        | MFN2    | Mitofusin-2 | hydrolase activity, acting on ester bonds; phosphatase activity | intracellular protein transport; organelle organization; mitochondrion organization |
| 10  | 2.19        | NDRG3   | Protein NDRG3 |  |  |
| 11  | 2.19        | UNC13D  | Protein unc-13 homolog D |  | intracellular protein transport; exocytosis |
| 12  | 2.20        | GOLPH3  | Golgi phosphoprotein 3 |  |  |
| 13  | 2.20        | SLC22A13| Solute carrier family 22 member 13 | | cation transport; anion transport; extracellular transport; carbohydrate transport; carbohydrate metabolic process |
| 14  | 2.20        | USP54   | Inactive ubiquitin carboxyl-terminal hydrolase 54 | ubiquitin-protein ligase activity | protein metabolic process |
| 15  | 2.22        | NMI     | N-myc-interactor | DNA binding; transcription factor activity; transcription cofactor activity | response to interferon-gamma; intracellular signaling cascade; signal transduction; cellular defense response |
| 16  | 2.22        | PDCD1   | Programmed cell death protein 1 |  |  |
| 17  | 2.22        | SNRPN   | Small nuclear ribonucleoprotein-associated protein N | RNA splicing factor activity; transesterification mechanism; RNA binding | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
| 18  | 2.23        | SPS81   | SPRY domain-containing SOCS box protein 1 |  |  |
| 19  | 2.24        | ADAMTS15| A disintegrin and metalloproteinase with thrombospondin motifs 15 | peptidase activity; protein binding; peptidase inhibitor activity | fertilization; signal transduction; cell-matrix adhesion; cell-cell adhesion; protein metabolic process; signal transduction; ectoderm development; mesoderm development; skeletal system development; angiogenesis; nervous system development; muscle organ development |
| 20  | 2.24        | MTRF1L  | Peptide chain release factor 1-like, mitochondrial | translation factor activity, nucleic acid binding; translation release factor activity | protein metabolic process |
| 21  | 2.24        | TOMM7   | Mitochondrial import receptor subunit TOM7 homolog | transmembrane transporter activity | intracellular protein transport |
| 22  | 2.25        | ATG7    | Autophagy-related protein 7 | ligase activity | intracellular signaling cascade; coenzyme metabolic process; protein metabolic process; signal transduction |
| 23  | 2.25        | C19orf52| Uncharacterized protein C19orf52 |  |  |
| Rank | Log2FC | Gene Symbol | Description | Cellular Component and Process |
|------|--------|-------------|-------------|--------------------------------|
| 24   | 2.25   | USF2        | Upstream stimulatory factor 2 | DNA binding; transcription factor activity; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; lipid metabolic process |
| 25   | 2.26   | POGK        | Pogo transposable element with KRAB domain | DNA binding; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; ectoderm development; nervous system development |
| 26   | 2.26   | ZNF804B     | Zinc finger protein 804B | ubiquitin-protein ligase activity; receptor activity; DNA binding; receptor binding; transcription factor activity; transcription cofactor activity; immune system process; neurotransmitter secretion; intracellular protein transport; exocytosis; signal transduction; synaptic transmission; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; cell-cell signaling; organelle organization; establishment or maintenance of chromatin architecture; mesoderm development; mammary gland development; response to stress; cellular defense response |
| 27   | 2.27   | CD274       | Programmed cell death 1 ligand 1 | kinase activity; cell cycle; intracellular signaling cascade; protein metabolic process; cell cycle; signal transduction |
| 28   | 2.27   | FAM132B     | Protein FAM132B | cell cycle; intracellular signaling cascade; protein metabolic process; cell cycle; signal transduction |
| 29   | 2.27   | STK32C      | Serine/threonine-protein kinase 32C | cell cycle; intracellular signaling cascade; protein metabolic process; cell cycle; signal transduction |
| 30   | 2.28   | CADM4       | Cell adhesion molecule 4 | receptor activity |
| 31   | 2.28   | GLIPR2      | Golgi-associated plant pathogenesis-related protein 1 | immune system process |
| 32   | 2.28   | GPR160      | Probable G-protein coupled receptor 160 | immune system process |
| 33   | 2.29   | GALNT2      | Polypeptide N-acetylgalactosaminyltransferase 2 soluble form | transferase activity, transferring glycosyl groups; carbohydrate metabolic process; protein metabolic process |
| 34   | 2.29   | KRT20       | Keratin, type I cytoskeletal 20 | structural constituent of cytoskeleton; cellular component morphogenesis; cellular component morphogenesis; ectoderm development; cellular component morphogenesis |
| 35   | 2.29   | PTPN6       | Tyrosine-protein phosphatase non-receptor type 6 | hydrolase activity, acting on ester bonds; phosphatase activity; receptor activity; immune system process; intracellular protein transport; mitosis; cell surface receptor linked signal transduction; intracellular signaling cascade; cell-matrix adhesion; cell-cell adhesion; protein metabolic process; cytokinesis; cell motion; mitosis; signal transduction; nervous system development; cellular glucose homeostasis |
| 36   | 2.29   | SOHLH1      | Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 1 | calcium ion binding; calmodulin binding; small GTPase regulator activity; visual perception; sensory perception; cell surface receptor linked signal transduction; signal transduction |
| 37   | 2.31   | CENPM       | Centromere protein M | |
| 38   | 2.31   | HPCAL1      | Hippocalcin-like protein 1 | calcium ion binding; calmodulin binding; small GTPase regulator activity; visual perception; sensory perception; cell surface receptor linked signal transduction; signal transduction |
| 39   | 2.31   | TMEM81      | Transmembrane protein 81 | |
| 40   | 2.32   | HOXA1       | Homeobox protein Hox-A1 | DNA binding; transcription factor activity; female gamete generation; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; segment specification; ectoderm development; gut mesoderm development; embryonic development; skeletal system development; angiogenesis; nervous system development; muscle organ development |
|   |   |   |   |   |
|---|---|---|---|---|
| **Table 3 Down-regulated genes in cancer cells grown as a co-culture with CAFs (Continued)** |   |   |   |   |
|   | 2.32 | SMO | Smoothened homolog | G-protein coupled receptor activity; receptor binding | cell surface receptor linked signal transduction; cell-cell signaling |
|   | 2.33 | LAMP2 | Lysosome-associated membrane glycoprotein 2 | lysosomal transport; intracellular protein transport; protein metabolic process |
|   | 2.33 | RNF121 | RING finger protein 121 | ubiquitin-protein ligase activity | protein metabolic process |
|   | 2.34 | PLA2G2E | Group II secretory phospholipase A2 | hydrolase activity, acting on ester bonds | signal transduction; lipid metabolic process; signal transduction |
|   | 2.34 | POU6F2 | POU domain, class 6, transcription factor 2 | DNA binding; transcription factor activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |
|   | 2.34 | PPP2R4 | Serine/threonine-protein phosphatase 2A regulatory subunit B | protein binding; phosphatase activator activity; phosphatase regulator activity | protein metabolic process |
|   | 2.34 | STX5 | Syntaxin-5 | SNAP receptor activity | neurotransmitter secretion; intracellular protein transport; exocytosis; endocytosis; synaptic transmission; cell-cell signaling |
|   | 2.35 | DMC1 | Meiotic recombination protein DMC1/LIM15 homolog | hydrolase activity; DNA binding | immune system process; meiosis; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; response to stress |
|   | 2.35 | ERRFI1 | ERBB receptor feedback inhibitor 1 | signal transduction; signal transduction |
|   | 2.36 | TSPYL4 | Testis-specific Y-encoded-like protein 4 | protein binding; phosphatase inhibitor activity; phosphatase regulator activity | apoptosis; cell cycle; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; cell cycle; organelle organization; establishment or maintenance of chromatin architecture |
|   | 2.37 | USF1 | Upstream stimulatory factor 1 | DNA binding; transcription factor activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; lipid metabolic process |
|   | 2.40 | ARFGAP3 | ADP-ribosylation factor GTPase-activating protein 3 | nucleic acid binding; protein binding; small GTPase regulator activity | cell surface receptor linked signal transduction; cell adhesion |
|   | 2.40 | CNBP | Cellular nucleic acid-binding protein | nucleic acid binding | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; lipid metabolic process |
|   | 2.40 | NKD1 | Protein naked cuticle homolog 1 | protein metabolic process |
|   | 2.41 | MRP51 | 39S ribosomal protein L51, mitochondrial | structural constituent of ribosome; nucleic acid binding | protein metabolic process |
|   | 2.41 | OPRK1 | Kappa-type opioid receptor | G-protein coupled receptor activity | sensory perception; cell surface receptor linked signal transduction; synaptic transmission; cell motion; signal transduction; cell-cell signaling |
|   | 2.41 | PTX3 | Pentraxin-related protein PTX3 | immune response; response to stress; defense response to bacterium |
|   | 2.42 | GPRC5B | G-protein coupled receptor family C group 5 member B | G-protein coupled receptor activity | cell surface receptor linked signal transduction; signal transduction |
|   | 2.42 | NGLY1 | Peptide-N(4)-(N-acetyl-beta-glucosaminyl)asparagine amidase | hydrolase activity | protein metabolic process |
|   | 2.43 | CAPN12 | Calpain-12 | peptidase activity; calcium ion binding; calmodulin binding; calcium-dependent phospholipid binding | induction of apoptosis; intracellular signaling cascade; protein metabolic process; signal transduction |
|   | 2.43 | POLD1 | DNA polymerase delta catalytic subunit | DNA-directed DNA polymerase activity; hydrolase activity, acting on ester bonds; nucleic acid binding | cell cycle; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; cell cycle |
|   | 2.44 | TBC1D8 | TBC1 domain family member 8 | hydrolase activity; protein binding; small GTPase regulator activity | intracellular protein transport; exocytosis; cellular component morphogenesis |
|   | 2.45 | DHRS11 | Dehydrogenase/reductase SDR family member 11 | oxidoreductase activity | visual perception; sensory perception; lipid metabolic process |
| No.  | log2FC | Gene Name   | Full Description                                                                 | Process                                                                                     |
|------|--------|-------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| 64   | 2.46   | LZTR1       | Leucine-zipper-like transcriptional regulator 1                                  | structural constituent of cytoskeleton; DNA binding; chromatin binding; protein binding; small GTPase regulator activity; transcription factor activity spermatogenesis; immune system process; intracellular protein transport; vesicle-mediated transport; cell cycle; nitrogen compound metabolic process; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; protein metabolic process |
| 65   | 2.46   | OR6V1       | Olfactory receptor 6 V1                                                           |                                                                                             |
| 66   | 2.47   | C12orf65    | Uncharacterized protein C12orf65                                                 | translation factor activity; nucleic acid binding; translation release factor activity        | protein metabolic process                                                                  |
| 67   | 2.47   | C2orf56     | Protein midA homolog, mitochondrial                                               |                                                                                             |
| 68   | 2.47   | RBM42       | RNA-binding protein 42                                                            | RNA splicing factor activity, transcriptification mechanism; DNA binding; RNA binding         | spermatogenesis; neurological system process; cell cycle; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; protein metabolic process; ectoderm development; nervous system development |
| 69   | 2.48   | DPM2        | Dolichol phosphate-mannose biosynthesis regulatory protein                        |                                                                                             |
| 70   | 2.48   | TDRD12      | Tudor domain-containing protein 12                                                 | RNA helicase activity; translation factor activity, nuclei acid binding; translation initiation factor activity | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; protein metabolic process |
| 71   | 2.53   | CCDC85B     | Coiled-coil domain-containing protein 85B                                         |                                                                                             |
| 72   | 2.54   | MATN1       | Cartilage matrix protein                                                          | extracellular matrix structural constituent                                                  | immune system process; sensory perception of sound; sensory perception; signal transduction; cell-cell adhesion; cellular component morphogenesis; mesoderm development; skeletal system development; blood coagulation |
| 73   | 2.55   | FLYWCH1     | FLYWCH-type zinc finger-containing protein 1                                      |                                                                                             |
| 74   | 2.55   | PLA2G2D     | Group IID secretory phospholipase A2                                               | hydrolase activity, acting on ester bonds                                                   | signal transduction; lipid metabolic process; signal transduction                           |
| 75   | 2.56   | TMEM38A     | Trimeric intracellular cation channel type A                                       |                                                                                             |
| 76   | 2.58   | RGS11       | Regulator of G-protein signaling 11                                               | protein binding; small GTPase regulator activity                                            | cell surface receptor linked signal transduction; signal transduction; dorsal/ventral axis specification |
| 77   | 2.61   | BAH1D1      | Bromo adjacent homology domain-containing 1 protein                               | DNA binding                                                                                 | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process                       |
| 78   | 2.67   | CHDS        | Chromodomain-helicase-DNA-binding protein S                                       | DNA helicase activity; nucleic acid binding                                                 | nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; organelle organization; establishment or maintenance of chromatin architecture |
| 79   | 2.67   | CHDS        | Tryptophan-rich protein;                                                          | structural constituent of ribosome; nucleic acid binding                                   | protein metabolic process                                                                  |
| 80   | 2.67   | CYP39A1     | Cytochrome P450 39A1                                                              | oxidoreductase activity                                                                     | respiratory electron transport chain; lipid metabolic process                              |
| 81   | 2.68   | SUDS3       | Sin3 histone deacetylase corepressor complex component S3                          | cell cycle                                                                                  |                                                                                             |
| 82   | 2.69   | C16orf62    | UFF0505 protein C16orf62                                                          |                                                                                             |
| 83   | 2.69   | FLOT2       | Flotillin-2                                                                      | intracellular protein transport; vesicle-mediated transport                                 |                                                                                             |
observations and those of Swanson et al. [47], we suppose that the MAG up-regulation in cancer cells grown with the CAFs and its binding to the MUC1 may contribute to the adhesion between tumour cells and Schwann cells promoting metastasis to the nervous system.

We also found a down-regulation of 5 key genes associated with adhesion. Subject literature suggests 3 of them play a role in cancer development. The down-regulation of these genes is associated with poor prognosis and cancer metastases. One of these genes is the ADAMTS15 (a disintegrin and metalloproteinase with thrombospondin motif 15) which is an anti-angiogenic factor [48]. Our study also revealed a down-regulation of the CADM4. Nagata et al. [49] found decrease in the CADM4 expression in most of renal cell carcinomas and the cancer cell lines. Moreover, the CADM4 expression was decreased in carcinomas with vascular infiltration, suggesting that loss of the CADM4 is involved in tumour angiogenesis and invasion. We have also found a down-regulation of the MATN1 gene which has been defined an angiogenesis inhibitor [50].

In the current study we showed a significant over-manifestation of genes involved in the oxytocin receptor mediated signaling pathway, the thyrotropin-releasing hormone receptor signaling, the Beta 2 and Beta1 adrenergic receptor signaling, and the histamine H1 receptor mediated signaling in cancer cells grown with the CAFs. Entschladen et al. [51] described the role of
They found that similarly to chemokines, neurotransmitters are regulators of cell migration. Sadly though, we noticed that only a few results are available on the expression of neurotransmitter receptors in tumour tissues. Among them the best understood is the role of catecholamines in carcinogenesis and tumour progression. These are the stress hormones, whereas stress in turn is a major risk factor for the development of cancer. Norepinephrine has been shown to strongly induce the migration of tumor cells [52,53], whereas epinephrine was found a modulator for the carcinogenesis in the lung [54].

An interesting gene in cancer cells grown as a co-culture with the CAFs is the up-regulated protocadherin 19 (PCDH19) (Table 2). Up-to-date there is no information available on the involvement of this gene in tumor progression or metastasis. However, a PCDH19 mutation was found to be responsible for epilepsy and mental retardation confined to females (EFMR) [55]. There has been an on-going debate about the relationship between epilepsy and cancer. It has been hypothesized that the incidence of cancer is increased in people with epilepsy owing to the cancer promotion by antiepileptic drugs [56]. Perhaps the increased risk of cancer in epileptic patients is caused by the PCDH19 mutation and over-
expression, not however related to drugs toxicity. This hypothesis requires further studies.

Conclusions

The results of the current study showed that the co-culture of cancer cells and the CAFs caused significant changes in expression of genes involved in adhesion, angiogenesis and the EMT that take part in developmental processes.

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Authors’ contributions

MK: research design, experimental design, FACS cell sorting, CAFs isolation, microarray analyses; KS: real-time qPCR; HM statistical analysis of manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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