BBA - Molecular Cell Research 1868 (2021) 118912

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Research paper

Proprotein convertases blockage up-regulates specifically metallothioneins coding genes in human colon cancer stem cells☆

Daniela Gerovska [a,b,1], Patricia García-Gallastegi [c,d,1], Jean Descarpentrie [d,1], Olatz Crende [c], María Casado-Andrés [c,d], Ander Martín [c], Jokin Eguia [c], Abdel-Majid Khatib [d,2], Marcos J. Araúzo-Bravo [a,b,e,f,*,2], Iker Badiola [c,**,2]

[a] Computational Biology and Systems Biomedicine Group, Biodonostia Health Research Institute, Calle Doctor 8 Beguiristain s/n, 20014 San Sebastián, Spain
[b] Computational Biomedicine Data Analysis Platform, Biodonostia Health Research Institute, C/ Doctor 8 Beguiristain s/n, 20014 San Sebastián, Spain
[c] Department of Cell Biology and Histology, Faculty of Medicine and Nursing, University of the Basque Country, 48940 Leioa, Spain
[d] Univ. Bordeaux, INSERM, LAMC, U1029, F-33600 Pessac, France
[e] IKERBASQUE, Basque Foundation for Science, C/ María Díaz Harokoa 3, 48013 Bilbao, Spain
[f] CIBER of Frailty and Healthy Aging (CIBERfes), Madrid, Spain

ARTICLE INFO

Keywords:
Cancer stem cells
Colon cancer
Proprotein convertases
Computational biology
RNA-seq
Metallothioneins
Chromosome landscape analysis

ABSTRACT

Despite continuous exertion made, colon cancer still represents a major health problem and its incidence continues being high worldwide. There is growing evidence in support of the cancer stem cells (CSCs) being central in the initiation of this cancer, and CSCs have been the focus of various studies for the identification of new ways of treatment. Lately, the proprotein convertases (PCs) were reported to regulate the maturation and expression of various molecules involved in the malignant phenotype of colon cancer cells, however, the identity of the molecules regulated by these serine proteases in CSCs is unknown. In this study, we used the general PCs inhibitor, the Decanoyl-RVKR-chloromethylketone (Decanoyl-RVKR-CMK) that inhibits all the PCs found in the secretory pathway, and analyzed its effect on CSCs using RNA-seq analysis. Remarkably, from the only 9 up-regulated genes in the human SW620-derived sphere-forming cells, we identified 7 of the 11 human metallothioneins, all of them localized on chromosome 16, and zinc related proteins as downstream effectors of the PCs. The importance of these molecules in the regulation of cell proliferation, differentiation and chemoresistance, and their reported potential tumor suppressor role and loss in colon cancer patients associated with worse prognosis, suggests that targeting PCs in the control of the malignant phenotype of CSCs is a new potential therapeutic strategy in colon cancer.

1. Introduction

The existence of a subpopulation of non-differentiated cancer cells in the tumor mass has been postulated with the key study in this field conducted by Pierce and Speers in 1961 [1]. A tumor has been described as an aberration of normal tissue development and formulated as a hierarchical model with a population of Cancer Stem Cells (CSCs). In 1994, Dick and colleagues isolated for the first time leukemia stem cells [2]. Later, the CSCs were identified in solid tumors [3–6], and nowadays it is widely accepted that tumor masses have a subpopulation of cells able to mediate metastases that are resistant to therapies, and with capacity to maintain the bulk of tumor mass [7–9]. These properties make CSCs candidates to be the cells responsible for tumor initiation and progression. The critical feature of this subopulation of tumor cells is their ability to eject the therapeutic compounds through a protein complex called ABC transporter (ATP-binding cassette) that exports

☆ Reprogramming Tumor Activity and The Associated-Microenvironment edited by Khatib Abdel-Majid, Juan Rosado and Klaudia Brix

* Correspondence to: M.J. Araúzo-Bravo, Computational Biology and Systems Biomedicine Group, Biodonostia Health Research Institute, Calle Doctor 8 Beguiristain s/n, 20014 San Sebastián, Spain.
** Corresponding author.
E-mail addresses: marcos.arauzo@biodonostia.org (M.J. Araúzo-Bravo), iker.badiola@ehu.eus (I. Badiola).

[1] Equal contribution.
[2] Shared corresponding.

https://doi.org/10.1016/j.bbamcr.2020.118912
Received 9 September 2020; Received in revised form 16 November 2020; Accepted 19 November 2020
Available online 26 November 2020

drugs out of the cells [10]. The lack of efficient therapies against CSCs makes the development of such ones the great priority in cancer research. In colon cancer, that constitutes the third most common type of cancer and the second leading cause of tumor related death in the western world [11], the colon CSCs can be principally derived from well-differentiated intestinal cells or intestinal stem cells (ISCs) following alteration of several genes that are able to induce carcinogeneis [12]. In addition to their self-renewal potential, the colon CSCs show high tumorigenicity and resistance to various therapies, including radiation [13]. Additionally, these cells have atypical activation of signaling pathways promoting proliferation that maintain their stemness and drug resistance such as Wnt [14], Notch, Hedgehog [15] and TGF-β [16]. These proteins involve directly or indirectly the proprotein convertases (PCs) in their expression and/or activation [17].

PCs are a family of enzymes that cleavage many unfunctional precursos of proteins turning them in active molecules [17]. Till now, nine members have been described: PC1/3 (PCSK1), PC2 (PCSK2), Furin (PCSK3), PC4 (PCSK4), PC5/6 (PCSK5), PACE4 (PCSK6), PC7 (PCSK7), PCSK8 (MBTPS1) and PCSK9 (NARC1), where PCSK stands for Proprotein Convertase Subtilisin/Kexin. They are implicated in the processing of wide range of protein precursors, including hormones [18,19], proteases, growth factors [20], receptors [21] and adhesion molecules [22] at multibasic recognition sites displaying the general motif (K/R)-(X)n-(K/R)↓, where X is any amino acid except Cys and n = 0, 2, 4, or 6 amino acids. The relation between cancer and PCs has been extensively studied [23]. Evidently, cancer is a phenomenon that occurs in part due to overexpression of growth factors, cytokines and receptors, therefore the activation or expression of these oncogenic molecules by the PCs directly involve these enzymes in various processes required for tumor progression and metastasis including angiogenesis, inflammation and immune responses [17]. Furthermore, altered expression of various PCs has been described in various cancers including colon cancer [24]. For example, Furin has been reported in head and neck cancer [25], PC5 and PC7 are involved in angiogenesis of colon cancer [26], and PACE4 in colon cancer cells metastasizing to liver [27]. In all cases, there is a dysregulation of PCs expression or function. Cancer cells express altered PCs that activate more signaling molecules and maintain tumor growth and progression [25]. It is well known that the requirements and transduction signals of CSCs compared to the differentiated tumor cell populations are different [28], and protein maturation enzymes, such as PCs, could play a yet unknown important role. Here, using the general PCs inhbitor decanoyl-RVKR-cholromethyl ketone (Decanoyl-RVKR-CMK) and whole transcriptome analysis by RNA-seq, we assessed the effect of PCs inhibition on gene expression in the human SW620-derived sphere-forming cells. We discovered a staggering specificy: From only nine up-regulated genes by PCs in the CSCs, we found that seven are metallothioneins (seven out of the eleven human metallothioneins), all localized on chromosome 16. Also, we found genes coding for zinc related proteins among the genes up-regulated by PCs in these CSCs. The reported potential tumor supressor role and upregulated expression of various metallothioneins in colon cancer cell lines and patients [29] directly involves the PCs in the control of the malignant phenotype of CSCs and their potential use in colon cancer treatment.

2. Materials and methods

2.1. Cell culture

SW620 human colon carcinoma cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were routinely maintained in Dulbecco's minimal essential medium (DMEM) (Gibco, Saint Aubin, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate, 0.1 mg/mL streptomycin, 100 U/mL penicillin at 37 °C in a 5% CO_2-humidified atmosphere. For tumorspheres formation mimicking CSC condition [30], SW620 cells were cultured in DMEM/F-12 supplemented with N2, B27 supplements (Thermo Fisher Scientific, Waltham, MA, USA), and growth factors [20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF)] (Sigma, St. Louis, MO, USA) in non-adherent plastic (Sarstedt, Germany) for 10 days.

2.2. RNA extraction, reverse transcription and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from cells using the RNeasy Kit (Qiagen, Hilden, Germany) and checked for purity by measuring the 260/280 ratio in the Nanodrop Synergy HT instrument (Biotek, Winooski, VT, USA). cDNA (50 ng/μL) was obtained by reverse transcription of total extracted RNA using the iScript cDNA Kit (BioRad, Hercules, CA, USA) with the following reagents: iScript reverse Transcriptase (1 μL), 5× iScript Reaction Mix (4 μL) and Nuclease Free Water (variable) to a final volume of 20 μL. Quantitative Real-Time PCR experiments were conducted in an iCycler My iQTM Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA, USA), using 4.5 μL of Power SYBR® Green PCR Master Mix 2× (Applied Biosystems, Carlsbad, CA, USA), 0.5 μL of primers (0.3125 M), 0.3 μL of cDNA (1.5 ng/μL) and Nuclease free water for a total volume reaction of 10 μL. All primers were designed and checked for optimal efficiency (>90%) in the qPCR reaction under our experimental conditions. The relative expression of each gene was calculated using the standard $2^{-\Delta Ct}$ method [31], normalized with respect to the average of β-ACTIN as internal housekeeping control gene. All reactions were performed in triplicate. qPCR was run on a CFX96® Thermo Cycler (BioRad, Hercules, CA, USA). We assessed that all qPCR reactions yielded only one amplification product using the melting curve method. We used the following primer pairs for different human gene transcripts validated by the National Center for Biotechnology Information (NCBI) Primer-Blast method. The experiments were performed 3 times.

2.3. Decanoyl-RVKR-chloromethylketone treatment

SW620 derived CSCs were treated with Decanoyl-RVKR-CMK 50 μM (Bachem AG, Bubendorf, Switzerland) following previous experiences [32], for 24 h and cells were collected for total RNA isolation using RNeasy Plus Mini Kit (QIAGENT, Hilden, Germany) according to manufacturer's instruction. RNA concentration was determined using the Nanodrop Synergy HT (Biotek, Winooski, Vermont, USA) and purity was calculated by 260/280 nm absorbance ratio. Pure RNA samples from Decanoyl-RVKR-CMK- and non-treated cells were sequenced. The experiment was performed using 3 replicates for each condition.

2.4. Measurement of proprotein convertases activity

Proprotein convertases activity in cancer cells was assessed as previously described [22,33] by measuring the digestion of the universal PCs substrate, the fluorogenic peptide pERTKR-MCA. In brief, Decanoyl-RVKR-CMK- and non-treated cells were incubated with pERTKR-MCA (100 μmol/L) at various time periods (0 to 30 min) in the presence of 25 mmol/L Tris, 25 mmol/L methyl-ethane-sulfonic acid, and 2.5 mmol/L $CaCl_2$, pH 7.4 at 37 °C, and the enzymatic activity was determined using a spectrofluorometer (FLUOstar OPTIMA; BMG Labtech). The general PCs-inhibitor decanoyl-RVKR-cholromethyl ketone (DECANOYL-RVKR-CMK) was obtained from Calbiochem (San Diego, USA). The experiment was performed in three different and independent cell cultures.

2.5. RNA sequencing

RNA-seq libraries were generated by using the TruSeq RNA sample Preparation Kit (Illumina, USA). Briefly, mRNA was enriched by using poly-T oligo-attached magnetic beads, followed by mRNA fragmentation by acoustic shearing. First-strand cDNA was synthesized by using

reverse transcriptase and random hexamers, and second-strand cDNA by using DNA polymerase I and RNase H. cDNA was subject to adapter ligation, and then enriched with PCR to prepare cDNA library. cDNA libraries were sequenced on HiSeq 2000 (Illumina) to obtain approximately 100 million paired-end reads (2 × 101 bp).

2.6. RNA-seq data analysis

Three biological replicates of CSC-CON (SW620 CSC non-treated control) and CSC–CMK (SW620 CSC treated with Decanoyl-RVKR-CMK) were used for the transcriptomics analysis. HISAT2 [34] was used to align the 151 bp paired-end RNA-seq reads for each of the samples to the human reference genome GRCh38. The Cufflinks [35] was used to assemble the mapped reads into possible transcripts and generate a transcriptome assembly, and the counts of aligned reads to each gene were evaluated using HTSeq [36]. In-house software was used to merge the expression results into a single text file to be used in the downstream analysis [37] in Matlab (MathWorks). Quantile normalization was performed to equalize the data and the data was further stabilized through the $\log_2$ transform of the data plus one.

2.7. Selection of statistically significant Differentially Expressed Genes (DEGs)

To find the statistically significant DEGs between two groups of samples, we calculated the mean values of each probe across all the samples of each of the two groups. Next, we filtered out all the probes whose absolute value of difference of mean values between the two groups was less than a selection fold change threshold FC_{DEG} (in $\log_2$ scale), and we applied the Student's *t*-test to find the statistically significant DEGs. The multitest effect was tackled through control of the False Discovery Rate (FDR) using the Benjamini-Hochberg method for correcting the initial *p*-values with significance threshold $\alpha_{DEG} = 0.05$.

2.8. Chromosome landscape analysis of statistically significant DEGs

To evaluate the chromosomal enrichment of lists of DEGs, we mapped each DEG onto its corresponding chromosome, and we compared the number of DEGs mapped onto a chromosome with the number of genes on each chromosome. We estimated the statistical significance of the enrichment by the *p*-value calculated using the hypergeometric distribution using a significance threshold. The multitest effect was tackled through control of the False Discovery Rate (FDR) using the Benjamini-Hochberg method for correcting the initial *p*-values with significance threshold $\alpha_{LAN} = 0.01$. To analyze the way the gene *loci* group on a chromosome, we applied our one-dimensional clustering technique as previously described [38] that searches for groups of gene *loci* located at a distance smaller than $D_{clu} = 2{,}000{,}000$ bps, and considers them as a cluster if they are populated by a minimum number $N_{min} = 4$ genes.

2.9. Gene Ontology (GO) statistically significant enrichment analysis of DEGs

The GO terms were taken from the curated collection of molecular signatures (gene set collection C5) of version 3.0 of the Molecular Signatures Database (MSigDB) [39]. The statistical significance of the GO terms was analyzed as previously described [38] using an enrichment approach based on the hypergeometric distribution to estimate the significance (*p*-value) of the gene set enrichment. The multitest effect influence was tackled through control of the False Discovery Rate (FDR) using the Benjamini-Hochberg method for correcting the initial *p*-values with significance threshold $\alpha_{GO} = 0.05$.

2.10. Gene-coverage count track plots

We sorted the alignment bam files with samtools [40] and produced the bed files with bedtools [41]. We used our previously developed function in Matlab (MathWorks) [42] that for each gene of interest takes the exon boundary information from the basic annotation file in gtf format from Gencode (https://www.gencodegenes.org/human/) version 33, and plots the gene-coverage count track plots, preserving the same scale for the tracks of the same gene in all the samples.

3. Results

3.1. Inhibition of the proprotein convertases activity in SW620 colon CSCs by Decanoyl-RVKR-CMK

To evaluate the effect of the Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Decanoyl-RVKR-CMK) on the total PCs activity in colon CSCs, sphere-forming subgroup of cells were derived from the human colon cancer cell line SW620 (Fig. 1A) and were treated with Decanoyl-RVKR-CMK prior to their PCs enzymatic activity measurement using a chromogenic activity assay at the indicated time periods. As we can observe in Fig. 1A, tumorsphere size was reduced after DECANOYL-RVKR-CMK treatment and provoked reduction of CSC self-renewal capacity. The enzymatic activity was determined based on the ability of cell lysates to digest the universal PC substrate, the fluorogenic peptide pERTKR-MCA. While PCs activity in control CSCs (CSC-CON) was increased with time, cells treatment with Decanoyl-RVKR-CMK (CSC-CMK) inhibited the enzymatic activity in these cells at all the tested time periods (Fig. 1B–C). Using specific primers for all the PCs found in the secretory pathway, namely PACE4, PC5, PC7 and Furin, real time PCR analysis revealed that the SW620-derived sphere-forming cells express all these PCs (Fig. 1D).

3.2. Narrow dysregulation of colon CSCs gene expression by the proprotein convertases activity

Previously, the activity of the PCs was reported to be involved in the malignant phenotype of various cancer cells including colon cancer cells by mediating the maturation and/or activation of various protein precursors and the regulation of genes required for tumor progression and metastasis [17] [24]. On the other hand, the capacity of colon CSCs to induce tumor recurrence or metastasis and/or resistance to therapies were associated with altered expression of several genes involved in these processes [43]. To evalaute the role of the PCs in the regulation of colon CSCs gene expression, sphere-forming SW620 cells were treated with Decanoyl-RVKR-CMK prior mRNA isolation and sequencing. The transcriptome analysis by RNA-seq showed very small dysregulation (Fig. 2A) between control (CSCs-CON) and Decanoyl-RVKR-CMK-treated (CSC-CMK) cells, with a Pearson's correlation coefficient as high as $\rho = 0.996$ (Fig. 2B). These means that the effect of Decanoyl-RVKR-CMK is very small over the transcriptome, however, it is shown in the following analysis that this effect is extremely specific. For a fold change FC_{DEG} of 2 in $\log_2$ scale, we found only 11 down-regulated and 24 up-regulated genes.

From the 11 down-regulated genes, only two (*SKA1* and *SNORD14E*) were statistically significant at a significance threshold $\alpha_{DEG} = 0.01$ (Fig. 2B,C). *SKA1* spindle and kinetochore associated complex subunit 1 is a component of the SKA1 complex, a microtubule-binding subcomplex of the outer kinetochore that is essential for proper chromosome segregation that facilitates the processive movement of microspheres along a microtubule in a depolymerization-coupled manner [44]. *SNORD14E*, small nucleolar RNA, C/D box 14E, is associated with the cyto-/nucleoplasmatic 70-kDa heat-shock protein HSP70 [45].

From the 24 up-regulated genes, only 9 were statistically significant, namely *KIAA0430*, *SLC30A2* and 7 metallothioneins (MTs) (Fig. 2B,C). The official gene name of *KIAA0430* is *MARF1*, Meiosis Regulator And

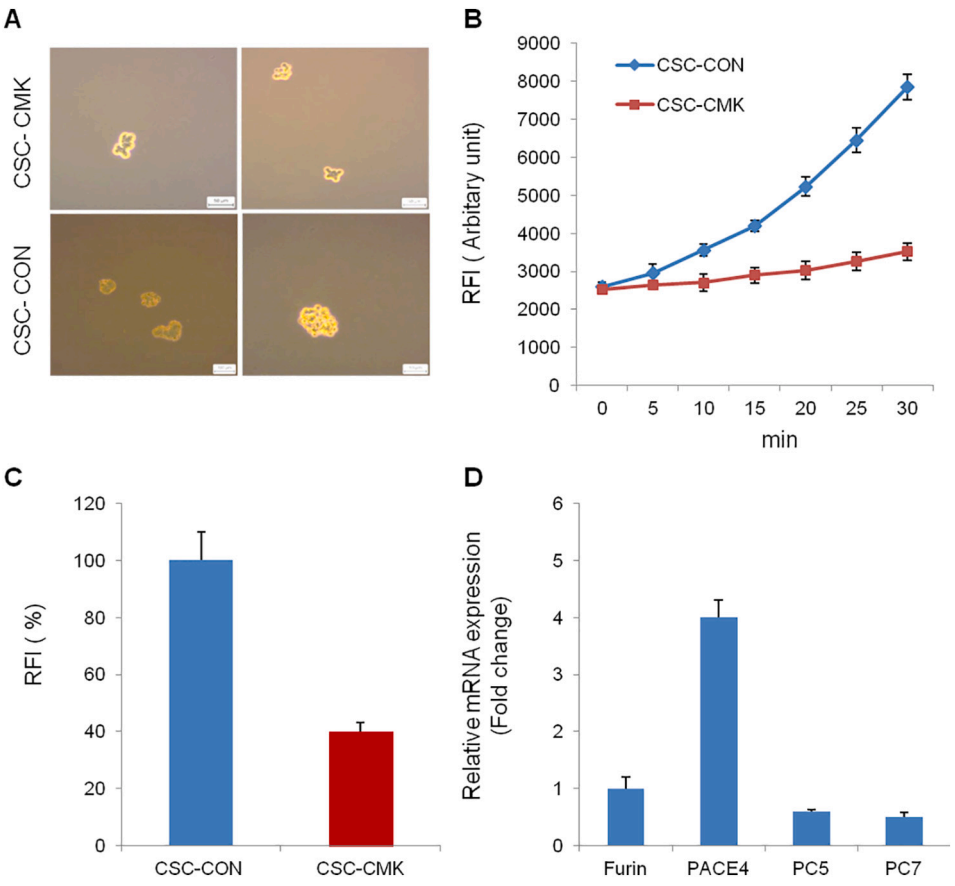


Fig. 1. Inhibition of the proprotein convertases activity in SW620 colon CSCs by Decanoyl-RVKR-CMK. (A) Sphere-forming subgroup of cells derived from the SW620 colon cancer cell line in the absence (CSC-CON) and presence (CSC-CMK) of Decanoyl-RVKR-CMK. (B) PCs activity assessed by evaluating the ability of equal amount of cell lysates of CSC-CON and CSC-CMK to digest the universal PCs substrate, the fluorogenic peptide pERTKR-MCA at the indicated time points. Note that exposure to Decanoyl-RVKR-CMK represses PC activity expressed as relative fluorescence intensity (RFI). (C) The corresponding percentages of PCs activity measured following pERTKR-MCA digestion after 20 min. (D) PCs expression in CSCs derived from SW620 measured by RT-PCR. Following total RNA extraction from 10^4 × cells, real-time PCR analysis was performed using specific primers for indicated PCs as described in Materials and methods (Table 1). Results are expressed as the percentage of the indicated transcripts relative to Furin transcript assigned to 1. Results are representative of three experiments and data are mean ± standard error (SE) performed in triplicate. *p-value <0.05 (Student's t-test).

Table 1
Primers used for real-time PCR of PCs.

Primers	Sequence 5′-3′	Annealing (°C)	Amplicon (bp)
PACE4	F: GTACCTCAACTTGGGCCAGA	59.31	393
	R: TCGTAGCTGGCGTAGGAATC	59.33	
PC5	F: GAAGCCAATCCGTTTCTGAC	57.01	375
	R: GTAGATGGCCAGGTCTCC	57.03	
PC7	F: GTGTGGGACAGGCTCTTCT	58.94	400
	R: CGAAGTCCTCTTCCCAACTG	58.94	
FURIN	F: GCAACACCTGGTGGTACAGA	59.89	399
	R: TCTGCGGAGTAGTCATGTGG	59.18	
βACTIN	F: CATGTACGTTGCTATCCAGGC	59.13	250
	R: CTCCTTAATGTCACGCACGAT	58.46	

mRNA Stability Factor, encodes a putative peroxisomal protein, is an endoribonuclease that interacts with the DCP1:2 decapping complex and degrades target mRNAs [46], and is localized on chromosome 16 p13.11. *SLC30A2* is the solute carrier family 30 member 2 encoding a zinc (Zn) transporter (ZnT2) that imports Zn into vesicles in highly-specialized secretory cells and whose expression is regulated by the metal regulatory transcription factor MTF1 [47]. Noteworthy, out of these very few dysregulated genes, 8 are on chromosome 16, and seven are MTs (Fig. 2D).

3.3. CSCs from human colon cancer SW620 treated with PC-inhibitor Decanoyl-RVKR-CMK show very specific up-regulated expression of metallothioneins compared to untreated CSCs

The transcriptomics analysis by RNA-seq showed that from the very few, nine genes up-regulated in Decanoyl-RVKR-CMK treated CSCs compared to non-treated CSCs, seven were from the family of the eleven human MTs, namely *MT2A*, *MT1X*, *MT1E*, *MT1G*, *MT1F*, *MT1M* and *MT1H*. This is a staggering specificity, with a p-value $= 2.067 \times 10^{-23}$ of statistical significance calculated with the hypergeometric test for the enrichment of these seven genes over a background of 20.358 genes that have at least one RNA count in at least one of the samples. The MTs are small cysteine-rich proteins that play important role in metal homeostasis and protection against heavy metal toxicity, DNA damage, and oxidative stress [48]. Accordingly, we found significantly enriched terms showing up-regulation in ion-related responses pathways (Fig. 3A).

We checked for the expression of the 11 MTs across the three biological replicates of the two analyzed samples. Fig. 3B shows the results for the statistically significant up-regulated MTs, and Fig. 3C for the statistically not significant up-regulated MTs. MT1H is especially affected by DECANOYL-RVKR-CMK, changed from being not expressed to middle-level expression. In the case of the statistically not significant up-regulated MTs, *MT1B* is up-regulated in CSC-CMK, *MT1A* is down-regulated, and *MT4* is not expressed in all the samples (Fig. 3C). To zoom into the expression of the 11 MTs from the MT cluster on chromosome 16, we draw the gene-coverage count track plot where we observe that *MT2A* has a very high peak of reads in all the samples and spreads its support to the *MT1A locus* of the second replicate of the Decanoyl-RVKR-CMK-treated samples (Fig. 3D).

4. Discussion

Previously, CSCs were described as self-renewing cells and resistant to chemotherapy and radiation [49]. In addition, various cancer studies indicate that many malignancies are directly linked to CSCs. Thereby, the ability of CSCs to potentially constitute the basis of tumor formation were proposed as potential target for efficient future cancer therapies. Regarding colon cancer, it was reported to originate from a small fraction of tumor cells in the colon that display self-renewal, pluripotency

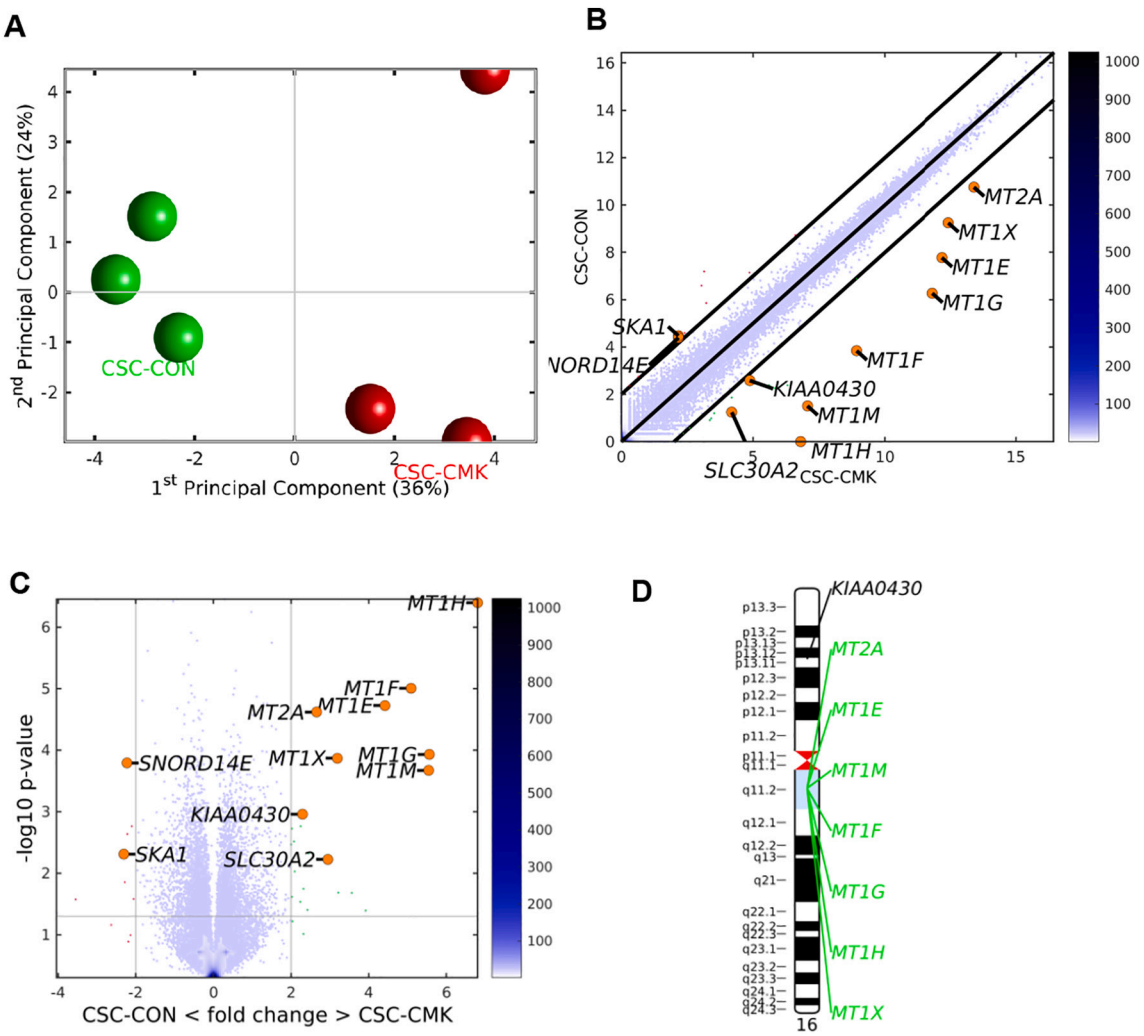
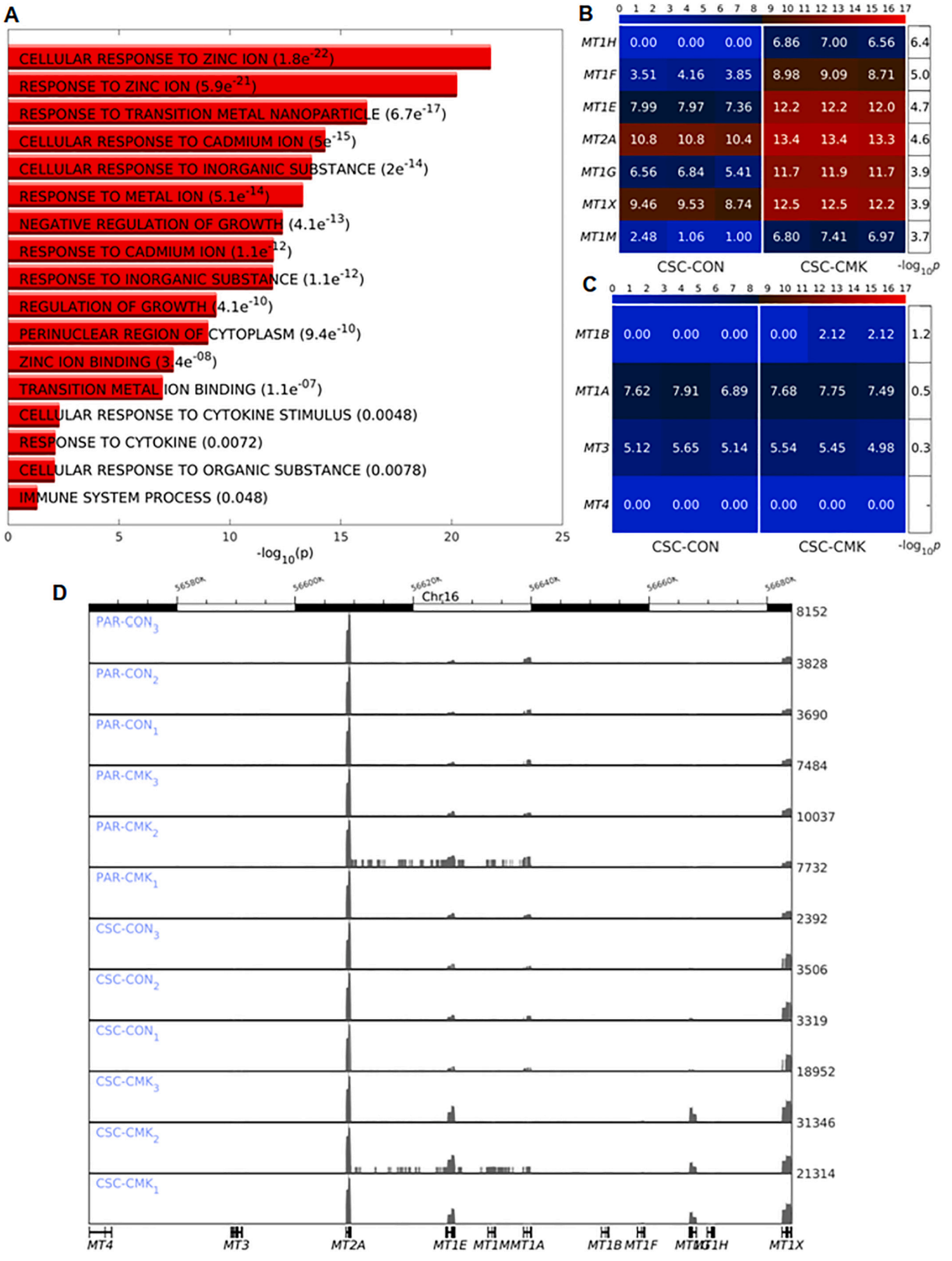


Fig. 2. RNA-seq transcriptomics analysis of Decanoyl-RVKR-CMK-treated CSCs *versus* untreated SW620 CSCs. (A) Principal Component Analysis (PCA) of global RNA-seq expression data. The CSC-CMK populations are depicted by red spheres, the CSC-CON populations by green spheres. (B) Pairwise scatter plots of CSC-CON *vs* CSC-CMK. The black lines are the boundaries of the 2-fold changes in gene expression levels between the paired samples. Transcripts up-regulated in ordinate samples compared with abscissa samples, are shown with red dots; those down-regulated, with green. The colour bar indicates the scattering density. Darker blue colour corresponds to higher scattering density. The transcript expression levels are $\log_2$ scaled. (C) Volcano plot of CSC-CON *vs* CSC-CMK. The fold change is shown in the abcissas in $\log_2$ scale. The $-\log_{10}(p\text{-values})$ in the ordinates. The two grey vertical lines mark the fold change boundaries for a fold change of $FC_{DEG} = 2$ in $\log_2$ scale. The grey horizontal line marks the $\alpha_{DEG} = 0.05$ significance boundary in $-\log_{10}$ scale. The positions of all statistically significant DEGs are shown as orange dots. (D) Ideogram of chromosome 16 with the *loci* of all the DEGs. The green lines and gene names mark a cluster of metallothioneins.

and ability to initiate and maintain tumor development [50]. Therefore, the identification of molecules that regulate CSCs functions is essential for the development of new approaches in order to prevent resistance to chemotherapy and radiotherapy in patients with colon cancer. Such strategy may include potential therapies that interfere with induction of CSCs differentiation or targeting genes that control self-renewal and/or resistance to therapy. Indeed, previously, various pathways in CSCs were reported to be involved in colon CSCs self-renewal and their repression was proposed to target colon cancer. These include Wnt, Notch, PTEN, and Hedgehog pathway. Inhibition of these pathways was previously proposed as potential therapeutic approaches against colorectal cancer [51,52]. Interestingly, many of these CSCs self-renewal pathway proteins directly or indirectly require PCs activity to exhibit their secretion or full biological activity [17]. Thereby inhibition of the PCs in CSCs may affect wide range of protein precursor maturation and/or genes expression involved in the malignant phenotype of CSCs. We found that suppression of PC activity following the treatment of the colon CSCs SW620 by the PCs inhibitor Decanoyl-RVKR-CMK and transcriptomic analyses showed an overexpression of the metallothioneins (MTs) *MT2A*, *MT1X*, *MT1E*, *MT1G*, *MT1F*, *MT1H* and *MT1M*, and the zinc-related gene *SLC30A2*. The most important discovery of our work is that although the effect of Decanoyl-RVKR-CMK is very small over the transcriptome, it has an extremely specific effect, *i.e.* from the only nine up-regulated genes, seven of them are from the family of the eleven MTs. This implies a staggering specificity (p-value $= 2.067 \times 10^{-23}$). MTs play important role in cell proliferation, differentiation, genotoxicity and apoptosis [53]. Metallothioneins up-regulation is induced by zinc elevation producing a scavenging effect and preventing zinc excess into the cell. Another important point of the zinc uptake and MT up-regulation is the chemoresistance. These two events are related with failure of treatment [53], one of the most important characteristics of CSCs. Another important inductor of MTs expression is oxidative stress by the direct action of reactive oxygen strains (ROS) [54]. The antioxidative properties enable MTs decrease the DNA damage caused by hydroxyl radicals, transferring from cytoplasm to nucleus and supporting the progress of the cell cycle [55]. Although increased oxidative stress is related to cancer cell sustained proliferation, these cells have higher antioxidant capacity expressing more molecules with this



(caption on next page)

Fig. 3. Up-regulated genes in human colon cancer SW620 CSCs treated with PC-inhibitor Decanoyl-RVKR-CMK. (A) Bar plot of the $-\log_{10}(p\text{-values})$ of the significantly enriched GO terms of the up-regulated genes. Longer bars correspond to higher statistical significance of the enrichment (*p*-values inside parentheses). (B) Heatmap of the expression of the seven statistically significant up-regulated metallothioneins for a fold change in decreasing order of significance. The color bar codifies gene expression in $\log_2$ scale. Higher gene expression corresponds to redder color. The $-\log_{10}(p\text{-value})$ of the significance of the up-regulation is presented in the table to the right of the heatmap. (C) Heatmap of the expression of the four metallothioneins that are not statistically significantly up-regulated genes for a fold change $FC_{DEG} = 2$ in $\log_2$ scale and significance level $\alpha_{DEG} = 0.05$. (D) Gene-coverage count track plots for the 11 MTs of the MT cluster of chromosome 16.

property. The high oxidative stress status of cancer cell is limited and they cannot exceed certain thresholds [56]. In this case, the reduction of PC's function by Decanoyl-RVKR-CMK could provoke an excessive oxidative stress by deregulation of antioxidant molecules and CSCs would respond with MTs expression. Another important role of MTs is regulation of apoptosis. Overexpression of MTs provokes zinc arrest, and zinc is essential for p53 mediated-apoptosis process [57]. PACE4 inhibition increases apoptosis in cancer cells [58] and MTs upregulation could be a counterbalance mechanism to avoid apoptosis. Another fact related to MTs and apoptosis is the regulation of NF-κB. Overexpression of MT1/2 positively regulates the p50 subunit of NF-κB, protecting breast cancer cells and cervical cancer cells from apoptosis, activating antiapoptotic and pro-oncogenic molecules [59]. It is also important to note the role of MT overexpression in cell differentiation. Increased expression of MTs has been observed in less differentiated tumors [60] and MTs regulate negatively T-cell differentiation [61]. The relation of MTs overexpression with undifferentiation is important since CSCs are non-differentiated cells. Inhibition of PCs by Decanoyl-RVKR-CMK treatment in human nasal epithelial cells promotes cell differentiation inhibiting Notch pathway [62], one of the most important pathways for stemness maintenance of CSCs [15]. Inactivation of stemness canonical pathways in CSCs using Decanoyl-RVKR-CMK could activate substitute mechanism based on MTs to maintain stemness. Recently it has been found that MT1M upregulation in cancer cells significantly inhibits the colony formation, proliferation, migration, and invasion of various cancer cells through the modulating of the expression of N-cadherin and vimentin [63–65]. In addition, downregulation of the mRNA levels of various MT genes was found to significantly correlate with a high risk of poor prognosis in patients with colon cancer whereas high expression of *other MTs* was significantly correlated with good prognosis in these patients [29]. Accordingly, MT induction and zinc administration was reported to sensitize colorectal cancer chemotherapeutic agents [66], suggesting that MTs might act as a tumor suppressor gene in colon cancer and probably in other cancers [67]. In the current study, we revealed that the relation between PCs activity and the expression of various MTs is tightly correlated, and could play a role in CSC biology and maintenance. More studies will be needed to clarify the role of PCs in CSCs and a possible therapeutic application through MTs expression.

CRediT authorship contribution statement

Daniela Gerovska: Methodology, Analysis, Investigation, Writing and Editing. **Patricia García-Gallastegi:** Methodology, Experimentation, Investigation, Writing and Editing. **Jean Descarpentrie** Methodology, Experimentation, Investigation, Writing and Editing. **Olatz Crende**: Experimentation, Writing. **María Casado**: Experimentation, Investigation, Writing. **Ander Martín:** Experimentation, Writing. **Jokin Eguia**: Editing; Writing. **Abdel-Majid Khatib**: Conceptualization, Methodology, Analysis, Investigation, Editing, Writing. **Marcos J. Araúzo-Bravo**: Conceptualization, Methodology, Analysis, Investigation, Writing, Editing. **Iker Badiola:** Conceptualization, Methodology, Analysis, Investigation, Writing, Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by doctoral program Euskampus. Further funding was provided by the University of the Basque Country, Spain, Project ref.: PPGA20/22. D.G. and M.J.A.-B. have been supported by Grants of the Ministry of Economy and Competitiveness, Spain, MINECO Grant BFU2016-77987-P and Instituto de Salud Carlos III (AC17/00012) Grant co-funded by the European Union (Eracosysmed/H2020 Grant Agreement No. 643271) and European Union (H2020-FETOPEN, Project 899417). D.G., M.J. A.-B. and I.B. have been supported by Grants Health Department of the Basque Government (Spain), RIS3 call, Exp. No. 2020333039 and 2020333001. This research was also funded by SIRIC-Brio, La Ligue Contre le Cancer and Region Nouvelle Aquitaine to AMK.

References

[1] G.B. Pierce, W.C. Speers, Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation, Cancer Res. 48 (1988) 1996–2004.
[2] T. Lapidot, C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, J.E. Dick, A cell initiating human acute myeloid leukaemia after transplantation into SCID mice, Nature. 367 (1994) 645–648, https://doi.org/10.1038/367645a0.
[3] T. Schatton, G.F. Murphy, N.Y. Frank, K. Yamaura, A.M. Waaga-Gasser, M. Gasser, Q. Zhan, S. Jordan, L.M. Duncan, C. Weishaupt, R.C. Fuhlbrigge, T.S. Kupper, M. H. Sayegh, M.H. Frank, Identification of cells initiating human melanomas, Nature. 451 (2008) 345–349, https://doi.org/10.1038/nature06489.
[4] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3983–3988, https://doi.org/10.1073/pnas.0530291100.
[5] S.K. Singh, I.D. Clarke, M. Terasaki, V.E. Bonn, C. Hawkins, J. Squire, P.B. Dirks, Identification of a cancer stem cell in human brain tumors, Cancer Res. 63 (2003) 5821–5828.
[6] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, Nature. 414 (2001) 105–111, https://doi.org/10.1038/35102167.
[7] C. Massard, E. Deutsch, J.-C. Soria, Tumour stem cell-targeted treatment: elimination or differentiation, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 17 (2006) 1620–1624, https://doi.org/10.1093/annonc/mdl074.
[8] B. Bao, A.S. Prasad, Targeting CSC in a most aggressive subtype of breast cancer TNBC., Adv. Exp. Med. Biol. 1152 (2019) 311–334. doi:https://doi.org/10.1007/978-3-030-20301-6_17.
[9] S.Y. Lee, E.K. Jeong, M.K. Ju, H.M. Jeon, M.Y. Kim, C.H. Kim, H.G. Park, S.I. Han, H.S. Kang, Induction of metastasis, cancer stem cell phenotype, and oncogenic metabolism in cancer cells by ionizing radiation, Mol. Cancer 16 (2017) 10, https://doi.org/10.1186/s12943-016-0577-4.
[10] C. Naujokat, R. Steinhart, Salinomycin as a drug for targeting human cancer stem cells, J. Biomed. Biotechnol. 2012 (2012) 950658, https://doi.org/10.1155/2012/950658.
[11] R.L. Siegel, K.D. Miller, A. Goding Sauer, S.A. Fedewa, L.F. Butterly, J.C. Anderson, A. Cercek, R.A. Smith, A. Jemal, Colorectal cancer statistics, 2020, CA Cancer J. Clin. 70 (2020) 145–164, https://doi.org/10.3322/caac.21601.
[12] S. Basu, G. Haase, A. Ben-Ze'ev, Wnt signaling in cancer stem cells and colon cancer metastasis., F1000Research. 5 (2016). doi:10.12688/f1000research.7579.1.
[13] M.C. Cleophas, L.A. Joosten, L.K. Stamp, N. Dalbeth, O.M. Woodward, T. R. Merriman, ABCG2 polymorphisms in gout: insights into disease susceptibility and treatment approaches, Pharmgenomics. Pers. Med. 10 (2017) 129–142, https://doi.org/10.2147/PGPM.S105854.
[14] A.S. Jaiswal, B.P. Marlow, N. Gupta, S. Narayan, Beta-catenin-mediated transactivation and cell-cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells, Oncogene. 21 (2002) 8414–8427, https://doi.org/10.1038/sj.onc.1205947.
[15] N. Takebe, L. Miele, P.J. Harris, W. Jeong, H. Bando, M. Kahn, S.X. Yang, S.P. Ivy, Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update., Nat. Rev. Clin. Oncol. 12 (2015) 445–464. doi:https://doi.org/10.1038/nrclinonc.2015.61.
[16] J. Wang, B. Zhang, H. Wu, J. Cai, X. Sui, Y. Wang, H. Li, Y. Qiu, T. Wang, Z. Chen, Q. Zhu, H. Xia, W. Song, A.P. Xiang, CD51 correlates with the TGF-beta pathway and is a functional marker for colorectal cancer stem cells, Oncogene. 36 (2017) 1351–1363, https://doi.org/10.1038/onc.2016.299.
[17] G. Siegfried, J. Descarpentrie, S. Evrard, A.-M. Khatib, Proprotein convertases: key players in inflammation-related malignancies and metastasis, Cancer Lett. 473 (2020) 50–61, https://doi.org/10.1016/j.canlet.2019.12.027.

[18] F. Soulet, C. Bodineau, K.B. Hooks, J. Descarpentrie, I. Alves, M. Dubreuil, A. Mouchard, M. Eugenie, J.-L. Hoepffner, J.J. López, J.A. Rosado, I. Soubeyran, M. Tomé, R. V Durán, M. Nikolski, B.O. Villoutreix, S. Evrard, G. Siegfried, A.-M. Khatib, ELA/APELA precursor cleaved by furin displays tumor suppressor function in renal cell carcinoma through mTORC1 activation., JCI Insight. 5 (2020). doi:https://doi.org/10.1172/jci.insight.129070.

[19] F. Adam, A.-M. Khatib, J.J. Lopez, C. Vatier, S. Turpin, A. Muscat, F. Soulet, A. Aries, I. Jardin, R. Bobe, A. Stepanian, D. de Prost, C. Dray, J.A. Rosado, P. Valet, B. Feve, G. Siegfried, Apelin: an antithrombotic factor that inhibits platelet function, Blood. 127 (2016) 908–920, https://doi.org/10.1182/blood-2014-05-578781.

[20] Z. He, L. Thorrez, G. Siegfried, S. Meulemans, S. Evrard, S. Tejpar, A.-M. Khatib, J. W.M. Creemers, The proprotein convertase furin is a pro-oncogenic driver in KRAS and BRAF driven colorectal cancer, Oncogene. 39 (2020) 3571–3587, https://doi.org/10.1038/s41388-020-1238-z.

[21] Z. He, A.-M. Khatib, J.W.M. Creemers, Loss of the proprotein convertase Furin in T cells represses mammary tumorigenesis in oncogene-driven triple negative breast cancer, Cancer Lett. 484 (2020) 40–49, https://doi.org/10.1016/j.canlet.2020.05.001.

[22] N. Scamuffa, G. Siegfried, Y. Bontemps, L. Ma, A. Basak, G. Cherel, F. Calvo, N. G. Seidah, A.-M. Khatib, Selective inhibition of proprotein convertases represses the metastatic potential of human colorectal tumor cells, J. Clin. Invest. 118 (2008) 352–363, https://doi.org/10.1172/JCI32040.

[23] A.J. Klein-Szanto, D.E. Bassi, Proprotein convertase inhibition: paralyzing the cell's master switches, Biochem. Pharmacol. 140 (2017) 8–15, https://doi.org/10.1016/j.bcp.2017.04.027.

[24] M. Tomé, A. Pappalardo, F. Soulet, J.J. López, J. Olaizola, Y. Leger, M. Dubreuil, A. Mouchard, D. Fessart, F. Delom, V. Pitard, D. Bechade, M. Fonck, J.A. Rosado, F. Ghiringhelli, J. Déchanet-Merville, I. Soubeyran, G. Siegfried, S. Evrard, A.-M. Khatib, Inactivation of proprotein convertases in T cells inhibits PD-1 expression and creates a favorable immune microenvironment in colorectal cancer, Cancer Res. 79 (2019) 5008–5021, https://doi.org/10.1158/0008-5472.CAN-19-0086.

[25] D.E. Bassi, H. Mahloogi, R. Lopez De Cicco, A. Klein-Szanto, Increased furin activity enhances the malignant phenotype of human head and neck cancer cells, Am. J. Pathol. 162 (2003) 439–447, https://doi.org/10.1016/S0002-9440(10)63838-2.

[26] G. Siegfried, A. Basak, J.A. Cromlish, S. Benjannet, J. Marcinkiewicz, M. Chrétien, N.G. Seidah, A.-M. Khatib, The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis, J. Clin. Invest. 111 (2003) 1723–1732, https://doi.org/10.1172/JCI17220.

[27] F. Sfaxi, N. Scamuffa, C. Lalou, J. Ma, P. Metrakos, G. Siegfried, H. Ragg, A. Bikfalvi, F. Calvo, A.-M. Khatib, Repression of liver colorectal metastasis by the serpin Spn4A a naturally occurring inhibitor of the constitutive secretory proprotein convertases., Oncotarget. 5 (2014) 4195–4210. doi:10.18632/oncotarget.1966.

[28] S.R. Singh, P. Aggarwal, S.X. Hou, Cancer stem cells and stem cell tumors in drosophila., Adv. Exp. Med. Biol. 1167 (2019) 175–190. doi:https://doi.org/10.1007/978-3-030-23629-8_10.

[29] K.-C. Hung, T.-C. Huang, C.-H. Cheng, Y.-W. Cheng, D.-Y. Lin, J.-J. Fan, K.-H. Lee, The expression profile and prognostic significance of metallothionein genes in colorectal cancer., Int. J. Mol. Sci. 20 (2019). doi:https://doi.org/10.3390/ijms20163849.

[30] L. Ricci-Vitiani, D.G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle, R. De Maria, Identification and expansion of human colon-cancer-initiating cells, Nature. 445 (2007) 111–115, https://doi.org/10.1038/nature05384.

[31] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods. 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[32] A. Guentsch, C. Hirsch, W. Pfister, B. Vincents, M. Abrahamson, A. Sroka, J. Potempa, S. Eick, Cleavage of IgG1 in gingival crevicular fluid is associated with the presence of Porphyromonas gingivalis, J. Periodontal Res. 48 (2013) 458–465, https://doi.org/10.1111/jre.12027.

[33] J. Ma, S. Evrard, I. Badiola, G. Siegfried, A.-M. Khatib, Regulation of the proprotein convertases expression and activity during regenerative angiogenesis: role of hypoxia-inducible factor (HIF), Eur. J. Cell Biol. 96 (2017). doi:https://doi.org/10.1016/j.ejcb.2017.06.001.

[34] M. Pertea, D. Kim, G.M. Pertea, J.T. Leek, S.L. Salzberg, Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown, Nat. Protoc. 11 (2016) 1650–1667, https://doi.org/10.1038/nprot.2016.095.

[35] C. Trapnell, A. Roberts, L. Goff, G. Pertea, D. Kim, D.R. Kelley, H. Pimentel, S. L. Salzberg, J.L. Rinn, L. Pachter, Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks, Nat. Protoc. 7 (2012) 562–578, https://doi.org/10.1038/nprot.2012.016.

[36] S. Anders, P.T. Pyl, W. Huber, HTSeq—a Python framework to work with high-throughput sequencing data., Bioinformatics. 31 (2015) 166–169. doi:https://doi.org/10.1093/bioinformatics/btu638.

[37] D. Gerovska, G. Larrinaga, J.D. Solano-Iturri, J. Márquez, P. García Gallastegi, A.-M. Khatib, G. Poschmann, K. Stühler, M. Armesto, C.H. Lawrie, I. Badiola, M.J. Araúzo-Bravo, An integrative omics approach reveals involvement of BRCA1 in hepatic metastatic progression of colorectal cancer., Cancers (Basel). 12 (2020). doi:https://doi.org/10.3390/cancers12092380.

[38] D. Gerovska, M.J. Araúzo-Bravo, Computational analysis of single-cell transcriptomics data elucidates the stabilization of Oct4 expression in the E3.25 mouse preimplantation embryo., Sci. Rep. 9 (2019) 8930. doi:https://doi.org/10.1038/s41598-019-45438-y.

[39] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15545–15550, https://doi.org/10.1073/pnas.0506580102.

[40] H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, The sequence alignment/map format and SAMtools., Bioinformatics. 25 (2009) 2078–2079. doi:https://doi.org/10.1093/bioinformatics/btp352.

[41] A.R. Quinlan, I.M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features, Bioinformatics. 26 (2010) 841–842, https://doi.org/10.1093/bioinformatics/btq033.

[42] H. Lee, H.Y. Lee, B.E. Lee, D. Gerovska, S.Y. Park, H. Zaehres, M.J. Araúzo-Bravo, J.-I. Kim, Y. Ha, H.R. Schöler, J.B. Kim, Sequentially induced motor neurons from human fibroblasts facilitate locomotor recovery in a rodent spinal cord injury model., Elife. 9 (2020). doi:https://doi.org/10.7554/eLife.52069.

[43] L. Song, Y. Li, the role of stem cell DNA methylation in colorectal carcinogenesis., Stem Cell Rev. Reports. 12 (2016) 573–583. doi:https://doi.org/10.1007/s12015-016-9672-6.

[44] J.P.I. Welburn, E.L. Grishchuk, C.B. Backer, E.M. Wilson-Kubalek, J.R. 3rd Yates, I. M. Cheeseman, The human kinetochore Ska1 complex facilitates microtubule depolymerization-coupled motility., Dev. Cell. 16 (2009) 374–385. doi:https://doi.org/10.1016/j.devcel.2009.01.011.

[45] S.A. Rensing, U.G. Maier, Phylogenetic analysis of the stress-70 protein family, J. Mol. Evol. 39 (1994) 80–86, https://doi.org/10.1007/BF00178252.

[46] T. Nishimura, H. Fakim, T. Brandmann, J.-Y. Youn, A.-C. Gingras, M. Jinek, M.R. Fabian, Human MARF1 is an endoribonuclease that interacts with the DCP1:2 decapping complex and degrades target mRNAs., Nucleic Acids Res. 46 (2018) 12008–12021. doi:https://doi.org/10.1093/nar/gky1011.

[47] Y.-J. Lu, Y.-C. Liu, M.-C. Lin, Y.-T. Chen, L.-Y. Lin, Coordinative modulation of human zinc transporter 2 gene expression through active and suppressive regulators, J. Nutr. Biochem. 26 (2015) 351–359, https://doi.org/10.1016/j.jnutbio.2014.11.009.

[48] M. Si, J. Lang, The roles of metallothioneins in carcinogenesis, J. Hematol. Oncol. 11 (2018) 107, https://doi.org/10.1186/s13045-018-0645-x.

[49] H.-R. Sun, S. Wang, S.-C. Yan, Y. Zhang, P.J. Nelson, H.-L. Jia, L.-X. Qin, Q.-Z. Dong, Therapeutic strategies targeting cancer stem cells and their microenvironment, Front. Oncol. 9 (2019) 1104, https://doi.org/10.3389/fonc.2019.01104.

[50] K. Ganesan, B. Xu, Molecular targets of vitexin and isovitexin in cancer therapy: a critical review, Ann. N. Y. Acad. Sci. 1401 (2017) 102–113, https://doi.org/10.1111/nyas.13446.

[51] M. Todaro, M.G. Francipane, J.P. Medema, G. Stassi, Colon cancer stem cells: promise of targeted therapy, Gastroenterology. 138 (2010) 2151–2162, https://doi.org/10.1053/j.gastro.2009.12.063.

[52] M. Lepourcelet, Y.-N.P. Chen, D.S. France, H. Wang, P. Crews, F. Petersen, C. Bruseo, A.W. Wood, R.A. Shivdasani, Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex, Cancer Cell 5 (2004) 91–102, https://doi.org/10.1016/s1535-6108(03)00334-9.

[53] S. Krizkova, M. Ryvolova, J. Hrabeta, V. Adam, M. Stiborova, T. Eckschlager, R. Kizek, Metallothioneins and zinc in cancer diagnosis and therapy, Drug Metab. Rev. 44 (2012) 287–301, https://doi.org/10.3109/03602532.2012.725414.

[54] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, Free radicals, metals and antioxidants in oxidative stress-induced cancer, Chem. Biol. Interact. 160 (2006) 1–40, https://doi.org/10.1016/j.cbi.2005.12.009.

[55] Y. Takahashi, Y. Ogra, K.T. Suzuki, Nuclear trafficking of metallothionein requires oxidation of a cytosolic partner, J. Cell. Physiol. 202 (2005) 563–569, https://doi.org/10.1002/jcp.20158.

[56] M. Fiocchetti, V.S. Fernandez, E. Montalesi, M. Marino, Neuroglobin: a novel player in the oxidative stress response of cancer cells, Oxidative Med. Cell. Longev. 2019 (2019) 6315034, https://doi.org/10.1155/2019/6315034.

[57] E. Palecek, M. Brázdová, H. Cernocká, D. Vlk, V. Brázda, B. Vojtesek, Effect of transition metals on binding of p53 protein to supercoiled DNA and to consensus sequence in DNA fragments, Oncogene. 18 (1999) 3617–3625, https://doi.org/10.1038/sj.onc.1202710.

[58] C. Levesque, F. Couture, A. Kwiatkowska, R. Desjardins, B. Guérin, W.A. Neugebauer, R. Day, PACE4 inhibitors and their peptidomimetic analogs block prostate cancer tumor progression through quiescence induction, increased apoptosis and impaired neovascularisation., Oncotarget. 6 (2015) 3680–3693. doi:10.18632/oncotarget.2918.

[59] E. Papouli, M. Defais, F. Larminat, Overexpression of metallothionein-II sensitizes rodent cells to apoptosis induced by DNA cross-linking agent through inhibition of NF-kappa B activation, J. Biol. Chem. 277 (2002) 4764–4769, https://doi.org/10.1074/jbc.M108447200.

[60] M.G. Cherian, A. Jayasurya, B.-H. Bay, Metallothioneins in human tumors and potential roles in carcinogenesis, Mutat. Res. 533 (2003) 201–209, https://doi.org/10.1016/j.mrfmmm.2003.07.013.

[61] C. Wu, C. Pot, L. Apetoh, T. Thalhamer, B. Zhu, G. Murugaiyan, S. Xiao, Y. Lee, M. Rangachari, N. Yosef, V.K. Kuchroo, Metallothioneins negatively regulate IL-27-induced type 1 regulatory T-cell differentiation, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 7802–7807, https://doi.org/10.1073/pnas.1211776110.

[62] S.-N. Lee, I.-S. Choi, H.J. Kim, E.J. Yang, H.J. Min, J.-H. Yoon, Proprotein convertase inhibition promotes ciliated cell differentiation - a potential mechanism for the inhibition of Notch1 signalling by decanoyl-RVKR-chloromethylketone, J. Tissue Eng. Regen. Med. 11 (2017) 2667–2680, https://doi.org/10.1002/term.2240.

[63] Y. Chen, R. Quan, A. Bhandari, Z. Chen, Y. Guan, J. Xiang, J. You, L. Teng, Low metallothionein 1M (MT1M) is associated with thyroid cancer cell lines progression, Am. J. Transl. Res. 11 (2019) 1760–1770.
[64] L. Changjun, H. Feizhou, P. Dezhen, L. Zhao, M. Xianhai, MiR-545-3p/MT1M axis regulates cell proliferation, invasion and migration in hepatocellular carcinoma, Biomed. Pharmacother. 108 (2018) 347–354, https://doi.org/10.1016/j.biopha.2018.09.009.
[65] J.M. Arriaga, A.I. Bravo, J. Mordoh, M. Bianchini, Metallothionein 1G promotes the differentiation of HT-29 human colorectal cancer cells, Oncol. Rep. 37 (2017) 2633–2651, https://doi.org/10.3892/or.2017.5547.
[66] J.M. Arriaga, A. Greco, J. Mordoh, M. Bianchini, Metallothionein 1G and zinc sensitize human colorectal cancer cells to chemotherapy, Mol. Cancer Ther. 13 (2014) 1369–1381, https://doi.org/10.1158/1535-7163.MCT-13-0944.
[67] J. Mao, H. Yu, C. Wang, L. Sun, W. Jiang, P. Zhang, Q. Xiao, D. Han, H. Saiyin, J. Zhu, T. Chen, L.R. Roberts, H. Huang, L. Yu, Metallothionein MT1M is a tumor suppressor of human hepatocellular carcinomas., Carcinogenesis. 33 (2012) 2568–2577. doi:https://doi.org/10.1093/carcin/bgs287.