Metallothionein 1G promotes the differentiation of HT-29 human colorectal cancer cells

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Received September 27, 2016; Accepted November 16, 2016

DOI: 10.3892/or.2017.5547

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Abstract. Metallothioneins (MTs) are a family of low-molecular-weight, cysteine-rich proteins involved in zinc and redox metabolism, that are epigenetically downregulated during colorectal cancer (CRC) progression, but may be re-induced with a variety of agents. Since loss of MT expression is associated with a worse prognosis, in the present study we investigated the effects of overexpression of the most significantly downregulated isoform in CRC, namely MT1G, on the HT-29 cell line. Overexpression of MT1G resulted in xenograft tumors with an aberrant morphology, characterized by an evident increase in mucin-containing cells that were identified as goblet cells under electron microscopy. Immunohistochemical detection of CDX2 and cytokeratin 20 was also increased, as were goblet-cell and enterocyte-specific genes by qRT-PCR. Microarray analysis of gene expression confirmed the alteration of several differentiation signaling pathways, including the Notch pathway. Using sodium butyrate and post-confluent growth as inducers of differentiation, we demonstrated that MT1G does indeed play a functional role in promoting goblet over enterocyte differentiation in vitro. Labile zinc is also induced upon differentiation of CRC cells, functionally contributing to goblet over enterocyte differentiation, as revealed using zinc-specific chelating agents. Overall, our results uncover a new tumor-suppressor activity of MT1G in promoting the differentiation of at least some CRC tumors, and implicate MTs and zinc signaling as new players in colorectal differentiation. This further contributes to the hypothesis that re-induction of MTs may have therapeutic value by diminishing the aggressiveness of CRC tumors.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, having a mortality rate near 50% (1). Recent studies have shown that these tumors retain multilineage differentiation processes similar to those of the normal intestinal epithelium, mainly the goblet cell and enterocyte lineages (2). Furthermore, molecular classifications representing these cellular phenotypes can have prognostic value and be predictive of response to different therapeutic agents (3).

Metallothioneins (MTs) are a family of low-molecular-weight, cysteine-rich proteins involved in zinc and redox metabolism. By chelating zinc ions through redox-active thiol groups, they have the capacity to regulate the exchangeable, loosely-bound pool of intracellular zinc, termed the ‘labile’ pool, which participates in zinc transfer reactions and intracellular signaling. Thus MTs have been implicated in many aspects of tumor biology, such as proliferation, differentiation, apoptosis, angiogenesis, redox and zinc homeostasis, anti-inflammatory reactions and immunomodulation (4-7). The human genome encodes at least 11 functional MT isoforms that share structural and functional similarities. Due to their structural similarity, commercially available antibodies do not distinguish between individual MT isoforms, and therefore their individual mRNA expression levels can be measured by qRT-PCR. However, due to the fact that they are variably expressed in tissues and induced by several stimuli, it is possible that different tumors express distinct MT genes, which could help explain the conflicting data on MT function in different tumor types (6,7). We and others have previously shown that multiple MT isoforms and MT2A are downregulated during CRC progression (especially isoform MT1G) mainly through epigenetic mechanisms, and that this is associated with shorter patient survival (8-11). Several agents such as DNA methyltransferase inhibitors, histone deacetylase inhibitors or zinc are capable of re-inducing MT expression in colorectal tumors, which can

Corrections and Abbreviations: BUT, sodium butyrate; CRC, colorectal cancer; FZ, fluozin 3-AM; MMPs, matrix metalloproteinases; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine; MTs, metallothioneins

Key words: metallothioneins, labile zinc, colorectal cancer, goblet cells, differentiation


slow down in vivo tumor growth and sensitize these tumors to chemotherapeutic agents (12).

In order to help understand the phenotypic consequences of MT induction, in the present study we investigated the effects of stable overexpression of the most downregulated isoform in CRC, namely MT1G, on the HT-29 CRC cell line. We uncovered a new role for this isoform in modulating tumor differentiation and thus expand the mechanisms by which this gene may act as a tumor suppressor in CRC.

Materials and methods

Reagents and cell lines. The MT1G cDNA was cloned into the pcDNA3.1/myc-His(-) vector expression vector, resulting in an MT1G-myc fusion protein as previously described (12). Sodium butyrate and N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA), and FluoZin-3-AM (FZ) from Invitrogen (San Diego, CA, USA). The human CRC cell lines HT-29 and HCT116 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), maintained as previously described (8), and subjected to STR profiling for authentication after all experiments were finalized. For post-confluent growth, day 0 was considered the day when cells reached 100% confluence, and fresh medium was replaced every 1-2 days thereafter.

Animal studies and histological procedures. Eight-to 10-week-old male nude mice were subcutaneously injected (2x10⁶ cells each) with two independent clones of MOCK or MT1G cells (5 mice/group) and tumor size was measured to 10-week-old male nude mice were subcutaneously injected (2x10⁶ cells each) with two independent clones of MOCK or MT1G cells (5 mice/group) and tumor size was measured with citrate buffer antigen retrieval and the following antibodies: anti-cytokeratin 20 (KS 20.8; Dako Corporation, Carpinteria, CA, USA) and anti-CDX2 (clone EPR2764Y; Cell Marque, Rocklin, CA, USA). For transmission electron microscopy, freshly xenografted tumors were cut into small (~1-mm thick) pieces and promptly fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 2 h, washed and fixed for 90 min in 1% osmium tetroxide in phosphate-buffered saline (PBS), dehydrated in acetone gradients and included in resin. Semi- (0.5 µm) and ultra-thin (70 nm) sections were cut and contrasted in 2.5% uranyl-acetate, and visualized using a Zeiss EM 109T microscope coupled to a digital CCD Gatan ES1000W camera.

Gene expression profile analysis and qRT-PCR. Total RNA was extracted, and mRNA expression was analyzed using an Agilent Custom microarray 8x15K (Agilent Technologies, Palo Alto, CA, USA), which contained 15,744 oligonucleotide probes representing >8,200 different human transcripts. Two samples from each group were used to detect mRNA expression; each biological replicate was run in duplicate, and the fluorochromes were swapped to reduce dye-bias; in total eight 15K microarrays were scanned using the Axon Confocal Scanner 4000B (Molecular Devices, Sunnyvale, CA, USA) with optimized settings: dye channel, 635 nm, PMT=720, laser power, 30%, scan resolution, 10 nm; dye channel, 532 nm, PMT=540, laser power, 30%, scan resolution, 10 nm; line average, 4 lines. The data were analyzed using GenePix® Pro 6 Microarray Acquisition and Analysis Software (Molecular Devices) and normalized with the MIDAS v2.2: Microarray Data Analysis System (TIGR’s Microarray Data Analysis System). Normalization was necessary to compensate for variability between slides and fluorescent dyes. To this end we employed a locally weighted linear regression [Lowess (13,14)]; data were filtered using low-intensity cutoff and replicate consistency trimming.

The differentially expressed genes among the MT1G+, and control (MOCK) sets were identified using the significant analysis of microarray (SAM) statistical software from MultiExperiment Viewer (MeV) (TIGR’s Microarray Data Analysis System). In the comparisons of MT1G+ vs. MOCK, the genes that were all upregulated in the comparisons were identified as the persistently upregulated genes, and the genes that were all downregulated in the comparisons were defined as the persistently downregulated genes.

The gene annotation enrichment analysis using Gene Ontology (GO) (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) data for gene sets was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (15,16). A Benjamini p-value of 0.05 was used in the analysis.

Quantitative reverse-transcription PCR (qRT-PCR) was used to quantify mRNA levels as previously described (8). Briefly, PCR runs were carried out using SYBR Universal Master Mix (Applied Biosystems, Carlsbad, CA, USA), and relative expression levels were determined by the ΔΔCt method using ACTB gene expression to normalize all samples. The primers used are listed in Table I.

Alkaline phosphatase activity measurement. The activity of this enzyme was used as a marker of differentiation of HT-29 cells (17). For this purpose, confluent cell lines were lysed in 10 mM Tris (pH 7.4), 1 mM MgCl₂, 20 µM ZnCl₂, 0.2% Triton X-100 + protease inhibitors, and incubated with NBT-BCIP as the chromogenic substrate for 16 h at 37°C. The resulting brown precipitate was solubilized in 10% SDS, 10% HCl and absorbance was measured at 595 nm.

siRNA transfection. Two siRNAs targeting the MT1G isoform (siG.1 and siG.2) and one targeting all functional MT-1 and MT-2 isoforms were previously validated (12), and transfected at 125 nM using LF2000 (Invitrogen) as described by the manufacturer. After 24 h of siRNA treatment, medium was replaced...
with or without 2 mM sodium butyrate for 48 h, and cells were collected for RNA extraction or ALP activity measurement.

**Scratch assays and gelatin zymography.** We used the scratch assay to estimate the migration capacities of MOCK and MT1G+ cell lines, which were plated in triplicate in 24-well plates until they reached confluence. Two perpendicular scratches were made with a pipette tip, after which the cells were washed thrice in PBS and replaced with 1% fetal bovine serum (FBS) medium. Areas with the same wound length were selected and photographed until complete wound closure. Wound closure at a given time t was calculated as: (initial wound length - wound length at time t)/initial wound length x 100.

To determine gelatinase activity of matrix metalloproteinases (MMPs), upon reaching confluence medium was replaced with serum-free Dulbecco's modified Eagle's medium (DMEM) for 24 h, and the conditioned medium was centrifuged at 1,200 x g for 5 min, and immediately loaded into 10% polyacrylamide electrophoretic gels with or without 2.5 mg/ml gelatin (Sigma-Aldrich) as described in (18). Coomasie Blue staining of the non-gelatin gels were used as a loading control.

**Measurement of intracellular labile zinc.** For this purpose we employed the cell-permeable zinc-specific fluorophore FZ as described in (12). Briefly, cells were plated in triplicate in sterile plastic coverslips (for fluorescence microscopy) or in 96-well plates (for fluorimetric analysis), and incubated for 30 min at room temperature with 2 µM FZ in PBS, washed in PBS and incubated a further 30 min in PBS at room temperature. Propidium iodide staining was used to control for plating differences and data are expressed as normalized fluorescence FZ = (F - FTPEN)/(FZn - FTPEN), so as to get values relative to a ‘maximum’ intensity given by pretreatment with zinc 400 µM for 8 h (FZn, resulting in FZ=1) and a ‘minimum’ intensity given by 20 µM TPEN treatment during the final 30 min incubation of fluozin (FTPEN, resulting in FZ=0). This score allowed us to better compare results of the different experiments.

**Statistical analysis.** Data are expressed as mean ± SEM and p-values <0.05 were considered significant. Comparison of means was carried out using Student's t-test, with one-way ANOVA followed by Dunnett's post hoc t-test for three or more groups, or with two-way ANOVA followed by Bonferroni's post hoc t-test for two variables. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for analysis.

**Results**

**MT1G overexpression in the HT-29 CRC cell line.** We stably expressed MT1G as a myc-epitope fusion protein in HT-29 cells. When grown in vivo as subcutaneous xenografts on nude mice, these MT1G+ cells grew at similar rates compared to the empty-vector ('MOCK')-transfected cells (data not shown), in stark contrast to the antiproliferative effects we had previously observed using the HCT116 cell line (12). However, hematoxylin and eosin (H&E) staining (Fig. 1A) showed that MT1G + tumors contained a higher number of mucin-containing, Alcian Blue-positive cells (Fig. 1B) that were confirmed to be goblet cells by transmission electron microscopy (Fig. 1C). Nuclear expression of the intestine-specific homeobox transcription factor CDX2 was markedly enhanced in the MT1G + tumors, as shown by immunohistochemical staining on Fig. 1D, as was also the intensity of cytokeratin 20 (Fig. 1E). The latter also suggests that commitment to the enterocyte lineage may be enhanced as well. Indeed, both goblet-associated (TFF3, ATOH1 and MUC2) and enterocyte-associated genes (HSI, CA2 and ALPI) were overexpressed in the MT1G+ tumors by qRT-PCR analysis (Fig. 1F), suggesting that MT1G+ tumors are more differentiated than MOCK controls.

**Gene expression analysis of HT-29 MT1G+ tumors using cDNA microarrays.** We then used cDNA microarrays to profile the mRNA expression of MOCK and MT1G+ HT-29 xenografts, derived from two different MT1G+ or MOCK clonal cell lines (MT1G-1 and MT1G-2, or MOCK-1 and

Table I. Primer sequences.

| Gene   | Forward primer          | Reverse primer          |
|--------|-------------------------|-------------------------|
| MT1G   | CTTCTCGCTTGGGAACCTCTA   | AGGGGTCAAGATTGTAGCAAA   |
| MT2A   | GCAACCTGTCGCCGACTCTAG   | TTGCAGGGATGTCAATTG     |
| ACTB   | GCCATCTCTGTCTCAGTGCTCCAG| ATGTTTGAGACCTTCAACACCCC|
| CDKN1A | AAGACCATGTTGGACCTGT    | GGTAGAAATCTGTCATGCT    |
| HSI    | GAGGACACTGCGTGGAGAC     | ATCCACGCGGTACAGATG     |
| HALPI  | GACACCTCACCATGCTCTCTCTT| TCACGCGCTGATGTGAA      |
| CA2    | CCCGGGACACAGTGTCAGCG    | CCAATCGTCAGGTCGGTTGT   |
| CA1    | CAGACATACATGTTGGAGGAGTCAA| GGCCTCACAACCTTCTATCA   |
| K20    | AAAGCCTCGGATGTCCTCTTG  | ACTCTCCTCAGTGTCCTT     |
| ATOH1  | CCCGGGAGCATCTTGG       | GGGACCAGGCGAGATTG      |
| TFF3   | CTCCAGCCTGTCGTGAGGAGT  | GCTTGAACACAGGCACT      |
| HMUC2  | CAGCACCGATTGGTCTGGT    | GCTTGCATCATTAGGCCAG    |
| CDX2   | GATGCTGATGACTCAGTGA    | CTCGGCAGCAGATGAAAAC    |

**Statistical analysis.** Data are expressed as mean ± SEM and p-values <0.05 were considered significant. Comparison of means was carried out using Student's t-test, with one-way ANOVA followed by Dunnett's post hoc t-test for three or more groups, or with two-way ANOVA followed by Bonferroni's post hoc t-test for two variables. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for analysis.
Gene expression profiles of the biological replicates were reproducible and highly correlated (Pearson's correlation coefficient 0.81). Analysis of data with Rank product analysis revealed significant gene expression differences among the groups, with a total of 305 known genes found to be consistently upregulated or downregulated in the MT1G+ tumors (Table II). GO analysis indicated that several functional categories were enriched by DAVID, and included upregulated genes associated with cell differentiation, cell fate commitment and Notch signaling pathway, as well as downregulated genes in the categories of regulation of apoptosis, cell migration and cell proliferation (Table III). Differentially expressed genes were also analyzed for KEGG pathway enrichment and two significantly enriched pathways were identified between upregulated or downregulated genes: the Notch signaling pathway and pathways in cancer, respectively.

Given the finding of downregulated genes in the cell migration category, we performed migration 'scratch' assays in the HT-29 and HCT116 cell lines overexpressing MT1G, and found in both cell lines a statistically significant reduction in migration rates upon MT1G overexpression (Fig. 2A and B). Gelatin zymography using conditioned media from these cells, however, revealed no differences in MMP2 activity (Fig. 2C).

Next, in order to further investigate the involvement of MT1G in the differentiation of HT-29 cells, we used two different and well-known cell culture conditions to stimulate the in vitro differentiation of these cells: sodium butyrate (BUT) treatment (19) and post-confluent cell growth (20). We used TFF3 and MUC2 mRNA expression as surrogate markers for the goblet cell lineage, and HS1 and CA1 mRNAs, along with enzymatic alkaline-phosphatase activity (ALP) for enterocytes.

Involvement of MT1G in butyrate-mediated differentiation of HT-29 cells. Sodium butyrate is a well-known inducer of differentiation in CRC cell lines (21), and indeed, as shown in
Table II. List of all significantly differentially expressed genes in MT1G+ vs. MOCK HT-29 xenografts.

A, Upregulated genes

| Gene reference | Gene symbol | Name                                      | Mean | P-values (Up) | RP-values (Up) |
|----------------|-------------|-------------------------------------------|------|---------------|----------------|
| NM_138444      | KCTD12      | Potassium channel tetramerisation domain containing 12 | 2.65 | 2.81E-06      | 80.00          |
| NM_000051      | ATM         | Ataxia telangiectasia mutated             | 2.52 | 4.68E-06      | 91.77          |
| NM_175698      | SSX2        | Synovial sarcoma, X breakpoint 2           | 3.06 | 6.56E-06      | 99.19          |
| NM_031964      | KRTAP17-1   | Keratin-associated protein 17-1            | 2.35 | 2.25E-05      | 141.15         |
| NM_003357      | SCGB1A1     | Secretoglobulin, family 1A, member 1       | 2.19 | 2.72E-05      | 155.94         |
| NM_005430      | WNT1        | Wingless-type MMTV integration site family, member 1 | 2.40 | 2.81E-05      | 156.29         |
| NM_001031672   | CYB5RL      | Cytochrome b5 reductase-like               | 2.37 | 3.18E-05      | 164.29         |
| NM_000546      | TP53        | Tumor protein p53                          | 2.12 | 3.75E-05      | 168.99         |
| NM_001123065   |             | Chromosome 7 open reading frame 65         | 2.04 | 4.59E-05      | 181.40         |
| NM_001443      | FABP1       | Fatty acid binding protein 1, liver        | 2.27 | 5.99E-05      | 197.89         |
| NM_000364      | TNNT2       | Troponin T type 2 (cardiac)                | 2.01 | 8.24E-05      | 216.07         |
| NM_001201      | BMP3        | Bone morphogenetic protein 3               | 2.17 | 1.01E-04      | 224.84         |
| NM_031310      | PLVAP       | Plasmalemma vesicle-associated protein      | 2.02 | 1.22E-04      | 239.78         |
| NM_182981      | OSGIN1      | Oxidative stress induced growth inhibitor 1 | 1.89 | 1.44E-04      | 249.22         |
| NM_139211      | HOPX        | HOP homeobox                               | 1.88 | 1.65E-04      | 256.18         |
| NM_017774      | CDKAL1      | CDK5 regulatory subunit-associated protein 1-like | 1.86 | 2.00E-04      | 271.00         |
| NM_001077195   | ZNF436      | Zinc finger protein 436                    | 1.96 | 2.62E-04      | 289.52         |
| NM_000067      | CA2         | Carbonic anhydrase II                     | 1.76 | 2.82E-04      | 295.37         |
| NM_015894      | STMN3       | Stathmin-like 3                           | 1.17 | 2.86E-04      | 296.25         |
| NM_014237      | ADAM18      | ADAM metalloproteinase domain 18          | 2.19 | 2.95E-04      | 298.48         |
| NM_182705      | FAM101B     | Family with sequence similarity 101, member B | 1.81 | 4.28E-04      | 330.71         |
| NM_025191      | EDEM3       | ER degradation enhancer, α-mannosidase-like 3 | 1.78 | 4.56E-04      | 337.06         |
| NM_020639      | RIPK4       | Receptor-interacting serine-threonine kinase 4 | 1.70 | 4.56E-04      | 337.43         |
| NM_004557      | NOTCH4      | Notch 4                                   | 1.70 | 4.77E-04      | 340.80         |
| NM_005618      | Dll1        | δ-like 1 (Drosophila)                     | 1.70 | 5.05E-04      | 346.21         |
| NM_004001      | FCGR2B      | Fc fragment of IgG, low affinity IIb, receptor (CD32) | 1.71 | 5.22E-04      | 349.94         |
| NM_001008225   | CNOT4       | CCR4-NOT transcription complex, subunit 4 | 1.66 | 6.17E-04      | 367.80         |
| NM_170664      | OTOA        | Otoancorin                                | 1.64 | 6.23E-04      | 368.88         |
| NM_019845      | RPRM        | Reprimo, TP53-dependent G2 arrest mediator candidate | 1.39 | 6.24E-04      | 369.37         |
| NM_033409      | SLC52A3     | Chromosome 20 open reading frame 54       | 1.65 | 6.26E-04      | 369.79         |
| NM_001010879   | ZIK1        | Zinc finger protein interacting with K protein 1 homolog (mouse) | 1.59 | 6.68E-04      | 376.61         |
| NM_007365      | PAD2        | Peptidyl arginine deiminase, type II      | 1.98 | 6.99E-04      | 381.36         |
| NM_007314      | ABL2        | v-abl Abelson murine leukemia viral oncogene homolog 2 | 0.99 | 7.21E-04      | 385.01         |
| NM_001080519   | BAHCC1      | BAH domain and coiled-coil containing 1    | 1.58 | 7.93E-04      | 397.25         |
| NM_000584      | CXCL8       | Interleukin 8                             | 1.68 | 9.46E-04      | 419.56         |
| NM_002649      | PIK3CG      | Phosphoinositide-3-kinase, catalytic, γ-polypeptide | 1.74 | 1.07E-03      | 437.34         |
| NM_178311      | GGTL1       | γ-glutamyltransferase light chain 1       | 1.28 | 1.09E-03      | 440.27         |
| NM_001124756   | PABPC1L     | Poly(A) binding protein, cytoplasmic 1-like | 1.51 | 1.11E-03      | 442.81         |
| NM_001010926   | HES5        | Hairy and enhancer of split 5 (Drosophila) | 1.09 | 1.15E-03      | 446.74         |
| NM_152643      | KNDC1       | Kinase non-catalytic C-lobe domain (KIND) containing 1 | 1.85 | 1.18E-03      | 449.71         |
| Gene reference | Gene symbol | Name                                           | Mean  | P-values (Up) | RP-values (Up) |
|----------------|-------------|------------------------------------------------|-------|---------------|----------------|
| NM_152279      | ZNF585B     | Zinc finger protein 585B                        | 1.30  | 1.18E-03      | 450.06         |
| NM_003018      | SFTPC       | Surfactant protein C                            | 1.51  | 1.20E-03      | 452.03         |
| NM_003460      | ZP2         | Zona pellucida glycoprotein 2 (sperm receptor)  | 1.79  | 1.23E-03      | 456.86         |
| NM_022101      | RAD21L1     | RAD21-like 1 (S. pombe)                         | 0.84  | 1.30E-03      | 464.79         |
| NM_001136566   | CHST7       | Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7 | 1.49  | 1.43E-03      | 477.78         |
| NM_002410      | MGAT5       | Mannosyl (α-1,6)-glycoprotein β-1,6-N-acetyl-glucosaminyltransferase | 1.37  | 1.49E-03      | 484.02         |
| NM_001130715   | PLAC8       | Placenta-specific 8                            | 1.47  | 1.51E-03      | 485.05         |
| NM_012368      | OR2C1       | Olfactory receptor, family 2, subfamily C, member 1 | 1.42  | 1.60E-03      | 493.64         |
| NM_198463      | C3ORF67     | Chromosome 3 open reading frame 67             | 1.55  | 1.72E-03      | 503.50         |
| NM_080647      | TBX1        | T-box 1                                        | 1.01  | 1.74E-03      | 504.66         |
| NM_001136003   | C2CD4D      | C2 calcium-dependent domain containing 4D       | 1.38  | 1.83E-03      | 512.27         |
| NM_014909      | VASH1       | Vasohibin 1                                    | 1.38  | 1.84E-03      | 512.54         |
| NM_002318      | LOXL2       | Lysyl oxidase-like 2                           | 1.44  | 1.91E-03      | 518.96         |
| NM_031457      | MS4A8       | Membrane-spanning 4-domains, subfamily A, member 8B | 1.36  | 2.17E-03      | 538.29         |
| NM_001146190   | ZNF407      | Zinc finger protein 407                        | 1.35  | 2.20E-03      | 541.10         |
| NM_004375      | COX11       | COX11 cytochrome c oxidase assembly homolog (yeast) | 1.37  | 2.36E-03      | 552.91         |
| NM_004375      | COX11       | COX11 cytochrome c oxidase assembly homolog (yeast) | 1.37  | 2.36E-03      | 552.91         |
| NM_001040462   | BTNL8       | Butyrophilin-like 8                            | 0.84  | 2.39E-03      | 554.54         |
| NM_001265      | CDX2        | Caudal type homeobox 2                         | 1.33  | 2.44E-03      | 558.57         |
| NM_001013661   | VSIG8       | V-set and immunoglobulin domain containing 8   | 1.33  | 2.50E-03      | 563.31         |
| NM_019119      | PCDHB9      | Protocadherin-β9                                | 1.32  | 2.51E-03      | 564.49         |
| NM_001144875   | DOK3        | Docking protein 3                               | 1.29  | 2.54E-03      | 566.33         |
| NM_003722      | TP63        | Tumor protein p63                               | 1.38  | 2.56E-03      | 569.10         |
| NM_006138      | MS4A3       | Membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific) | 1.58  | 2.73E-03      | 580.41         |
| NM_005427      | TP73        | Tumor protein p73                               | 1.37  | 2.88E-03      | 589.30         |
| NM_003106      | SOX2        | SRY (sex determining region Y)-box 2           | 1.07  | 3.12E-03      | 604.10         |
| NM_033318      | SMDT1       | Chromosome 22 open reading frame 32             | 0.80  | 3.14E-03      | 605.59         |
| NM_012426      | SF3B3       | Splicing factor 3b, subunit 3, 130 kDa          | 1.31  | 3.31E-03      | 615.60         |
| NM_002458      | MUC5B       | Mucin 5B, oligomeric mucus/gel-forming          | 1.53  | 3.45E-03      | 623.75         |
| NM_001001411   | ZNF676      | Zinc finger protein 676                         | 1.45  | 3.48E-03      | 625.90         |
| NM_000362      | TIMP3       | TIMP metalloproteinase inhibitor 3              | 1.33  | 3.55E-03      | 629.94         |
| NM_014751      | MTSS1       | Metastasis suppressor 1                         | 1.23  | 3.62E-03      | 633.32         |
| NM_201442      | C1S         | Complement component 1, subcomponent 1         | 0.91  | 3.63E-03      | 633.68         |
| NM_005961      | MUC6        | Mucin 6, oligomeric mucus/gel-forming           | 1.21  | 3.92E-03      | 647.05         |
| NM_001002758   | PRY2        | PTPN13-like, Y-linked 2                         | 1.47  | 3.99E-03      | 650.48         |
| NM_001135654   | PABPC4      | Poly(A) binding protein, cytoplasmic 4 (inducible form) | 1.31  | 4.01E-03      | 651.26         |
| NM_014030      | GIT1        | G protein-coupled receptor kinase interacting ArfGAP 1 | 1.17  | 4.13E-03      | 657.53         |
| NM_001083537   | FAM86B1     | Family with sequence similarity 86, member B1   | 1.29  | 4.16E-03      | 658.91         |
| NM_001645      | APOC1       | Apolipoprotein C-I                             | 1.20  | 4.27E-03      | 664.10         |
| NM_003226      | TFF3        | Trefoil factor 3 (intestinal)                   | 1.19  | 4.29E-03      | 664.92         |
| NM_005172      | ATOH1       | Atonal homolog 1 (Drosophila)                   | 1.26  | 4.31E-03      | 665.93         |
Table II. Continued.

| Gene reference | Gene symbol | Name | Mean (Up) | P-values (Up) | RP-values (Up) |
|----------------|-------------|------|-----------|---------------|---------------|
| NM_003708      | RDH16       | Retinol dehydrogenase 16 (all-trans) | 0.92 | 4.41E-03 | 670.22 |
| NM_002917      | RFNG        | RFNG O-fucosylpeptide 3-β-N-acetylglucosaminyltransferase | 1.28 | 4.56E-03 | 677.43 |
| NM_016585      | THEG        | Theg homolog (mouse) | 1.19 | 4.63E-03 | 681.11 |
| NM_007058      | CAPN11      | Calpain 11 | 1.51 | 4.73E-03 | 684.84 |
| NM_003759      | SLC4A4      | Solute carrier family 4, sodium bicarbonate co-transporter, member 4 | 1.19 | 4.74E-03 | 685.17 |
| NM_020299      | AKR1B10     | Aldo-keto reductase family 1, member B10 (aldose reductase) | 1.17 | 4.77E-03 | 686.57 |
| NM_032133      | MYCBPAP     | MYCBP-associated protein | 0.92 | 4.95E-03 | 693.39 |
| NM_001631      | ALPI        | Alkaline phosphatase, intestinal | 1.25 | 4.98E-03 | 695.09 |
| NM_002486      | NCBP1       | Nuclear cap binding protein subunit 1, 80 kDa | 1.23 | 5.09E-03 | 699.73 |
| NM_001105659   | LRRIQ3      | Leucine-rich repeats and IQ motif containing 3 | 1.18 | 5.13E-03 | 702.05 |
| NM_014276      | RBPIJL      | Recombination signal binding protein for immunoglobulin-kappa region-like | 1.15 | 5.29E-03 | 708.75 |
| NM_015461      | ZNF521      | Zinc finger protein 521 | 0.91 | 5.35E-03 | 711.10 |
| NM_001105662   | ALPI        | Alkaline phosphatase, intestinal | 1.21 | 5.63E-03 | 722.91 |
| NM_001105662   | SIM1        | Single-minded homolog 1 (Drosophila) | 1.23 | 5.73E-03 | 726.21 |
| NM_018646      | TRPV6       | Transient receptor potential cation channel, subfamily V, member 6 | 1.04 | 6.05E-03 | 739.17 |
| NM_139026      | ADAMTS13    | ADAM metallopeptidase with thrombospondin type 1 motif, 13 | 0.84 | 6.31E-03 | 749.50 |
| NM_152749      | ATXN7L1     | Ataxin 7-like 1 | 0.75 | 6.31E-03 | 749.64 |
| NM_019034      | RHOF        | Ras homolog gene family, member F (in filopodia) | 1.21 | 6.35E-03 | 751.22 |
| NM_017592      | MED29       | Mediator complex subunit 29 | 0.92 | 6.38E-03 | 752.10 |
| NM_206965      | FTCD        | Formiminotransferase cyclodeaminase | 1.16 | 6.40E-03 | 752.88 |
| NM_020663      | BARHL2      | BarH-like homeobox 2 | 1.10 | 6.41E-03 | 753.43 |
| NM_016338      | IPO11       | Importin 11 | 0.74 | 6.51E-03 | 756.92 |
| NM_001109997   | KLHL33      | Kelch-like 33 (Drosophila) | 1.15 | 6.61E-03 | 761.02 |
| NM_004235      | KLF4        | Kruppel-like factor 4 (gut) | 0.96 | 6.64E-03 | 762.27 |
| NM_172365      | PPP1R36     | Protein phosphatase 1, regulatory subunit 36 | 0.93 | 6.74E-03 | 765.82 |
| NM_003665      | FCN3        | Ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen) | 1.23 | 6.86E-03 | 770.05 |
| NM_017910      | TRMT61B     | tRNA methyltransferase 61 homolog B (S. cerevisiae) | 0.97 | 7.11E-03 | 778.71 |
| NM_031459      | SESN2       | Sestrin 2 | 0.27 | 7.16E-03 | 780.18 |
| NM_203458      | NOTCH2NL    | Notch 2 N-terminal like | 0.59 | 7.16E-03 | 780.21 |
| NM_002203      | ITGA2       | Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor) | 1.20 | 7.16E-03 | 780.44 |
| NM_138337      | CLEC12A     | C-type lectin domain family 12, member A | 1.32 | 7.22E-03 | 782.42 |
| NM_020533      | MCOLN1      | Mucolipin 1 | 0.51 | 7.33E-03 | 786.12 |
| NM_022481      | ARAP3       | ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3 | 1.11 | 7.42E-03 | 789.36 |
| NM_001105578   | SYCE2       | Synaptonemal complex central element protein 2 | 1.13 | 7.66E-03 | 797.43 |
| NM_021969      | NR0B2       | Nuclear receptor subfamily 0, group B, member 2 | 1.16 | 7.68E-03 | 798.17 |
| NM_015852      | ZNF117      | Zinc finger protein 117 | 1.18 | 7.69E-03 | 798.86 |
| NM_023946      | LYNX1       | Ly6/neurotoxin 1 | 1.10 | 7.89E-03 | 805.77 |
| NM_001039887   | PROSER2     | Chromosome 19 open reading frame 55 | 1.17 | 7.94E-03 | 807.24 |
| NM_015184      | PLCL2       | Phospholipase C-like 2 | 1.06 | 8.02E-03 | 809.76 |
Table II. Continued.

| Gene reference | Gene symbol | Name                                             | Mean | P-values (Up) | RP-values (Up) |
|----------------|-------------|--------------------------------------------------|------|---------------|----------------|
| NM_004938      | DAPK1       | Death-associated protein kinase 1                | 0.54 | 8.06E-03      | 811.54         |
| NM_004755      | RPS6KA5     | Ribosomal protein S6 kinase, 90 kDa, polypeptide 5| 1.04 | 8.21E-03      | 816.35         |
| NM_001007532   | STH         | Saitohin                                         | 1.17 | 8.24E-03      | 817.51         |
| NM_002613      | PDPK1       | 3-Phosphoinositide-dependent protein kinase-1     | 1.10 | 8.34E-03      | 820.46         |
| NM_006620      | HBS1L       | HBS1-like (S. cerevisiae)                        | 1.04 | 8.46E-03      | 824.24         |
| NM_003382      | VIPR2       | Vasoactive intestinal peptide receptor 2         | 0.77 | 8.55E-03      | 826.94         |
| NM_203486      | DLL3        | β-like 3 (Drosophila)                           | 1.07 | 8.56E-03      | 827.15         |
| NM_018010      | IFT57       | Intraflagellar transport 57 homolog (Chlamydomonas)| 0.92 | 8.74E-03      | 833.66         |
| NM_001135816   | CXORF56     | CIQTNF9B antisense RNA 1 (non-protein coding)    | 0.87 | 8.76E-03      | 834.30         |
| NM_033133      | CNP         | 2',3'-Cyclic nucleotide 3' phosphodiesterase     | 1.02 | 8.84E-03      | 836.32         |
| NM_005199      | CHRNG       | Cholinergic receptor, nicotinic, γ                | 0.98 | 9.01E-03      | 841.20         |
| NM_182765      | HECTD2      | HECT domain containing 2                        | 0.79 | 9.12E-03      | 844.85         |
| NM_001145290   | SLC37A2     | Solute carrier family 37 (glycerol-3-phosphate transporter), member 2| 0.90 | 9.15E-03      | 845.70         |
| NM_001195252   | APTX        | Aprataxin                                        | 1.05 | 9.31E-03      | 850.77         |
| NM_001251964   | TP53AIP1    | Tumor protein p53-regulated apoptosis inducing protein 1 | 1.26 | 9.35E-03      | 851.82         |
| NM_198270      | NHS         | Nance-Horan syndrome (congenital cataracts and dental anomalies) | 1.13 | 9.53E-03      | 857.71         |
| NM_000578      | SLC11A1     | Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 | 1.06 | 9.63E-03      | 859.97         |
| NM_002139      | RBMX        | RNA binding motif protein, X-linked              | 1.06 | 9.65E-03      | 860.47         |
| NM_000435      | NOTCH3      | Notch 3                                          | 1.10 | 9.71E-03      | 862.14         |
| NM_033066      | MPP4        | Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)  | 1.12 | 9.87E-03      | 867.43         |

B. Downregulated genes

| Gene reference | Gene symbol | Name                                                  | Mean | P-values (Down) | RP-values (Down) |
|----------------|-------------|-------------------------------------------------------|------|-----------------|------------------|
| AJ298317       | MUC5AC      | Mucin 5AC, oligomeric mucus/gel-forming                | -2.54| 8.43E-06        | 112.88           |
| AF547222       | LOC280665   | Anti-CNG α1 cation channel translation product-like   | -2.76| 1.31E-05        | 123.92           |
| AK097187       | NQO2        | NAD(P)H dehydrogenase, quinone 2                       | -2.48| 3.75E-05        | 169.61           |
| AK128551       | RNF216      | Ring finger protein 216                                | -2.19| 6.09E-05        | 198.31           |
| BC062748       | EFCA8B10    | EF-hand calcium binding domain 10                     | -2.10| 1.14E-04        | 233.55           |
| NM_000639      | FASLG       | Fas ligand (TNF superfamily, member 6)                 | -1.61| 2.15E-04        | 274.72           |
| NM_001124      | ADM         | Adrenomedullin                                        | -1.48| 3.43E-04        | 311.18           |
| NM_000043      | FAS         | Fas (TNF receptor superfamily, member 6)              | -1.68| 4.22E-04        | 329.27           |
| BC065002       | EXD3        | Exonuclease 3'-5' domain containing 3                 | -2.04| 5.19E-04        | 349.20           |
| NM_004931      | CD8B        | CD8b molecule                                         | -1.24| 6.82E-04        | 378.73           |
| NM_021635      | PBOV1       | Prostate and breast cancer overexpressed 1            | -1.17| 7.31E-04        | 386.63           |
| NM_000093      | COL5A1      | Collagen, type V, α1                                  | -1.67| 8.08E-04        | 398.60           |
| NM_000429      | MAT1A       | Methionine adenosyltransferase 1, α                    | -1.67| 8.43E-04        | 403.54           |
| Gene reference | Gene symbol | Name | Mean (Down) | P-values (Down) | RP-values (Down) |
|----------------|-------------|------|-------------|----------------|-----------------|
| NM_000033      | ABCD1       | ATP-binding cassette, sub-family D (ALD), member 1 | -1.69          | 8.92E-04       | 411.33          |
| NM_000125      | ESR1        | Estrogen receptor 1 | -1.67          | 8.95E-04       | 411.64          |
| NM_000808      | GABRA3      | γ-Aminobutyric acid (GABA) A receptor, α3 | -1.60          | 9.27E-04       | 415.96          |
| NM_000595      | LTA         | Lymphotixin-α (TNF superfamily, member 1) | -1.63          | 9.95E-04       | 427.69          |
| NM_00197       | HSD17B3     | Hydroxysteroid (17-β) dehydrogenase 3 | -1.67          | 1.04E-03       | 432.79          |
| NM_001037442   | RUFY3       | RUN and FYVE domain containing 3 | -1.54          | 1.05E-03       | 434.60          |
| NM_000545      | HNF1A       | HNF1 homeobox A | -1.64          | 1.07E-03       | 437.31          |
| NM_001005490   | OR6C74      | Olfactory receptor, family 6, subfamily C, member 74 | -1.59 | 1.09E-03 | 439.40 |
| NM_001031848   | SERPINB8    | Serpin peptidase inhibitor, clade B (ovalbumin), member 8 | -1.54 | 1.15E-03 | 447.15 |
| NM_000612      | IGF2        | Insulin-like growth factor 2 (somatomedin A) | -1.63          | 1.16E-03       | 448.01          |
| NM_000517      | HBA2        | Hemoglobin, α2 | -1.64          | 1.19E-03       | 451.63          |
| NM_001130861   | CLDN5       | Claudin 5 | -1.44          | 1.24E-03       | 458.33          |
| NM_001004688   | OR2M2       | Olfactory receptor, family 2, subfamily M, member 2 | -1.59 | 1.24E-03 | 458.57 |
| NM_001030004   | HNF4A       | Hepatocyte nuclear factor 4, α | -1.56          | 1.26E-03       | 460.62          |
| NM_001033952   | CALCA       | Calcitonin-related polypeptide α | -1.54          | 1.26E-03       | 461.21          |
| NM_001010870   | TDRD6       | Tudor domain containing 6 | -1.58          | 1.32E-03       | 466.23          |
| NM_001018025   | MTCP1       | Mature T cell proliferation 1 | -1.57          | 1.41E-03       | 475.20          |
| NM_001012967   | DDX60L      | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like | -1.57 | 1.41E-03 | 475.20 |
| NM_0010185     | SERPINA3    | Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 3 | -1.49 | 1.42E-03 | 476.63 |
| NM_000633      | BCL2        | B-cell CLL/lymphoma 2 | -1.63          | 1.45E-03       | 479.53          |
| NM_001037666   | GATSL3      | GATS protein-like 3 | -1.52          | 1.52E-03       | 486.52          |
| NM_0011165     | BIRC3       | Baculoviral IAP repeat containing 3 | -1.42          | 1.58E-03       | 491.86          |
| NM_002247      | KCNMA1      | Potassium large conductance calcium-activated channel, subfamily M, α member 1 | -1.33 | 1.94E-03 | 521.16 |
| NM_173625      | C17orf78    | Chromosome 17 open reading frame 78 | -1.01          | 1.95E-03       | 521.61          |
| NM_001124759   | FRG2C       | FSHD region gene 2 family, member C | -1.44          | 2.00E-03       | 524.95          |
| NM_001080453   | INTS1       | Integrator complex subunit 1 | -1.51          | 2.00E-03       | 524.88          |
| NM_004613      | TGM2        | Transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase) | -1.24 | 2.14E-03 | 536.10 |
| NM_001044392   | MUC1        | Mucin 1, cell surface-associated | -1.51          | 2.31E-03       | 548.26          |
| NM_001195127   | WI2-2373i12 | Forkhead box L1-like | -1.39          | 2.41E-03       | 556.59          |
| NM_001243042   | HLA-C       | Major histocompatibility complex, class I, C | -1.38          | 2.43E-03       | 558.50          |
| NM_001083602   | PTCH1       | Patched 1 | -1.49          | 2.58E-03       | 570.46          |
| NM_207352      | CYP4V2      | Cytochrome P450, family 4, subfamily V, polypeptide 2 | -0.86 | 2.71E-03 | 579.03 |
| NR_029392      | KRT16P2     | Keratin 16 pseudogene 2 | -0.54          | 2.97E-03       | 594.00          |
| NM_001172646   | PLCB4       | Phospholipase C, β4 | -1.39          | 3.03E-03       | 598.52          |
| NM_002089      | CXCL2       | Chemokine (C-X-C motif) ligand 2 | -1.34          | 3.39E-03       | 620.43          |
| NM_001496      | GFRA3       | GDNF family receptor α3 | -1.38          | 3.40E-03       | 620.75          |
| NM_001668      | ARNT        | Aryl hydrocarbon receptor nuclear translocator | -1.37 | 3.42E-03 | 622.12 |
| NM_021151      | CROT        | Carnitine O-octanoyltransferase | -1.18          | 3.47E-03       | 624.70          |
Table II. Continued.

| Gene reference | Gene symbol | Name                        | Mean   | P-values   | RP-values   |
|----------------|-------------|-----------------------------|--------|------------|-------------|
| NM_001949      | E2F3        | E2F transcription factor 3  | -1.36  | 3.53E-03   | 628.70      |
| NM_002307      | LGALS7      | Lectin, galactoside-binding, soluble, 7 | -1.32  | 3.56E-03   | 630.07      |
| NM_001704      | BA13        | Brain-specific angiogenesis inhibitor 3 | -1.37  | 3.57E-03   | 630.78      |
| NM_001978      | DMTN        | Erythrocyte membrane protein band 4.9 (dematin) | -1.35  | 3.62E-03   | 633.47      |
| NM_183001      | SHC1        | SHC (Src homology 2 domain containing) transforming protein 1 | -0.90  | 3.64E-03   | 634.32      |
| NM_001185      | IL24        | Interleukin 24               | -1.39  | 3.71E-03   | 637.18      |
| NM_004048      | B2M         | β-2-microglobulin            | -1.27  | 3.73E-03   | 637.88      |
| NM_001004456   | OR1M1       | Olfactory receptor, family 1, subfamily M, member 1 | -1.60  | 3.85E-03   | 644.36      |
| NM_002133      | HMOX1       | Heme oxygenase (deceling) 1  | -1.33  | 3.97E-03   | 649.35      |
| NM_002457      | MUC2        | Mucin 2, oligomeric mucus/gel-forming | -1.31  | 4.02E-03   | 651.72      |
| NM_001198      | PRDM1       | PR domain containing 1, with ZNF domain | -1.39  | 4.05E-03   | 653.14      |
| NM_001136022   | NFATC4      | Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 4 | -1.43  | 4.06E-03   | 653.68      |
| NM_001454      | FOXJ1       | Horkhead box J1              | -1.38  | 4.11E-03   | 656.34      |
| NM_002006      | FGF2        | Fibroblast growth factor 2 (basic) | -1.35  | 4.11E-03   | 656.64      |
| NM_177996      | EPB41L1     | Erythrocyte membrane protein band 4.1-like 1 | -0.97  | 4.19E-03   | 659.94      |
| NM_004417      | DUSP1       | Dual specificity phosphatase 1 | -1.25  | 4.38E-03   | 669.42      |
| NM_201282      | EGFR        | Epidermal growth factor receptor | -0.88  | 4.53E-03   | 676.59      |
| NM_004416      | DTX1        | Deltex homolog 1 (Drosophila) | -1.25  | 4.68E-03   | 682.88      |
| NM_003128      | SPTBN1      | Spectrin, β, non-erythrocytic 1 | -1.29  | 4.75E-03   | 685.70      |
| NM_001807      | CEL         | Carboxyl ester lipase (bile salt-stimulated lipase) | -1.36  | 4.94E-03   | 693.06      |
| NM_207336      | ZNF467      | Zinc finger protein 467     | -0.86  | 4.95E-03   | 693.44      |
| NM_002381      | MATN3       | Matrinil 3                  | -1.32  | 5.00E-03   | 695.99      |
| NM_002317      | LOX         | Lysyl oxidase               | -1.32  | 5.00E-03   | 696.01      |
| NM_024766      | CAMKMT      | Calmodulin-lysine N-methyltransferase | -1.15  | 5.07E-03   | 699.15      |
| NM_003667      | LGR5        | Leucine-rich repeat containing G protein-coupled receptor 5 | -1.29  | 5.27E-03   | 707.84      |
| NM_002535      | OAS2        | 2′-5′-Oligoadenylate synthetase 2, 69/71 kDa | -1.30  | 5.27E-03   | 708.15      |
| NM_145041      | TMEM106A    | Transmembrane protein 106A  | -1.10  | 5.27E-03   | 708.30      |
| NM_003061      | SLIT1       | Slit homolog 1 (Drosophila)  | -1.30  | 5.36E-03   | 711.28      |
| NM_013292      | MYLPF       | Myosin light chain, phosphorylatable, fast skeletal muscle | -1.21  | 5.40E-03   | 717.21      |
| NM_004310      | RHOH        | Ras homolog gene family, member H | -1.26  | 5.55E-03   | 719.34      |
| NM_002483      | CEACAM6     | Carcinoembryonic antigen-related cell adhesion molecule 6 | -1.30  | 5.71E-03   | 725.32      |
| NM_005531      | IFI16       | Interferon, γ-inducible protein 16 | -1.23  | 5.87E-03   | 732.01      |
| NM_133471      | PPIR18      | Protein phosphatase 1, regulatory subunit 18 | -1.13  | 5.88E-03   | 732.45      |
| NM_006398      | UBD         | Ubiquitin D                 | -1.22  | 5.89E-03   | 732.86      |
| NM_004994      | MMP9        | Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) | -1.24  | 5.90E-03   | 733.00      |
| NR_003531      | MEG3        | Maternally expressed 3 (non-protein coding) | -0.79  | 5.98E-03   | 736.40      |
| NM_012171      | TSPAN17     | Tetraspanin 17              | -1.22  | 6.10E-03   | 741.06      |
| NM_032599      | FAM71F1     | Family with sequence similarity 71, member F1 | -1.14  | 6.13E-03   | 742.39      |
| NM_019074      | DLL4        | δ-like 4 (Drosophila)       | **-1.19** | **6.16E-03** | **743.53**  |
| NM_002405      | MFNG        | MFNG O-fucosylpeptide 3-β-N-acetylgalcosaminyltransferase | -1.31  | 6.30E-03   | 749.05      |
| NM_015000      | STK38L      | Serine/threonine kinase 38-like | -1.21  | 6.32E-03   | 750.10      |
| NM_018416      | FOXJ2       | Forkhead box J2             | -1.20  | 6.36E-03   | 751.65      |
| NM_016135      | ETV7        | Ets variant 7               | -1.21  | 6.38E-03   | 752.29      |
Table II. Continued.

| Gene reference | Gene symbol | Name | Mean   | P-values (Down) | RP-values (Down) |
|----------------|-------------|------|--------|-----------------|------------------|
| NM_015886      | PI15        | Peptidase inhibitor 15 | -1.21 | 6.39E-03        | 752.62           |
| NM_002543      | OLR1        | Oxidized low density lipoprotein (lectin-like) receptor 1 | -1.30 | 6.40E-03        | 752.88           |
| NM_005023      | PGGT1B      | Protein geranylgeranyltransferase type I, β-subunit | -1.24 | 6.53E-03        | 757.78           |
| NM_172390      | NFATC1      | Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 | -1.02 | 6.57E-03        | 759.52           |
| NM_017766      | CASZ1       | Castor zinc finger 1 | -1.20 | 6.78E-03        | 767.06           |
| NM_144633      | KCHN8       | Potassium voltage-gated channel, subfamily H (eag-related), member 8 | -1.12 | 6.86E-03        | 770.19           |
| NM_025125      | TMEM254     | Chromosome 10 open reading frame 57 | -1.14 | 6.87E-03        | 770.43           |
| NM_182909      | FILIP1L     | Filamin A interacting protein 1-like | -0.92 | 6.89E-03        | 771.28           |
| NM_173503      | EFCAB3      | EF-hand calcium binding domain 3 | -1.02 | 6.92E-03        | 772.10           |
| NM_144673      | CMTM2       | CKLF-like MARVEL transmembrane domain containing 2 | -1.12 | 6.95E-03        | 773.54           |
| NM_021819      | LMAN1L      | Lectin, mannose-binding, 1-like | -1.17 | 6.95E-03        | 773.62           |
| NM_022804      | SNURF       | SNRNP upstream reading frame | -1.16 | 6.99E-03        | 775.02           |
| NM_021633      | KLHL12      | Kelch-like 12 (Drosophila) | -1.17 | 7.01E-03        | 775.60           |
| NM_021966      | TCLI1A      | T cell leukemia/lymphoma 1A | -1.16 | 7.23E-03        | 782.50           |
| NM_032637      | SKP2        | S phase kinase-associated protein 2 (p45) | -1.14 | 7.27E-03        | 784.16           |
| NM_022648      | TNS1        | Tensin 1 | -1.16 | 7.32E-03        | 785.88           |
| NM_004213      | SLC28A1     | Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1 | -1.27 | 7.46E-03        | 790.45           |
| NM_033088      | STRIP1      | Family with sequence similarity 40, member A | -1.14 | 7.49E-03        | 791.43           |
| NM_022304      | HRH2        | Histamine receptor H2 | -1.16 | 7.62E-03        | 796.01           |
| NM_021105      | PLSCR1      | Phospholipid scramblase 1 | -1.18 | 7.65E-03        | 797.28           |
| NM_024768      | EFCC1       | Coiled-coil domain containing 48 | -1.15 | 7.66E-03        | 797.48           |
| NM_006290      | TNFAIP3     | Tumor necrosis factor, α-induced protein 3 | -1.22 | 7.68E-03        | 798.22           |
| NM_030639      | BHLHB9      | Basic helix-loop-helix domain containing, class B, 9 | -1.14 | 7.69E-03        | 798.53           |
| NM_004246      | GLP2R       | Glucagon-like peptide 2 receptor | -1.26 | 7.79E-03        | 802.00           |
| NM_032873      | UBASH3B     | Ubiquitin-associated and SH3 domain containing B | -1.14 | 7.79E-03        | 802.14           |
| NM_001963      | EGF         | Epidermal growth factor | -1.35 | 7.84E-03        | 803.92           |
| NM_052904      | KLHL32      | Kelch-like 32 (Drosophila) | -1.13 | 7.89E-03        | 805.79           |
| NM_006125      | ARHGAP6     | Rho GTPase activating protein 6 | -1.23 | 7.90E-03        | 806.11           |
| NM_032772      | ZNF503      | Zinc finger protein 503 | -1.14 | 7.95E-03        | 807.90           |
| NM_024886      | C10orf95    | Chromosome 10 open reading frame 95 | -1.15 | 7.99E-03        | 809.09           |
| NM_152703      | SAMD9L      | Sterile α motif domain containing 9-like | -1.09 | 8.02E-03        | 809.77           |
| NM_032752      | ZNF496      | Zinc finger protein 496 | -1.14 | 8.03E-03        | 810.31           |
| NM_138456      | BATF2       | Basic leucine zipper transcription factor, ATF-like 2 | -1.13 | 8.04E-03        | 810.45           |
| NM_172370      | DAOA        | D-amino acid oxidase activator | -1.04 | 8.07E-03        | 811.67           |
| NM_005747      | CELA3A      | Chymotrypsin-like elastase family, member 3A | -1.23 | 8.07E-03        | 811.75           |
| NM_033101      | LGALS12     | Lectin, galactoside-binding, soluble, 12 | -1.14 | 8.14E-03        | 813.87           |
| NM_012224      | NEK1        | NIMA (never in mitosis gene a)-related kinase 1 | -1.21 | 8.21E-03        | 816.40           |
| NM_020436      | SALL4       | Sal-like 4 (Drosophila) | -1.19 | 8.31E-03        | 819.74           |
| NM_138980      | MAPK10      | Mitogen-activated protein kinase 10 | -1.13 | 8.34E-03        | 820.62           |
| NM_020896      | OBPPL5      | Oxysterol binding protein-like 5 | -1.18 | 8.41E-03        | 822.84           |
Fig. 3A, treatment with this agent dose-dependently induced differentiation as assessed by ALP activity. Concordantly, this agent also induced \textit{MT1G} and \textit{MT2A} mRNA levels, in close correlation to ALP activity (Pearson $r$=0.993, $p=0.007$ for \textit{MT1G} and $r=0.999, p=0.0006$ for \textit{MT2A}; Fig. 3B). To determine whether the induction of MTs has a functional role in butyrate-induced differentiation, we used siRNAs to inhibit the induction of only \textit{MT1G} (si1G.1 and si1G.2) or of all MT1 and MT2 isoforms (siMTs), as previously described (12). Fig. 3C shows that siRNA pre-treatment partially mitigated \textit{MT1G} induction after BUT treatment and markedly, also diminished the induction of \textit{CDX2} (Fig. 3D), and of goblet-cell marker \textit{TFF3} (Fig. 3E). Notably, BUT treatment had no effect on mRNA levels of \textit{MUC2}, as has been previously reported by others (17) (data not shown). In contrast, although the enterocyte-specific markers \textit{HSI} and \textit{CA1} were markedly upregulated at 2 mM BUT, silencing of MTs had no effect on their induction (Fig. 3F and G), or on the cell-cycle arrest mediator \textit{CDKN1A/p21} (data not shown), whereas ALP activity was only slightly, but significantly reduced (Fig. 3H).

We next treated HT-29 MOCK and MT1G + cells with butyrate. Notably, as depicted in Fig. 4A, whereas \textit{CDX2}...
mRNA levels were similarly induced, MT1G overexpression markedly enhanced the induction of TFF3 (Fig. 4B), whereas it blunted that of HSI (Fig. 4C). Therefore, both silencing and overexpression of MT1G support the hypothesis that this gene favors goblet over enterocyte differentiation upon butyrate treatment of HT-29 cells.

Table III. Significant functional categories of upregulated and downregulated genes.

| A, Upregulated genes |   |   |   |
|----------------------|---------------------------------|
| GO category          | P-value | Bonferroni | Benjamini | FDR |
| Cell fate commitment | 7.2E-07 | 8.9E-04 | 8.9E-04 | 1.2E-03 |
| Negative regulation of cell differentiation | 2.9E-05 | 3.5E-02 | 1.2E-02 | 4.7E-02 |
| Differentiation      | 6.8E-05 | 1.7E-02 | 1.7E-02 | 8.8E-02 |
| Developmental protein| 9.2E-05 | 2.4E-02 | 1.2E-02 | 1.2E-01 |
| Notch signaling pathway | 1.0E-04 | 2.6E-02 | 8.7E-03 | 1.3E-01 |
| Intestine            | 2.4E-04 | 6.0E-02 | 1.5E-02 | 3.1E-01 |
| KEGG pathway         |   |   |   |
| Notch signaling pathway | 3.5E-05 | 2.7E-03 | 2.7E-03 | 3.7E-02 |

| B, Downregulated genes |   |   |   |
|------------------------|---------------------------------|
| GO category            | P-value | Bonferroni | Benjamini | FDR |
| Regulation of cell death| 5.9E-07 | 9.2E-04 | 4.6E-04 | 9.8E-04 |
| Regulation of cell proliferation | 1.8E-04 | 2.4E-01 | 1.9E-02 | 2.9E-01 |
| Regulation of cell migration | 2.0E-04 | 2.7E-01 | 1.9E-02 | 3.3E-01 |
| KEGG pathway           |   |   |   |
| Pathways in cancer     | 1.9E-04 | 1.6E-02 | 1.6E-02 | 2.1E-01 |

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

Figure 2. Wound healing ‘scratch’ assays in (A) HT-29 and (B) HCT116 MT1G+ cells showing decreased migration capacity, but no difference in MMP2 activity as measured by (C) gelatin zymography. *p<0.05, **p<0.01 and ***p<0.001.
Involvement of MT1G in post-confluent differentiation of HT-29 cells. Next, we studied the expression of MT1G in the post-confluent growth of HT-29 cells, where this cell line is known to differentiate poorly (20). In this setting, MT1G mRNA was transiently induced at day 1 post-confluence, after which its expression was significantly reduced (Fig. 5A). In contrast, CDX2 and enterocyte-specific genes HSI and CA1 were transiently induced at day 3, two days after MT1G induction (Fig. 5B-D). Notably, TFF3 expression mirrors MT1G expression, until day 14 when it is induced again (Fig. 5E). These time-course analyses again favored the association of MT1G induction with goblet over enterocyte differentiation. Notably, as with BUT treatment, MUC2 expression was not altered in this context (Fig. 5F). We were unable to perform Figure 3. Butyrate mediates the differentiation of HT-29 cells. (A) Assessment of ALP enzymatic activity, 72 h after treatment. (B) Induction of MT1G and MT2A mRNA expression measured by qRT-PCR 72 h after treatment. (C-G) Expression of MT1G, CDX2, TFF3, HSI and CA1 as well as (H) ALP activity after siRNA-mediated silencing of MT1G or all MTs. AFC, average fold-change; *p<0.05, **p<0.01 and ***p<0.001.
Figure 5. Expression of (A) MT1G, (B) CDX2, (C) HIS, (D) CA1, (E) TFF3 and (F) MUC2 upon differentiation stimulated by post-confluent growth of HT-29 cells. AFC, average fold-change; *p<0.05, **p<0.01 and ***p<0.001.

Figure 4. (A) CDX2, (B) TFF3 and (C) HIS expression in MOCK and MT1G+ cells upon butyrate treatment. AFC, average fold-change; *p<0.05, **p<0.01 and ***p<0.001.
siRNA-mediated silencing of MT1G in this setting, as cells did not survive in a totally confluent state for >1 day after transfection.

When growing HT-29 MOCK and MT1G+ cells post-confluence, we noted no difference in the induction of ALP activity between these two cell lines (Fig. 6A). Notably however, in the latter, CDX2 mRNA was induced at significantly higher levels (Fig. 6B) whereas HSI induction was abolished (Fig. 6C). While we noted no differences in the induction of TFF3 mRNA (data not shown), our data also implies a role for MT1G expression in counteracting enterocyte differentiation of HT-29 cells.

**Labile zinc levels in butyrate-treated and post-confluent HT-29 cells.** Given the close relationship between MTs and zinc biology, we analyzed the levels of intracellular labile zinc in both models of differentiation, using the zinc-specific fluorophore FZ. Notably, after 72 h of 2 mM BUT treatment, FZ intensity was significantly induced in the HT-29 cells (Fig. 7A). We used TPEN treatment to chelate intracellular labile zinc before the addition of butyrate, and found that this abolished both CDX2 and HSI mRNA induction (Fig. 7B and C), but had no effect on TFF3 levels (Fig. 7D). In the post-confluence model, as shown by fluorescence microscopy in Fig. 7E and by fluorimetry in Fig. 7F, FZ intensity was induced at day 2 and progressively increased thereafter. Given that TPEN exposure for >6 h is toxic to HT-29 cells, we used daily 5-h TPEN treatments to evaluate the effect of labile zinc on goblet and enterocyte markers. Notably, TFF3 mRNA expression was significantly induced at days 1-3 post-confluence in TPEN-treated cells (Fig. 7G), whereas there was no effect on either CDX2 or HSI levels (not shown).

In summary, labile zinc was induced in both models of intestinal differentiation, and its chelation by TPEN treatment either inhibited enterocyte differentiation (butyrate model) or induced the expression of goblet-cell markers (post-confluency model).

**Discussion**

In the present study, we uncovered a new role for MT1G in altering the differentiation properties of the HT-29 cell line. We previously showed that induction of MTs by HDACi agents such as trichostatin A and sodium butyrate (BUT) is at least partly responsible for their cytostatic effects on human CRC cell lines, and that exogenous MT1G overexpression in the colorectal HCT116 cell line resulted in growth inhibition in nude mouse xenografts (12). Notably, whereas MT1G overexpression did not alter the in vivo xenograft growth rate of HT-29 cells, it markedly increased the number of goblet cells and differentiation markers of these tumors, both of the goblet and the enterocyte lineages. These effects were not readily observed in 2D culture (data not shown), suggesting that additional signals from the tumor microenvironment may be needed to fulfill this effect. The reasons for the different observed phenotypic consequences of MT1G overexpression in these two cell lines are unclear, but a possible explanation may stem from the differences in endogenous MT1G expression: HCT116 cells do not express MT1G due to promoter hypermethylation and therefore the impact of MT1G overexpression may be stronger than that in HT-29 cells, which express low, but detectable mRNA levels (8).

In an effort to understand the molecular mechanisms underlying the altered differentiation of MT1G+ tumors, we performed mRNA expression profiling by cDNA microarrays. The expression of several genes involved in the regulation of cell differentiation was found to be altered, particularly in the Notch signaling pathway, whose inhibition is well known to stimulate goblet cell differentiation in the intestine through activation of ATOH1 (22). Notably, markers of different sets of intestinal stem cell markers were differentially dysregulated in MT1G+ tumors, with upregulation of HOPX (expressed in quiescent stem cells) and downregulation of Lgr5 (in crypt base columnar stem cells), again suggesting altered differentiation hierarchies (23,24). Further studies are warranted to explore this in further detail.

To further characterize the involvement of MT1G in colorectal differentiation, we relied on two well-studied cell culture conditions: sodium butyrate and post-confluent growth. We showed that endogenous MT1G induction was required for the induction of goblet cell markers by butyrate, and was temporally associated with such markers in the confluen
model. Moreover, stable exogenous MT1G overexpression favored goblet and blunted enterocyte differentiation in both models. Previous studies have shown MTs to be upregulated in vitro upon CRC differentiation (25), and demonstrated a role for MTs in modulating differentiation in different tissues, such as human salivary gland tumor cells (where MT1F overexpression resulted in slower growing and more differentiated tumors) (26), leukemic (27) neurons and glial (28), and T cells (29). However, to the best of our knowledge, this is the first study showing a direct functional involvement of a metallothionein isoform in CRC differentiation.

Labile zinc ions have been recognized as secondary messengers capable of transducing a wide variety of intracellular signals (30,31), including differentiation (32-34). MTs can regulate labile zinc concentrations and zinc transfer to different cellular organelles (35), as well as respond to changes in intracellular zinc ions (36). We showed in the present study that labile zinc was increased during differentiation induced both by butyrate and confluency, and that this was required for enterocyte differentiation by butyrate, whereas it blunted goblet marker induction in post-confluency. While the reason behind the differences observed in both models are unclear,
the overall effects of zinc induction favor an enterocyte over goblet differentiation. Notably, although labile zinc increases have already been reported to occur during butyrate-mediated differentiation of the HT-29 cell line and have been associated to defined stages of the cell cycle (37), in the present study, we reported for the first time a functional consequence of labile zinc induction in this process. Previous studies in other tissues have shown that MTS transiently translocate to the nucleus during early phases of differentiation to release the zinc ions necessary for zinc-dependent transcription factors to execute the differentiation programs of adipocytes and myoblasts (38,39). Although we previously showed that MTS in HT-29 are localized to the cytoplasm (8), we were not able to detect a nuclear shift in either of the differentiation models that we used in the present study (data not shown), although this possibility should be studied in further detail.

Taking into account our results, we hypothesize that MT1G induction during differentiation may play a role in the cholesterol and re-distribution of intracellular labile zinc, perhaps modulating the activity of zinc-requiring transcription factors and enzymes, and stimulating the differentiation program of colorectal cells. In vitro, our results showed that MT1G favors a goblet over enterocyte differentiation, although our mouse xenografts assays suggest that in vivo the differentiation into enterocytes is also stimulated, perhaps as a compensatory mechanism or in a non-cell autonomous manner. The precise mechanisms whereby this occurs and the participation of MT1G (and other MTS) in labile zinc redistribution during differentiation need to be studied in further detail. Moreover, tumor classifications based on gene signatures associated with different cell types suggest that tumors of the more differentiated 'goblet-' or 'enterocyte-like' subtypes have a better prognosis than undifferentiated 'stem-like' subtype, as different responses to therapeutic agents. Therefore, better understanding of the molecular mechanisms that govern the differentiation processes of tumor cells may be of clinical relevance.

Overall, in the present study, we unveiled a pro-differentiation effect of MT1G on various CRC cells, thus proposing a new mechanism whereby MT1G may act as a tumor suppressor in this tumor type. Moreover, we established a functional consequence of transient increases in labile zinc upon differentiation stimuli, and support the need of further studies relating zinc signaling and differentiation, that may ultimately underlie tumor cell phenotypes and response to therapies.

Acknowledgements

The present study was funded by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP no. 845-10 to M.B.), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (IP-PAE 2007, to J.M.), the Fundación Cáncer, the Fundación P. Mosoteguy, the Fundación Sales, and the Fundación María Calderón de la Barca, Buenos Aires, Argentina.

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