

## Original Article

# Transcriptomic changes induced by mycophenolic acid in gastric cancer cells

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**Abstract:** Background: Inhibition of inosine monophosphate dehydrogenase (IMPDH) by mycophenolic acid (MPA) can inhibit proliferation and induce apoptosis in cancer cells. This study investigated the underlying molecular mechanisms of MPA's anticancer activity. Methods: A gastric cancer cell line (AGS) was treated with MPA and gene expression at different time points was analyzed using Illumina whole genome microarrays and selected genes were confirmed by real-time RT-PCR. Results: Transcriptomic profiling identified 1070 genes with  $\geq 2$  fold changes and 85 genes with  $>4$  fold alterations. The most significantly altered biological processes by MPA treatment include cell cycle, apoptosis, cell proliferation and migration. MPA treatment altered at least ten KEGG pathways, of which eight (p53 signaling, cell cycle, pathways in cancer, PPAR signaling, bladder cancer, protein processing in ER, small cell lung cancer and MAPK signaling) are cancer-related. Among the earliest cellular events induced by MPA is cell cycle arrest which may be caused by six molecular pathways: 1) up-regulation of cyclins (*CCND1* and *CCNE2*) and down-regulation of *CCNA2* and *CCNB1*, 2) down-regulation of cyclin-dependent kinases (*CDK4* and *CDK5*); 3) inhibition of cell division related genes (*CDC20*, *CDC25B* and *CDC25C*) and other cell cycle related genes (*MCM2*, *CENPE* and *PSRC1*), 4) activation of p53, which activates the cyclin-dependent kinase inhibitors (*CDKN1A*), 5) impaired spindle checkpoint function and chromosome segregation (*BUB1*, *BUB1B*, *BOP1*, *AURKA*, *AURKB*, and *FOXM1*); and 6) reduction of availability of deoxyribonucleotides and therefore DNA synthesis through down-regulation of the *RRM1* enzyme. Cell cycle arrest is followed by inhibition of cell proliferation, which is mainly attributable to the inhibition of the PI3K/AKT/mTOR pathway, and caspase-dependent apoptosis due to up-regulation of the p53 and FAS pathways. Conclusions: These results suggest that MPA has beneficial anticancer activity through diverse molecular pathways and biological processes.

**Keywords:** MPA, drug repurposing, regulatory networks, microarray

## Introduction

Inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme for the *de novo* synthesis of guanosine nucleotides [1, 2], which play crucial roles in cell proliferation and other cellular functions [3]. In many tumor cells, the expression of IMPDH, particularly IMPDH2, is significantly up-regulated [4, 5]. Therefore, IMPDH is potentially a biomarker and target for cancer therapy. Mycophenolate mofetil (MMF) is the morpholinoethyl ester prodrug of mycophenolic acid (MPA), which is a potent uncompetitive inhibitor of IMPDH. It has been used for the prevention of acute graft rejection in trans-

plantation [6, 7]. MPA prevents graft rejection through blocking T and B lymphocyte proliferation and clonal expansion, and prevents the generation of cytotoxic T cells and other effector T cells. Therefore, it has long been hypothesized that MPA may also inhibit cancer cell proliferation. Indeed, a number of studies have reported the inhibitory role of MPA on cancer cell proliferation and induction of apoptosis in cancer cells [8-13]. We have recently evaluated the anticancer activity of MPA in 13 different cancer lines including stomach, colon, pancreas, liver, ovary and cervix cancer and leukemia [14]. Our results suggested that five cell lines (AGS, NCI-N87, HCT-8, A2780 and BxPC-3) were

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highly sensitive to MPA with  $IC_{50} < 0.5 \mu\text{g/ml}$ , four cell lines (Hs746T, PANC-1, HepG2 and MCF-7) are very resistant to MPA with  $IC_{50} > 20 \mu\text{g/ml}$  and the four other cell lines (KATO III, SNU-1, K562 and HeLa) have intermediate sensitivity. We and others also demonstrated the *in vivo* anticancer activity of MPA using xenograft mouse models [14].

Our comprehensive studies indicated that MPA can effectively induce cell cycle arrest and consequently inhibits cancer cell proliferation and eventually leading to cell death through caspase-dependent apoptosis. Our analyses using a targeted proteomics approach identified several proteins that may be implicated in MPA-induced cell cycle arrest, reduced proliferation and increased apoptosis [14]. However, our understanding of the molecular mechanism underlying MPA's anticancer activity is incomplete. In this study, global transcriptomic profiling was carried out to construct the overall molecular network underlying MPA's antitumor activity.

### Materials and methods

#### *Cell culture and reagents*

Two gastric cancer cell lines (AGS and Hs746T) were obtained from the American Type Culture Collection (ATCC). Both cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml of penicillin and 100  $\mu\text{g/ml}$  of streptomycin at 37°C with 5%  $\text{CO}_2$ . MPA was purchased from VWR. Approximately  $5 \times 10^4$  cells were seeded in 6-well plates and cultured overnight before MPA is added to the culture medium at a final concentration of 2  $\mu\text{g/ml}$ . Cells were collected after 24, 48 and 72 hours of treatment.

#### *Microarray experiments*

Total RNA was extracted from AGS cells using a magnetic beads RNA extraction kit (Jinfiniti Biosciences, Augusta, GA). Gene expression profiling was performed using the human Illumina HumanHT-12 v4 BeadChip (Illumina, San Diego, CA). An aliquot of 200 ng of total RNA was converted into double stranded cDNA (ds-cDNA) by using the Illumina TargetAmp-Nano labeling kit with an oligo-dT primer containing a T7 RNA polymerase promoter (Genset, St. Louis, MO). *In vitro* transcription was per-

formed on the above ds-cDNA using the Enzo RNA transcript labeling kit. Biotin-labeled cRNA was purified by using an RNeasy affinity column (Qiagen), and fragmented randomly to sizes ranging from 35-200 bases by incubating at 94°C for 35 min. The hybridization solutions contained 100 mM MES, 1 M  $\text{Na}^+$ , 20 mM EDTA, and 0.01% Tween 20. The final concentration of fragmented cRNA was 0.05  $\mu\text{g}/\mu\text{l}$  in hybridization solution. Target for hybridization was prepared by combining 40  $\mu\text{l}$  of fragmented transcript with sonicated herring sperm DNA (0.1 mg/ml), BSA and 5 nM control oligonucleotide in a buffer containing 1.0 M NaCl, 10 mM Tris.HCl (pH7.6), and 0.005% Triton X-100. Target was hybridized for 16h at 45°C in an Illumina hybridization oven. Chips were then washed at 50°C with stringent solution, then again at 30°C with non-stringent washes. Arrays were then stained with streptavidin-Cy3. The microarray data are MIAME compliant and have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE46671 (GEO reviewer link: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hdkfdoisykeuybo&acc=GSE46671>).

#### *Microarray data analysis*

All statistical analyses were performed using the R language and environment for statistical computing ([www.r-project.org](http://www.r-project.org)). The *lumi* package was used to preprocess microarray data. Differential expression analyses were conducted using the *limma* package from the Bioconductor project [15]. We used the false discovery rate (FDR) to adjust for multiple testing [16] B-statistics were calculated for each gene. A combination of adjusted *p*-value and absolute value of fold change (FC) were used for selecting the differentially expressed genes.

Cluster analysis was performed for grouping differentially expressed genes exhibiting similar expression patterns using the HPCluster program [17]. The Bioconductor package "GOstats" was used for testing the association of Gene Ontology terms (biological processes) to the differentially expressed genes [18]. A *p*-value based on the hypergeometric test was computed to assess whether the number of genes associated with the term is larger than expected. The *p*-values obtained were adjusted for multiple testing using the FDR. The microarray

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**Table 1.** Summary of differentially expressed genes

	12H	24H	48H	72H
>4 fold up	8	50	48	27
2-4 fold up	185	335	315	265
2-4 fold down	150	206	225	212
>4 fold down	5	9	11	9

Only those genes with false discovery rate (FDR)  $p$  values <0.05 are included in this table.

data was also analyzed using prebuilt KEGG pathways. We used "spia" (signaling pathway impact analysis) package to identify the pathways impacted by the observed changes in the gene expression [19]. Network analysis was performed to construct and visualize molecular interaction networks using the STRING database [20]. The networks were imported in simple interaction format to Cytoscape [21].

### Real-time RT-PCR analysis

An aliquot of total RNA (2  $\mu$ g per sample) were arrayed in 96-well plates and then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA products were used for quantitative real-time PCR using ready-to-use TaqMan gene expression assays from the Applied Biosystems. Five reference genes with relatively constant expression (GAPDH, ESD, GUSB, IPO8 and MRPL19) were used for normalizing RNA concentration. Real-time PCR was performed with 96x96 Fluidigm Expression chips.

### Western blotting

Cells were harvested and resuspended in PBS. After centrifugation at 2000 rpm for 5 min, the pellet was lysed in ice cold M-PER Mammalian Protein Extraction Reagent containing 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) for 30 min. The supernatant was collected after 10 min of centrifugation at 12000 rpm, equaled by spectrophotometry, denatured with sample loading buffer for 10 min at 95°C and stored at 4°C for future use. Proteins were separated by 10% SDS-polyacrylamide gels and transferred to PVDF membrane, and incubated with primary antibodies of interest at 4°C overnight. The appropriate horseradish-conjugated secondary antibody at a dilution of 1:10000 in blocking buffer (3% BSA-TBST) was added and incubated for

1h at room temperature. All antibodies were diluted in 3% BSA-TBST. Antibody for RRM1 was purchased from Cell Signaling.

## Results

### *MPA alters expression of genes in multiple cancer-relevant pathways*

The AGS cell line was used as a model to investigate the molecular mechanisms of MPA on cancer cells. A concentration of 2  $\mu$ g/ml MPA was selected to treat AGS cells for 0, 12, 24, 48 and 72 hours. Gene expression was analyzed using Illumina microarrays. A total of 1070 genes were altered by  $\geq 2$  fold by MPA treatment and 85 genes were altered by >4 fold at one or more time points (**Table 1**). The significantly altered genes by MPA treatment were functionally annotated to identify the molecular pathways and biological functions. Among the prebuilt KEGG pathways, at least ten pathways are significantly altered by MPA and eight of these pathways (p53 signaling, cell cycle, pathways in cancer, PPAR signaling, bladder cancer, protein processing in ER, small cell lung cancer and MAPK signaling) are well-known to be cancer-related (**Table 2**).

The most significantly altered biological processes by MPA treatment are shown in **Table 3**. The processes highly relevant to cancer include cell cycle, cell death, cell proliferation, cell migration, small molecule metabolic process and response to stress. For example, 14 cell proliferation genes are altered by >4-fold ( $p = 0.0027$ ) and 129 cell proliferation genes are altered by 2-4 fold ( $p = 1.5 \times 10^{-8}$ ). Similarly, 13 cell death genes are altered by >4-fold ( $p = 0.022$ ) and 142 cell death genes are altered by 2-4 fold ( $p = 5.2 \times 10^{-8}$ ). These results suggest that MPA treatment can significantly alter a number of cancer-related cell functions including induction of cell cycle arrest and apoptosis as well as inhibition of cell proliferation.

### *Molecular changes underlying MPA-induced cell cycle arrest*

The expression patterns of cell cycle genes altered by MPA treatment are shown in **Figure 1A**. These genes are clustered in three distinct groups. The cluster 1 genes are gradually up-regulated during MPA treatment and the cluster 2 genes are gradually and increasingly down-

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**Table 2.** Major KEGG pathways altered in MPA treated AGS cells

Pathway Name	Gene #	Gene # changed	pFDR-12	Status-12	pFDR-24	Status-24	pFDR-48	Status-48	pFDR-72	Status-72
p53 signaling pathway	68	22	6E-11	I	7E-11	I	8E-11	I	9E-11	I
Cell cycle	123	22	5E-06	I	5E-06	I	4E-06	I	3E-06	I
Pathways in cancer	328	35	4E-04	I	3E-04	A	3E-04	A	3E-04	I
Small cell lung cancer	87	15	5E-04	A	2E-04	A	7E-05	A	1E-04	A
PPAR signaling pathway	71	10	2E-03	I	1E-03	I	1E-03	I	2E-03	I
HTLV-I infection	259	27	2E-03	I	2E-03	I	2E-03	I	2E-03	I
Bladder cancer	42	9	7E-03	I	7E-03	A	6E-03	A	7E-03	A
Protein processing in ER	163	18	1E-02	I	6E-03	I	8E-03	I	8E-03	I
MAPK signaling pathway	266	24	1E-02	I	3E-02	I	2E-02	I	2E-02	I
Pathogenic E. coli infection	54	9	2E-02	I	1E-02	I	1E-03	I	1E-03	I

I: inhibited; A: activated; pFDR: FDR *p* value for each time point (12, 24, 48 and 72 hours).

**Table 3.** Major biological processes modified in MPA treated AGS cells

Processes	≥2 fold (1070) UP: 620; Down: 450			>4 fold (85) UP: 69; Down: 16		
	# Genes	<i>p</i> -value	OR	# Genes	<i>p</i> -value	OR
Angiogenesis	23	0.183643	1.25	4	0.05803	2.88
Lipid metabolic process	84	0.001398	1.46	10	0.01807	2.34
Response to stress	219	4.47E-09	1.64	23	0.00079	2.47
Cell cycle	112	6.61E-06	1.64	6	0.52851	1.04
Cell death	142	5.22E-08	1.72	13	0.02280	2.04
Cell proliferation	129	1.45E-08	1.81	14	0.00272	2.64
Cell migration	59	0.00419	1.49	10	0.00130	3.52
Regulation of phosphate metabolic process	77	0.000128	1.63	9	0.01561	2.52
Cellular response to stress	92	3.85E-05	1.63	6	0.33588	1.30
Small molecule metabolic process	195	1.09E-06	1.52	21	0.00187	2.35
Regulation of angiogenesis	12	0.098609	1.57	2	0.12597	3.36
Blood vessel morphogenesis	30	0.06548	1.38	6	0.00784	3.73
Cell motility	64	0.002427	1.51	11	0.00061	3.65
Regulation of protein metabolic process	115	3.40E-07	1.75	10	0.05236	1.92

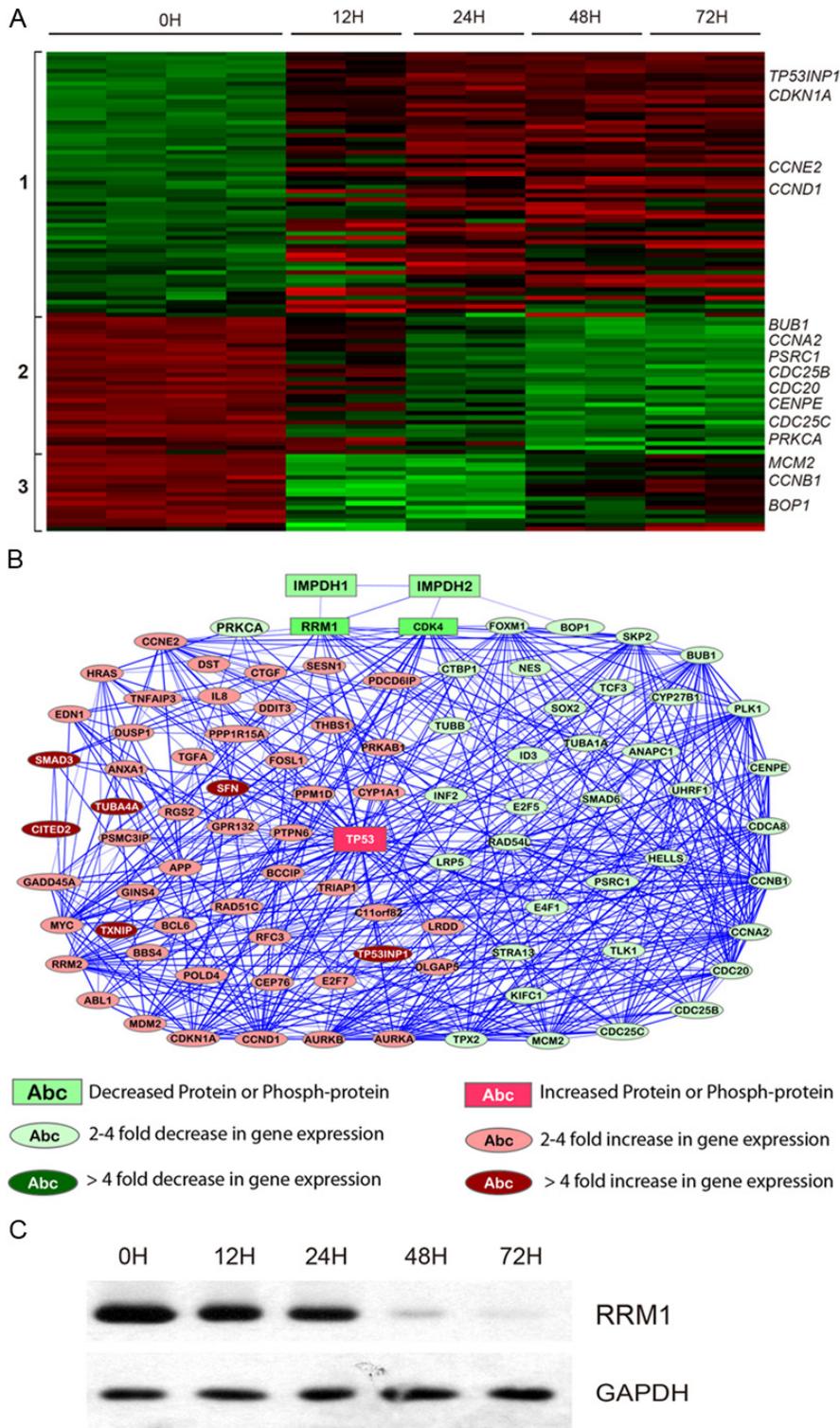
regulated over the course of treatment, while the cluster 3 genes are quickly down-regulated at the 12h but then up-regulated at the 48h time point. The most well-known cell cycle genes include cyclins, cyclin-dependent kinases (CDK), cell division cycle (CDC) genes and various cell cycle regulators.

Cyclins are a family of proteins that control the progression of cells through the cell cycle by activating cyclin-dependent kinases (CDK) [22, 23]. Several different cyclins are active in different phases of the cell cycle and they cause the CDKs to phosphorylate different substrates. Cyclin D1 encoded by the *CCND1* gene and Cyclin E encoded by the *CCNE2* gene are essen-

tial for the control of the cell cycle at the G1/S transition. Cyclin A (*CCNA2*) regulates transition from G1 to S phase. Cyclin B (*CCNB1*) is essential for the control of the cell cycle at the G2/M transition (mitosis). Our microarray data suggest that *CCND1* and *CCNE2* are increased, while *CCNA2* and *CCNB1* are decreased in MPA treated AGS cells.

The expression of the *CDK4* and *CDK5* genes was significantly decreased (**Table 4**), consistent with our previous observation on reduction of CDK4 protein expression by MPA treatment [14]. Several cell division related genes (*CDC20*, *CDC25B* and *CDC25C*) and cell cycle related genes (*MCM2*, *CENPE* and *PSRC1*) are also

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decreased by MPA treatment (Table 4).

The *cip/kip* family (CDK interacting protein/Kinase inhibitory protein) prevents the progression of the cell cycle. Because of their instrumental role in prevention of tumor formation, these genes are known as tumor suppressors. The *cip/kip* family includes p21, p27 and p57, which halt cell cycle in G1 phase, by binding to, and inactivating, cyclin-CDK complexes. The p21 protein encoded by the *CDKN1A* gene is a potent cyclin-dependent-kinase inhibitor (C-KI). The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. Although the microarrays used in this study do not contain a probe for the p53 gene (*TP53*), a gene encoding a p53-induced protein (*TP53INP1*) was significantly increased, consistent with incre-

**Figure 1.** Molecular changes related to cell cycle arrest induced by MPA treatment. A: Heatmap for genes differentially expressed after MPA treatment for 0h, 12h, 24h, 48h and 72h. Each sample is represented in a column and each gene is represented in a row. Increased expression is indicated as red and decreased expression is indicated as green. Representative genes are shown on the right panel and gene clusters are indicated on the left. B: A network illustrating the connectedness of the genes (>2-fold differences) involved in cell cycle regulation. C: Western blot for RRM1.

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**Table 4.** Selective genes differentially expressed after MPA treatment

Genes	R12h	R24h	R48h	R72h	p-12h	p-24h	p-48h	p-72h
<i>ABCA12</i>	1.0	2.3	5.4	4.2	9.7E-01	3.7E-09	8.1E-15	2.6E-13
<i>ABCB9</i>	2.3	3.4	4.3	2.9	6.2E-09	3.7E-12	1.4E-13	5.1E-11
<i>ATF3</i>	5.6	6.3	7.0	6.8	3.8E-13	2.0E-14	6.5E-15	1.1E-14
<i>CDH10</i>	1.5	3.1	4.8	7.4	2.6E-03	8.3E-10	2.8E-12	5.0E-14
<i>CEACAM1</i>	2.8	6.0	5.3	2.9	1.9E-10	4.2E-15	1.3E-14	6.0E-11
<i>CITED2</i>	2.2	2.2	4.6	3.5	1.5E-06	6.8E-07	1.9E-11	7.8E-10
<i>CYR61</i>	2.7	3.5	4.9	3.5	8.1E-09	5.3E-11	8.3E-13	7.6E-11
<i>GRIN2C</i>	5.9	8.3	9.6	10.6	6.4E-14	4.3E-16	1.3E-16	5.8E-17
<i>NOS3</i>	1.1	1.6	4.7	6.5	8.3E-01	4.3E-05	4.8E-14	1.9E-15
<i>NPPB</i>	2.9	8.9	17.0	11.6	1.4E-07	4.0E-13	3.1E-15	6.5E-14
<i>RN7SK</i>	3.6	12.1	81.6	176.9	3.7E-11	1.2E-16	6.2E-22	1.5E-23
<i>RNA28S5</i>	1.0	1.2	15.9	40.4	9.2E-01	3.6E-01	3.1E-15	2.9E-17
<i>SLC30A1</i>	5.3	6.3	3.8	3.4	7.6E-13	2.1E-14	6.3E-12	4.4E-11
<i>SLC30A1</i>	4.6	5.5	3.9	4.0	2.4E-13	6.5E-15	3.5E-13	3.7E-13
<i>SLC31A2</i>	2.8	4.1	2.9	3.4	4.9E-11	4.4E-14	7.3E-12	1.0E-12
<i>SMAD3</i>	1.6	4.3	3.6	2.7	1.5E-04	1.2E-12	1.5E-11	1.0E-09
<i>TP53I3</i>	5.4	12.0	11.0	7.7	1.2E-13	4.2E-17	5.7E-17	8.6E-16
<i>TP53INP1</i>	3.2	4.2	4.3	4.0	7.1E-11	6.0E-13	4.5E-13	1.8E-12
<i>TXNIP</i>	2.7	3.1	5.5	6.1	2.5E-09	9.3E-11	7.5E-14	3.1E-14
<i>ARRB1</i>	0.18	0.20	0.19	0.18	3.0E-14	8.1E-15	5.1E-15	2.8E-15
<i>CHN2</i>	0.38	0.37	0.27	0.32	2.4E-09	4.1E-10	3.5E-12	6.0E-11
<i>CLDN2</i>	0.53	0.37	0.23	0.22	5.9E-07	2.2E-10	2.0E-13	1.7E-13
<i>D2HGDH</i>	0.71	0.29	0.20	0.23	1.2E-03	3.6E-12	2.3E-14	2.0E-13
<i>ESRRG</i>	0.29	0.28	0.26	0.29	1.5E-08	4.7E-09	2.7E-09	1.3E-08
<i>HSPA5</i>	0.22	0.18	0.25	0.28	1.8E-12	2.8E-14	1.4E-12	8.2E-12
<i>IGFBP5</i>	0.14	0.10	0.10	0.09	1.2E-13	9.6E-16	1.0E-15	8.6E-16
<i>INF2</i>	0.25	0.22	0.24	0.24	4.9E-11	4.4E-12	1.1E-11	1.7E-11
<i>LRP8</i>	0.43	0.31	0.20	0.27	1.9E-08	4.6E-11	2.0E-13	7.2E-12
<i>NAV2</i>	0.18	0.17	0.13	0.12	1.3E-12	1.7E-13	1.0E-14	6.6E-15
<i>PAGE4</i>	0.63	0.34	0.14	0.10	2.9E-04	9.0E-10	2.3E-14	1.9E-15
<i>PDGFRA</i>	0.41	0.28	0.23	0.16	7.3E-09	5.1E-12	4.7E-13	1.1E-14
<i>PLA2G2A</i>	0.68	0.41	0.29	0.29	3.6E-03	5.6E-08	2.3E-10	4.4E-10
<i>PPARG</i>	0.56	0.33	0.28	0.35	2.2E-06	4.2E-11	2.3E-12	1.3E-10
<i>PRKCA</i>	0.31	0.35	0.28	0.27	3.7E-11	8.4E-11	2.8E-12	3.5E-12
<i>SNCAIP</i>	0.31	0.18	0.12	0.14	1.7E-10	7.3E-14	1.3E-15	5.4E-15
<i>AURKA</i>	0.73	0.68	0.64	0.54	1.3E-03	1.2E-04	1.6E-05	1.4E-07
<i>AURKB</i>	0.96	0.82	0.50	0.51	9.1E-01	1.8E-01	3.3E-06	5.0E-06
<i>FOXM1</i>	0.79	0.65	0.42	0.54	1.3E-01	1.6E-03	1.5E-07	2.1E-05
<i>BOP1</i>	0.48	0.34	0.41	0.57	3.4E-07	3.9E-10	8.6E-09	9.8E-06
<i>BUB1</i>	0.66	0.52	0.39	0.42	1.8E-04	2.7E-07	6.4E-10	2.7E-09
<i>BUB1B</i>	0.61	0.66	0.62	0.56	3.0E-05	1.9E-04	2.8E-05	2.5E-06
<i>SRC</i>	0.97	0.82	0.67	0.56	9.2E-01	1.4E-01	9.0E-04	1.1E-05

R12h, R24h, R48h and R72h denote gene expression ratios at the respective time point over untreated control (0h). p-12h, p-24h, p48h and p72h denote p-values for the comparison between untreated control (0h) and the respective time point.

ased expression of p53 protein after MPA treatment [14].

Several kinases implicated in cell cycle control are significantly altered by MPA treatment. A

prominently down-regulated gene is the *PRKCA* gene that plays an important role in cell cycle check point and many other cellular functions (Table 4). Furthermore, the *BUB1* and *BUB1B* genes are down-regulated by MPA treatment (Figure 1 and Table 4). These results are consistent with our proteomic results indicating a dramatic reduction of the BUB1 protein after MPA treatment [14]. *BUB1* and *BUB1B* encode two related mitotic checkpoint serine/threonine-protein kinases, which are involved in spindle checkpoint function. Many cancers have impaired spindle checkpoint function. The *AURKA* and *AURKB* genes encode the Aurora A and Aurora B kinases, which play important roles in mitosis. Aurora A kinase regulates spindle assembly and stability, while Aurora B kinase regulates chromosome segregation and cytokinesis. These two genes were significantly down-regulated by MPA treatment (Table 4).

Consistent with the protein expression changes in our previous study, the *FOXM1* gene is significantly down-regulated by MPA treatment (Table 4). *FOXM1* is known to play a key role in cell cycle progression. Endogenous *FOXM1* expression peaks at S and G2/M phases [24]. *FOXM1* knockout mice are neonatal lethal as a result of the development of polyploidy cardiomyocytes and hepatocytes, highlighting the role of *FOXM1* in mitotic division. *FOXM1* regulates expression of a large array of G2/M-specific genes including *PLK1* and *CENPE* that are down-regulated by MPA (Figure 1). It plays an important role in maintaining chromosomal segregation and genomic stability [25].

The *BOP1* gene encodes the ribosome biogenesis protein involved in rRNA processing, thereby controlling the cell cycle [26]. It is required for the maturation of the 25S and 5.8S ribosomal RNAs and it may serve as an essential factor in ribosome formation. The *BOP1* complex is involved in ribosome biogenesis and altered chromosome segregation. The expression of *BOP1* gene is significantly reduced by MPA treatment (Table 4). Consistent with the gene expression changes, the *BOP1* protein is also significantly down-regulated by MPA treatment [14].

Network analysis revealed that many of the cell cycle genes altered by MPA are connected to the *RRM1* gene. Although *RRM1* gene expression is not altered by MPA, Western blot showed

that the *RRM1* protein is drastically reduced by MPA treatment (Figure 1C). However, it is still unknown why the *RRM1* protein is significantly reduced, while the gene expression is not changed by MPA treatment. *RRM1* is the large subunit of ribonucleoside-diphosphate reductase that is essential for the production of deoxyribonucleotides prior to DNA synthesis in S phase of dividing cells.

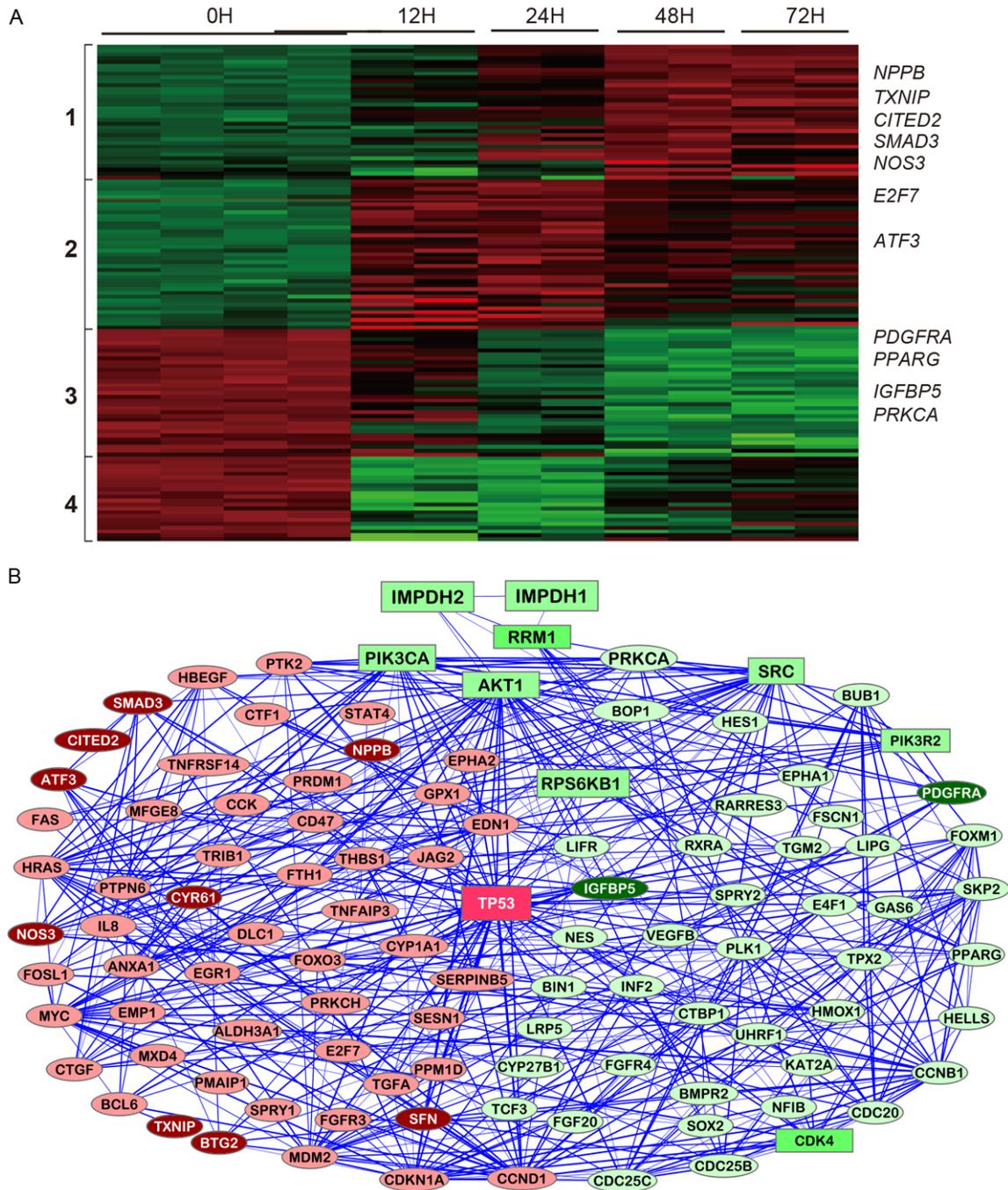
### *Molecular mechanisms underlying MPA-induced inhibition of cell proliferation*

The 143 proliferation genes with >2-fold differential expression can be clustered into four groups (Figure 2A) and the functional network of the proliferation genes is presented in Figure 2B. The cluster 1 genes are slightly up-regulated at the 24h time point and then more highly up-regulated at later time points, while the cluster 2 genes are already highly up-regulated at the 12h time point but the up-regulation is lower at later time points. The cluster 3 genes are gradually down-regulated starting at the 24h time point, while the cluster 4 genes are already highly down-regulated at the 12h time point but then the expression goes up at later time points. MPA treatment increased the expression of a number of proliferation-inhibitory genes such as *TXNIP*, *NOS3*, *NPPB*, *SMAD3* and transcription repressors *ATF3*, *E2F7* and *CITED2* and down-regulated several proliferation-promoting genes such as *IGFBP5* and *PPARG* (Figure 2A and Table 4). Six most altered genes implicated in cell proliferation (*IGFBP5*, *PPARG*, *PRKCA*, *TXNIP*, *PDGFRA* and *ATF3*) were selected for real-time RT-PCR confirmation (Figure 3). All six genes were confirmed to be altered in AGS cells which are sensitive to MPA treatment, while only two of these genes (*ATF3* and *TXNIP*) were altered in Hs746T cell that are resistant to MPA treatment (Figure 4).

### *MPA induces changes in genes implicated in apoptosis*

Figure 5 shows the heatmap and functional network for all 142 cell death genes with 2-4 fold differences and 13 genes with >4-fold differences after MPA treatment. Four distinct clusters of genes are recognized. The cluster 1 genes are quickly and highly up-regulated at the 12h and 24h time points but then down-regulated at the 48h and 72h time points, while

## Transcriptomic profiles associated with MPA's anticancer activity

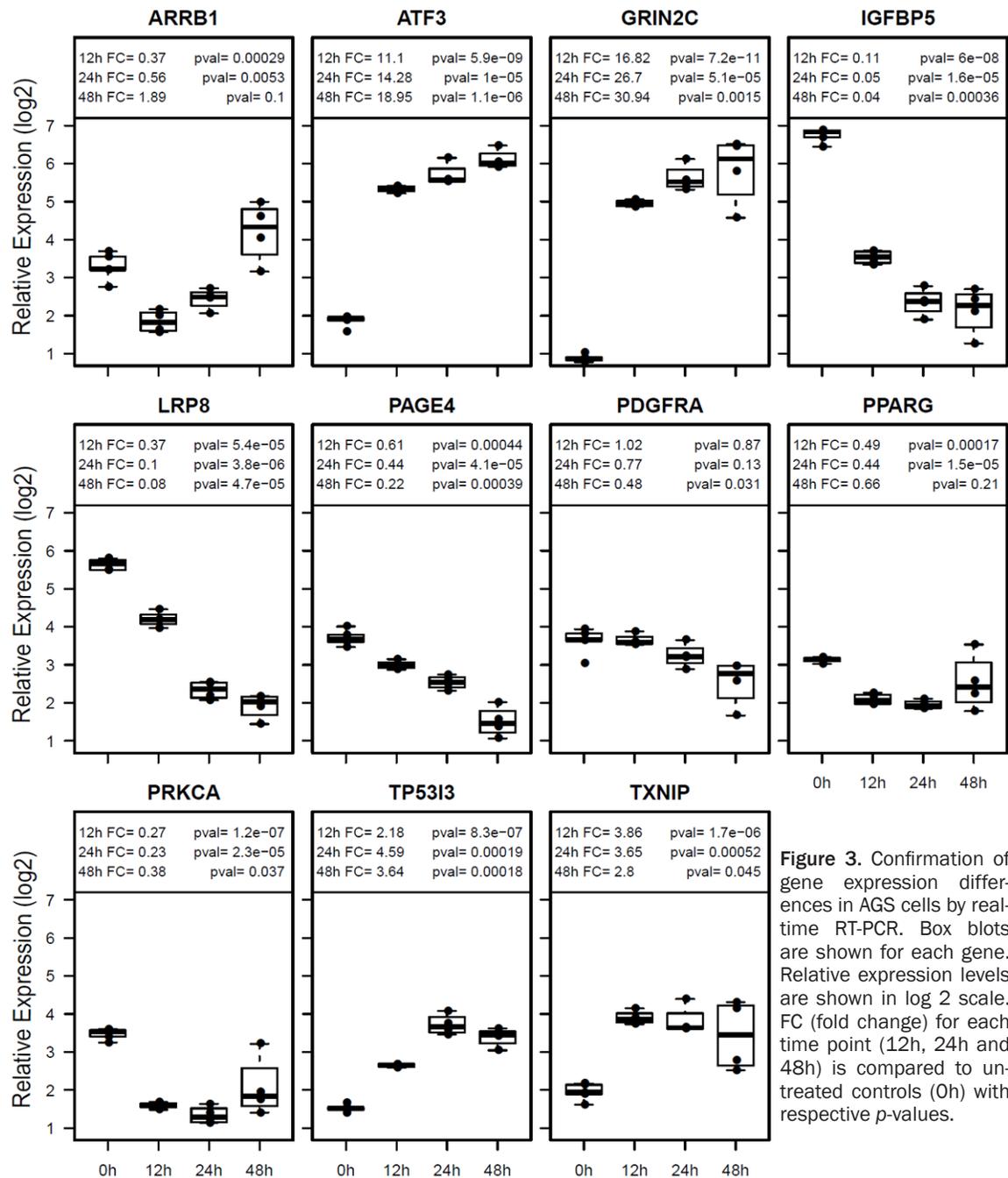


**Figure 2.** Molecular changes related to inhibition of cell proliferation induced by MPA treatment. A: Heatmap for genes differentially expressed after MPA treatment for 0h, 12h, 24h, 48h and 72h. B: A network illustrating the connectedness of the genes (>2-fold differences).

up-regulation becomes apparent only at the 48h time point for the cluster 2 genes. The cluster 3 genes are down-regulated starting at 24h time point, while the cluster 4 genes are quickly and highly down-regulated at the 12h and 24h time points but then slightly up-regu-

lated at later time points. Eight of the most altered genes (*TP53*, *TXNIP*, *PPARG*, *ARRB1*, *PAGE4*, *SNCAIP*, *IGFBP5* and *PRKCA*) were confirmed by real-time RT-PCR (Figure 3), while only one of these genes (*TXNIP*) was altered in the MPA-resistant Hs746T cells (Figure 4).

## Transcriptomic profiles associated with MPA's anticancer activity



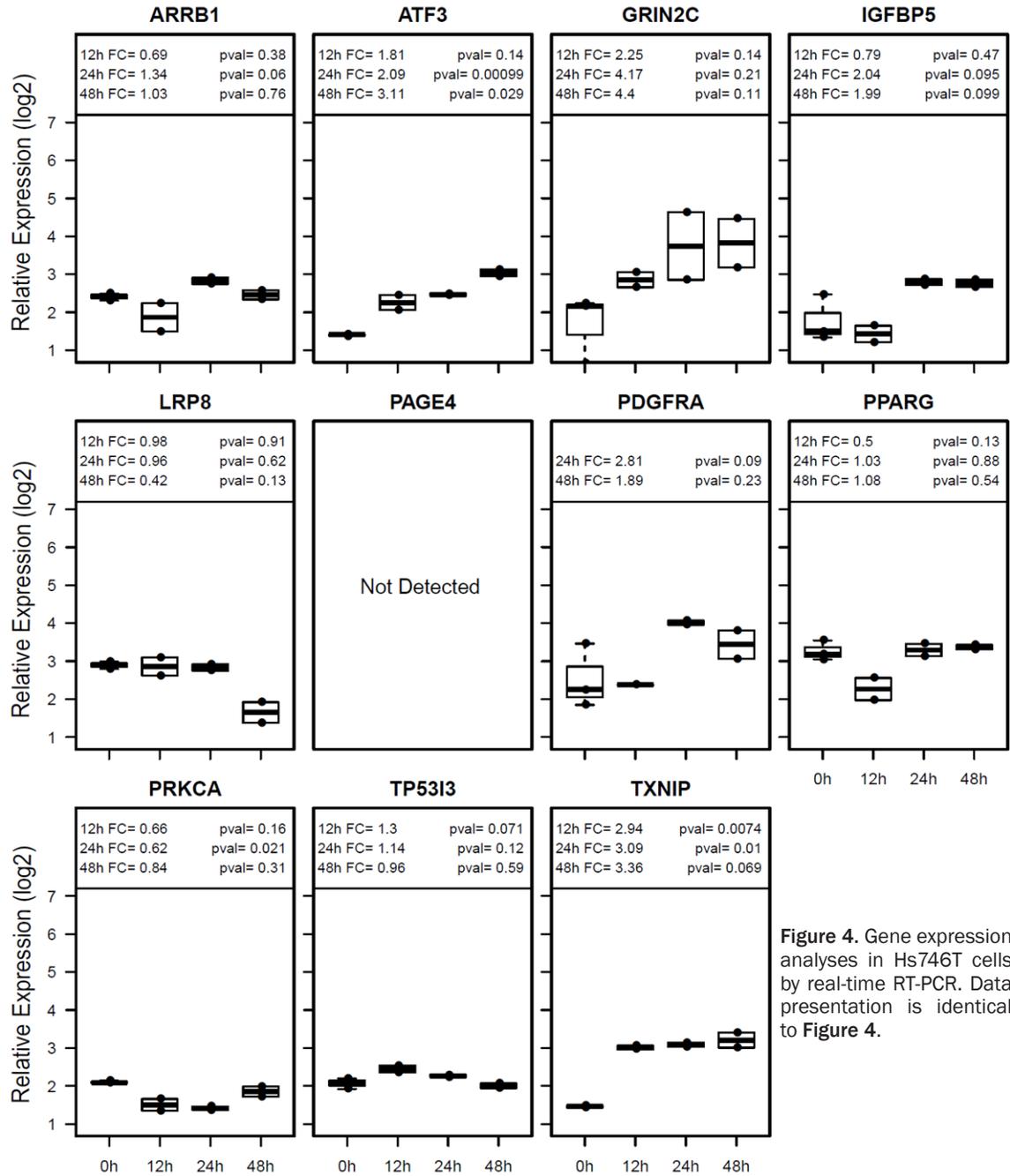
**Figure 3.** Confirmation of gene expression differences in AGS cells by real-time RT-PCR. Box blots are shown for each gene. Relative expression levels are shown in log<sub>2</sub> scale. FC (fold change) for each time point (12h, 24h and 48h) is compared to untreated controls (0h) with respective *p*-values.

*TP53I3* is the most up-regulated gene related to cell death (Table 4) and encodes a protein similar to oxidoreductases, which are enzymes involved in cellular responses to oxidative stresses and irradiation. This gene, which is induced by the tumor suppressor p53, is thought to be involved in p53-mediated cell death. *TP53INP1* (tumor protein 53-induced nuclear protein 1) is a tumor suppressor, whose expression is down-regulated in cancers from different organs. It is a p53 target gene involved

in cell death, cell-cycle arrest and cellular migration. *TP53INP1* could act as a tumor suppressor by inducing cell death by caspase-dependent autophagy [27]. *TP53NP1* is quickly up-regulated (~4-fold) and remains highly expressed during the course of MPA treatment (Table 4).

The thioredoxin-interacting protein (TXNIP) is a critical node in the terminal unfolded protein response (UPR). When unfolded proteins accu-

## Transcriptomic profiles associated with MPA's anticancer activity



**Figure 4.** Gene expression analyses in Hs746T cells by real-time RT-PCR. Data presentation is identical to Figure 4.

multate to irredeemably high levels within the endoplasmic reticulum (ER), the UPR signaling pathways become hyperactivated to cause programmed cell death [28]. The *TXNIP* gene is progressively up-regulated by MPA treatment as suggested by the microarray data and confirmed by real-time RT-PCR (Figure 3).

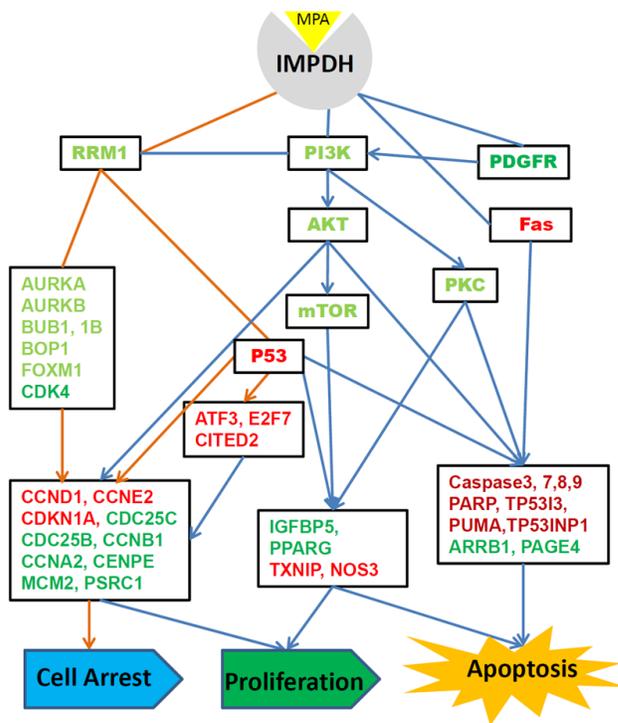
*NOS3* (NO synthase-3) is the third most up-regulated cell death gene by MPA treatment (Table 4). *NOS-3* overexpression increases oxidative/

nitrosative stress, p53 and CD95 expression, caspase-8 activation and cell death in HepG2 cells. *NOS-3* overexpression or NO donor injection into hepatoma-derived tumors reduced the size and increased p53 and cell death receptor expression in nude mice [29].

Prostate-associated gene 4 (*PAGE4*) is a member of the CT-X family of cancer/testis antigens that is highly expressed in testis and the fetal prostate and may also play an important role



## Transcriptomic profiles associated with MPA's anticancer activity



**Figure 6.** Overall molecular networks and biological functions altered by MPA treatment of AGS cells. Genes in light red and proteins in dark red are up-regulated. Genes in light green and proteins in dark green are down-regulated after MPA treatment.

sion of PAGE4 protects cells from stress-induced death [30]. PAGE4 is drastically down-regulated by MPA treatment.

The Fas receptor is a death receptor on the surface of cells. The expression of the gene encoding Fas (*TNFRSF6*) is already significantly increased at 12h of MPA treatment (**Figure 5** and **Table 4**).

### Discussion

Previous studies have demonstrated that MPA can exert potent antitumor activities including cell cycle arrest, inhibition of proliferation, and induction of apoptosis [14]. Although the anticancer activities of MPA are now well established, it is still unclear how this drug exerts its functions. In this study, we used transcriptomic technologies to elucidate the molecular and biological pathways activated or inhibited by MPA. Transcriptomic profiling is a powerful technology to assess global molecular changes. The major advantage of gene expression microarray is its ability to rapidly and economi-

cally evaluate the expression pattern of almost all genes expressed in the cells of interest. Transcriptomic comparison of AGS cells treated with MPA for 0, 12, 24, 48 and 72 hours revealed over 1000 significantly altered genes ( $\geq 2$ -fold and  $p < 0.05$ ). To validate our findings, we used real-time RT-PCR and confirmed the expression differences for genes selected for validation. Pathway analyses of the significantly altered genes allowed us to reveal the three main biological processes that were observed in the functional studies. Pathway analyses also identified ten molecular pathways that help account for MPA's biological functions.

Global assessment during the course of MPA treatment allowed us to deduce the cascade of molecular and biological events depicted in **Figure 6**. We have previously shown that cell cycle arrest already occurred at the 12h time point, while alterations in cell proliferation and apoptosis only became apparent at the 24h time point [14]. MPA-induced cell cycle arrest appears to be the first and most important mechanism underlying MPA's anticancer activity. Our microarray data presented in this manuscript as well as the confirma-

tion data with RT-PCR and proteomic data from our previous targeted proteomic analyses [14] suggest that MPA can induce cell cycle arrest through six major pathways: 1) up-regulation of cyclins (*CCND1* and *CCNE2*) and down-regulation of *CCNA2* and *CCNB1*, 2) down-regulation of cyclin-dependent kinases (*CDK4* and *CDK5*); 3) inhibition of cell division related genes (*CDC20*, *CDC25B* and *CDC25C*) and other cell cycle related genes (*MCM2*, *CENPE* and *PSRC1*), 4) activation of p53, which activates the cyclin-dependent kinase inhibitors (*CDKN1A*) and many other genes, 5) impaired spindle checkpoint function and chromosome segregation (*BUB1*, *BUB1B*, *BOP1*, *AURKA*, *AURKB*, and *FOXM1*); 6) reduction of availability of deoxyribonucleotides and therefore DNA synthesis through down-regulating the RRM1 enzyme. The drastically decreased RRM1 protein expression is of particular interest. RRM1 is an enzyme involved in the production of deoxyribonucleotides prior to DNA synthesis. As MPA's main function is to specifically inhibit the IMPDH enzyme, the rate-limiting enzyme for the de

*novo* synthesis of guanosine nucleotides, RRM1 protein reduction by MPA treatment should exacerbate its effect on DNA synthesis resulted from the reduced availability of guanosine nucleotides.

MPA treatment alters the expression of over 100 genes implicated in cell proliferation. The most important events induced by MPA include the increased expression of a number of proliferation-inhibitory genes such as *TXNIP*, *NOS3*, *NPPB*, *SMAD3* and transcription repressors *ATF3*, *E2F7* and *CITED2* and down-regulation of proliferation-promoting genes such as *IGFBP5* and *PPARG*. The functional network of the proliferation genes (**Figure 2B**) highlights the importance of the PI3K-AKT-mTOR pathway in the inhibition of AGS cell proliferation by MPA. The PI3K/AKT/mTOR pathway is activated by receptor tyrosine kinases (RTK). One of the key RTK involved in cancer is the PDGFR alpha which is encoded by the *PDGFRA* gene that was down-regulated by MPA treatment based on the microarray discovery data and RT-PCR confirmation data. Although the *PIK3R2* gene which encodes the PI3K p85beta protein and the *PIK3CA* gene which encodes a PI3K p110a protein, are not significantly changed by MPA treatment, we previously demonstrated that MPA treatment slightly decreased PI3K p85beta protein expression but drastically decreased phosphorylated PI3K p110a in a time-dependent manner [14]. The expression of the AKT genes was not altered by MPA treatment and the total AKT protein only showed slight change at later time points; however, phospho-AKT (Ser473) was severely decreased by MPA treatment [14]. Furthermore, it was shown that the phospho-p70S6 kinase (Thr389), an mTOR component, was also dramatically down-regulated by MPA treatment. These results suggest that inhibition of the PI3K/AKT/mTOR pathway is a major mechanism through which MPA inhibits AGS cell proliferation. In many cancers, the PI3K/AKT/mTOR pathway is overactive, allowing proliferation of cancer cells [31-33].

The third largest group of genes altered by MPA treatment is related to regulation of apoptosis. Several molecular pathways appear to play a critical role in MPA-induced apoptosis. Two p53 induced genes (*TP53I3* and *TP53INP1*) as well as the p53 protein [14] are up-regulated by MPA. The Fas pathway is another important cell death pathway that is activated by MPA treat-

ment. Fas is one of the two apoptosis pathways, the other being the mitochondrial (or intrinsic) pathway [34]. Fas forms the death-inducing signaling complex (DISC) upon ligand binding. The receptor complex is internalized, allowing the adaptor molecule FADD to bind Fas [35]. FADD then binds caspase-8 that can self-activate through proteolytic cleavage. Active caspase-8 is then released from the DISC into the cytosol, where it cleaves other effector caspases, eventually leading to DNA degradation, membrane blebbing, and other hallmarks of apoptosis. Indeed, caspase-8, caspase 3 and caspase 7 are all highly activated in AGS cells after MPA treatment as shown in our previous studies [14], suggesting that the Fas-mediated apoptosis pathway is activated by MPA treatment.

In summary, our microarray data, together with RT-PCR confirmation and previous proteomic data, suggest that MPA alters a number of molecular pathways implicated in inducing cell cycle arrest, reduced proliferation and increased apoptosis. Our functional and molecular data suggest that the FDA-approved immunosuppressive drug MMF may be a good candidate that may be repurposed for anticancer therapy.

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### Disclosure of conflict of interest

The authors have declared that no competing interests exist.

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