Suppression of ID1 expression in colon cancer cells increases sensitivity to 5-fluorouracil

Tomasz Przybyła¹, Monika Sakowicz-Burkiewicz¹², Izabela Maciejewska¹, Hanna Bielarczyk² and Tadeusz Pawelczyk¹

¹Department of Molecular Medicine, ²Department of Laboratory Medicine, Medical University of Gdańsk, Gdańsk, Poland

Adjuvant chemotherapy with 5-fluorouracil remains the basic treatment for patients with advanced colorectal carcinoma. The major obstacle in successful treatment is the ability of CRC cells to acquire chemoresistance. Here we examined the impact of ID1 silencing on the sensitivity of CRC cells to 5-FU. To suppress ID1 expression in HT-29 and HCT-116 cells the cells were transduced with a lentiviral vector carrying the ID1 silencing sequence. Cells with silenced ID1 showed altered expression of epithelial and mesenchymal markers and exhibited increased proliferation rate compared to the parental cells. HCT-116 cells with suppressed ID1 became sensitized to 5-FU and this was not observed in HT-29 cells. Silencing ID1 resulted in altered expression of genes encoding enzymes metabolizing 5-FU. HT-29 cells with suppressed ID1 had significantly reduced mRNA level for thymidine phosphorylase, thymidine kinase and uridine-cytidine kinase 2 and dihydropyrimidine dehydrogenase. ID1 suppression in HCT-116 cells resulted in an increase of mRNA level for thymidine phosphorylase, thymidine kinase and uridine-cytidine kinase 2 with concurrent drop of dihydropyrimidine dehydrogenase and thymidylate synthetase mRNA levels. In conclusion, ID1 expression impacts the sensitivity of colon cancer cells to 5-FU and may be considered as a potential predictive marker in CRC treatment.

Key words: colon cancer, ID1, 5-fluorouracil, TdMP, TK, UCK2, DYPD, TYMS.

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e-mail: ssak@gumed.edu.pl

Abbreviations: 5-FDHU, 5-fluoro-5,6-dihydropyrimidinol; 5-FdUTP, 5-fluoro-2’-deoxyuridine-5’-triphosphate; 5-FU, 5-fluorouracil; 5-FUTP, 5-fluorouridine-5’-triphosphate; 7-AAD, 7-aminoactinomycin D; CDH1, E-cadherin; CDH2, N-cadherin; CRC, colorectal carcinoma; CTNNβ1, beta-catenin; DMSO, dimethyl sulfoxide; DPYD, dihydropyrimidine dehydrogenase; DT, doubling time; dTMP, 2’-deoxythymidine-5’-monophosphate; EMT, epithelial to mesenchymal transition; F-BAL, fluoro-β-alanine; FBS, fetal bovine serum; FdUMP, 5-fluoro-2’-deoxyuridine-5’-monophosphate; FdUTR, 5’-deoxy-5-fluorouridine; FGF, fibroblast growth factor; FN1, fibronectin; FUMP, 5-fluorouridine-5’-monophosphate; FUR, 5-fluorouridine; GFP, green fluorescent protein; HLH, helix-loop-helix; ID1, inhibitor of differentiation 1; IL, leukemia inhibitory factor; MET, mesenchymal epithelial transition; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCF3, transcription factor 3; TK1, thymidine kinase 1; TP53, tumor protein p53; TWIST1, twist family, bHLH transcription factor 1; TdMP, thymidine phosphorylase; TYMS, thymidylate synthase; UCK2, uridine-cytidine kinase 2; UMPS, uridine monophosphate synthetase; UPP1, uridine phosphorylase 1; VIM, vimentin

INTRODUCTION

Colorectal carcinoma (CRC) is diagnosed in about 1 million people every year. It is the most frequent gastrointestinal neoplasm (cancer) and one of the most fatal cancers in general (Kelder et al., 2006). In most cases surgery remains the basic and sufficient treatment of patients with CRC at stage I and II, however, for patients with more advanced cancer (stage III and IV) the standard treatment following resection includes adjuvant chemotherapy. Implementation of such a treatment prolongs progression-free survival and overall survival (Lombardi et al., 2010). Adjuvant chemotherapy was also shown to highly improve 5-year survival in stage IV patients with isolated liver or lung metastases (Cassidy & Graham, 2012). 5-Fluorouracil (5-FU) is one of the earliest chemotherapeutic agents and it has been used successfully in CRC treatment for over 50 years (Hammond et al., 2016). The drug operates through mechanisms involving inhibition of DNA replication and cell death. 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP), the primary metabolite of 5-FU inhibits thymidylate synthase (TYMS), an enzyme producing 2’-deoxythymidine-5’-monophosphate (dTMP). Insufficient dTMP bioavailability disrupts DNA synthesis in the dividing cells. The alternative 5-FU metabolites, 5-fluorouridine-5’-triphosphate (5-FUTP) and 5-fluoro-2’-deoxyuridine-5’-triphosphate (5-FdUTP) undergo inclusion into DNA, which results in impaired translation and subsequent cell death (Cassidy & Graham, 2012; Hammond et al., 2016).

The major obstacle in successful treatment with 5-FU based adjuvant chemotherapy is the ability of CRC cells to acquire resistance (Hammond et al., 2016). Several mechanisms have been proposed to be responsible for the decrease in cancer cells sensitivity to 5-FU including: variable number of tandem repeats, microsatellite instability, single nucleotide polymorphisms occurring within genes encoding enzymes metabolizing 5-FU, and altered expression of 5-FU metabolizing enzymes (Hammond et al., 2016). Improvement of 5-FU adjuvant treatment efficacy remains one of the most important challenges in CRC management. One of the main approaches is identification of predictive biomarkers associated with specific response to chemotherapy, which firstly would allow to determine a group of patients benefiting from 5-FU treatment and could exclude the non-responsive ones. Secondly, it may be useful in development and application of novel therapies combining 5-FU and selective agents capable of targeting specific signaling pathways in tumor. It was initially shown that targeting vascular endothelial growth factor in addition to standard chemotherapy improved outcomes of patients with metastatic CRC. However, recent clinical trials demonstrated that patients with CRC stage III did not benefit from such combined regimen. Thus, understanding the tumor biology through identification of biomarkers, which determine the success or failure of treatment at specific
Cancer cells were seeded (Oyan, 2012). The inhibitor of differentiation 1 (ID1) belongs to the family of helix-loop-helix (HLH) transcription regulators. These proteins achieve transcriptional activity by forming heterodimers with other members of the family. ID1, however, lacks the ability to bind DNA and plays a role of dominant negative regulator of its dimerization partners (Ling et al., 2014). ID1 was shown to promote chemoresistance of esophageal and pancreatic cancer cells (Li et al., 2014). On the other hand, it was reported that ID1 is a favorable predictor for surgically treated non-small-cell lung cancer patients undergoing chemotherapy (Cheng et al., 2014). Research on the role of ID1 in breast and gastric cancer and glioma revealed that it is also involved in epithelial to mesenchymal transition (EMT) which is considered one of the mechanisms promoting chemoresistance of cancer cells (Tobin et al., 2011; Peng et al., 2014; Sánchez-Tilló et al., 2014). Still, the direct mechanisms of ID1 related chemoresistance in cancer cells are not known, moreover, not much attention in this context has been paid regarding colon cancer. The aim of this work was to examine the relation between the ID1 expression level and the sensitivity of colon cancer cells to 5-FU.

MATERIALS AND METHODS

Cell cultures. All experiments were conducted on colon cancer cell lines HT-29 and HCT-116 (ATCC, Rockville, MD, USA). Cells were cultured in DMEM enriched with 10% fetal bovine serum (FBS), penicillin (10 U/ml) and streptomycin (100 μg/ml) under standard conditions (37°C, 5% CO2). The culture medium was replaced twice a week and cells were passaged at 70% confluence.

Transduction procedure. ID1 silencing shRNA sequence CTCTACGCAATTTCAAGAGAATTGTGTA-GAGCAGCAGTCTTTTTC (Oligos, Warsaw, Poland) was subcloned into lentiviral vector pLL3.7 (Addgene, Cambridge, MA, USA) to generate pLL3.7-shID1 construct. Scrambled oligonucleotide GGTATATTGGCTC-GATCTCTA (Oligos, Warsaw, Poland) was used to obtain pLL3.7-scramble plasmid, which served as a negative control. The packaging cells HEK-293T (ATCC, Rockville, MD, USA) were transfected with envelope plasmid pMD2.G, and a packaging plasmid psPAX2 (Addgene, Cambridge, MA, USA) together with pLL3.7-shID1 or pLL3.7-scramble. X-treme Gene 9 (Roche, Mannheim, Germany) was used as a transfection agent. Transfected HEK-293T cells were then incubated for 48 hours to allow generation and replication of the virus. HEK-293T medium containing viral particles was subsequently used to transduce HT-29 and HCT-116 cells. Negative control virus generated control (parental) cells, while vector expressing shRNA targeting ID1 produced cells with suppressed ID1 expression (HT-29shID1 and HCT-116shID1). Successfully transduced cells expressed green fluorescent protein (GFP) and number of GFP positive cells was counted under fluorescent microscope (Leica, Wetzlar, Germany). When at least 80% of cancer cells exhibited green fluorescence, the transduction procedure was finished.

MTT assay. Cells viability in appropriate experiments was estimated on the basis of mitochondrial dehydrogenase activity. In living cells 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Poznan, Poland) is converted by the enzyme to insoluble formazan and the extent of conversion reflects the total number of viable cells (Berridge et al., 1996). After the end of particular experiment the cells were incubated for two hours in fresh DMEM containing 0,5 mg/ml MTT at 37°C. Next, the medium was removed and formazan crystals were dissolved in 0.2 ml of acidic isopropanol. Finally, the absorbance of formazan was measured in a microplate reader (1420 multilabel Counter VICTOR3, PerkinElmer, Waltham, MA, USA) at 570 nm and at 690 nm (background subtraction).

Proliferation assays. Cells were seeded onto 24-well plates at a density of 1.5×104/well and cultured in complete medium or medium lacking FBS. Medium was replaced every day. Measurement of cell viability with MTT test was conducted after 24 hours and repeated every day for 124-hour period. The growth curves were generated and population doubling times (DT) were calculated on the basis of exponential growth periods (see Statistical analysis).

5-FU dose response assays. Cancer cells were seeded onto 24-well plates at a density of 1.5×104/well and left for 24 hours to attach to plate bottom. Next day the medium was replaced with fresh one containing 5-FU (Sigma-Aldrich, St. Louis, MO, USA) at appropriate concentrations. The control cells were cultured in medium with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) at the concentration used to dissolve 5-FU. After 48 hours of incubation the cell culture medium was removed and cell viability was evaluated with MTT test. Concentration at which 50% reduction of cell viability occurred (IC50) was determined (see Statistical analysis).

Flow cytometric analysis. Cancer cells were seeded onto 60 mm plates at density of 1.5×106 cells/plate and cultured overnight. Next day the medium was replaced with fresh one containing 5-FU at appropriate concentrations. The control cells were cultured in medium with equal amount of DMSO. After 48 hours of incubation the experimental medium was removed and the cell viability was evaluated by flow cytometric analysis. Control and experimental cells were stained with 7-aminoactinomycin D (7-AAD) and annexin V-FITC from PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) according to manufacturer protocols. Analysis was conducted on FACSscan flow cytometer (BD Biosciences, San Diego, CA, USA). The cells negative for annexin V and 7-AAD staining were considered as viable, while early apoptotic cells were marked by decreased size and staining with annexin V. Late apoptotic cells exhibited diminished diameter and were positive both for annexin V and 7-AAD staining, while necrotic cells were defined by size ranging between normal and increased and presence/emission of 7-AAD signal.

Reverse transcription-quantitative PCR. Total RNA Prep Plus Kit (A&A biotechnology, Gdynia, Poland) was used to isolate RNA from the cells. The expression level of each gene was analyzed with One-Step Real-Time PCR carried out using LightCycler 2.0 (Roche, Basel, CH). Path-ID Multiplex One-Step RT-PCR Kit (ThermoFisher Scientific, Waltham, MA, USA) and appropriate probes from Universal ProbeLibrary (Roche, Basel, CH) were applied to prepare samples. Each transcript level was normalized to ACTIN BETA (ACTB) mRNA.

Table 1 shows the list of primer sequences, TaqMan probes and cycling conditions.

Protein analysis. Qualitative determination of ID1 level in parental and ID1 suppressed cancer cells was performed using Western Blot. Protein samples were prepared as described previously (Maciejewska et al., 2014) and loaded on gradient (4–20%) SDS-PAGE gel.
Table I. List of primers, TaqMan probes and cycling conditions used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>TaqMan probe</th>
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<tr>
<td>ID1</td>
<td>CTGGACGAGCAGCGAGGTAA</td>
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<td>CTTCAATCTTAAAGGCTTCTAA</td>
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<td>TTGGATTTCTATTAACATGTTGTGTAAA</td>
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<td>ATATCTTAACTTCCGAAGCCACCTTCA</td>
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<td>VIM</td>
<td>TAGCTAAGCTTCTCTGACAC</td>
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<tr>
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<td>GACCCGAGGAGGAGGAAGTCT</td>
<td>Probe # 56 (Roche)</td>
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<td>GG GCAGTTGGTCAACTCCCTGT</td>
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Reverse transcription: 48°C (10 min), 95°C (10 min). Amplification: 95°C (10 s), 60°C (45 s). *The human genes are listed according to HUGO Gene Nomenclature Committee.

RESULTS

The impact of ID1 gene suppression on ID1 protein content in the cell

Transduction procedure of colon cancer cells resulted in a significant reduction of ID1 expression (Fig. 1). The ID1 transcript level in parental HT-29 cells was 0.26±0.011 compared to 0.005±0.001 observed in HT-29shID1 cells (53-fold decrease). In HCT-116shID1 cells the relative ID1 transcript level was reduced by 68 fold compared to parental HCT116 cells (from 0.67±0.025 to 0.01±0.001) (Fig. 1B). Western blot analysis showed that the ID1 protein content decreased in transduced HT-29 and HCT-116 cell lines (Fig. 1C). Quantitative analysis of ID1 protein in parental HT-29 and HCT-116 cell lines yielded 2.6±0.30 and 2.8±0.26 pg of ID1 protein/10⁶ cells, respectively. In HT-29shID1 and HCT116shID1 cells the ID1 protein levels averaged 1.62±0.13 pg/10⁶ cells and 1.03±0.10 pg/10⁶ cells, respectively (Fig. 1D). The efficiency of ID1 suppression both at mRNA and protein level was higher in HCT-116shID1 cells and resulted in significantly lower amount of ID1 protein compared to HT-29shID1 cells.

Altered expression of epithelial and mesenchymal markers in cells with silenced ID1

To examine the impact of ID1 suppression on possible epithelial to mesenchymal transition (EMT) we analyzed expression of selected epithelial and mesenchymal markers. We observed that in cells with silenced ID1 the expression of epithelial markers i.e. E-cadherin (CDH1) and beta-catenin (CTNNB1) was altered differently (Fig. 2). The transcript level of CDH1 increased in both cell lines, however, the change was significant only in HCT-116shID1 cells. ID1 suppression resulted in significant elevation of CTNNB1 transcript levels in both cell lines, although greater degree of change was observed in HT-29shID1 compared to HCT-116shID1 cells (6.5-fold versus 1.3-fold, respectively). We noted that suppression of ID1 resulted in changes in expression of mesenchymal markers (N-cadherin, vimentin and fibronectin) in examined cells. The expression of N-cadherin (CDH2) decreased 4.9-fold, and increased 3.4-fold in HT-29shID1 and HCT-116shID1 cells, respectively (Fig. 2). The expression level of vimentin (VIM) changed differently in HT-29 and HCT-116 cells after ID1 silencing. HT-29shID1 cells showed 17-fold increase in VIM mRNA level compared to control cells, however, it should be pointed that the transcript level was very low before as well as after ID1 knockdown. 1.0×10⁴±1.0×10⁴ and 2.4×10⁴±1.0×10⁴, respectively. Low VIM transcript level was also observed in parental HCT-116 (7.0×10⁴±2.0×10⁴), and silencing ID1 expression resulted in a drop of this mRNA levels below the detection limit. The fibronectin (FN1) expression was undetectable in both parental HT-29 and...
2.6×10^{-3} \text{cells}^\text{−1}\text{ml}^{-1} \text{h}^{-1}

HCT-16 cells, however, suppression of ID1 resulted in a rise of this transcript level up to 1.3×10^{-3} \pm 1.6\times10^{-4} and 2.6\times10^{-3} \pm 1.0\times10^{-4} in HT-29shID1 and HCT-116shID1 cells respectively (Fig. 2).

**Suppression of ID1 expression affects cell proliferation**

In the next step of our investigation we examined the impact of ID1 suppression on cells proliferation rate. Comparison of both parental cell lines showed that HT-29 cells exhibited higher proliferation rate (DT=33 h; 95%CI: 29-40) compared to parental HCT-116 (DT=27 h; 95%CI: 21-24) (Fig. 3). Suppression of ID1 enhanced proliferation of investigated cells, the doubling time dropped to 23 h (95%CI: 21-24) and 30 h (95%CI: 27-33) in HT-29shID1 and HCT-116shID1 cell lines respectively. HT-29 and HCT-116 cell lines, independently of ID1 expression, showed rather similar progression of growth curves. Lag phase lasted approximately 48h and afterwards cells approached phase of exponential growth. However, only HT-29 cells, both parental and transduced, reached the stationary step, which occurred after 120 h. FBS is a source of growth factors boosting cellular proliferation. To examine whether ID1 suppression alters the proliferation rates of investigated cells irrespectively of exogenous growth stimuli we evaluated growth of cell cultures under serum-starvation conditions. In HT-29 line a peak of density occurred at 120th and 96th hour for parental and transduced cells respectively and was followed by constant decline. During the last three days of measurement parental HT-29 cells showed higher proliferation rate compared to HT-29shID1 cells (Fig. 3). Parental HCT116 cells as well as HCT-116shID1 cells were unable to grow without FBS supplementation. Nevertheless, none of the examined cell lines reached the exponential growth phase; thus, the DTs remained undetermined under serum-starvation conditions.

**Effect of ID1 suppression on the cells sensitivity to 5-fluorouracil**

We examined whether suppression of ID1 affected the colon cancer cell lines response to 5-FU. The cells underwent treatment with increasing concentrations of 5-FU ranging from 0.01 to 100 µM, and subsequently the concentration at which 50% of growth inhibition occurred (IC_{50}) was calculated. Parental and transduced HT-29 cells did not show significant differences in response to 5-FU and shared IC_{50} value was 6.2 µM (95%CI: 4.0–9.9) (Fig. 4).

We observed that ID1 expression level modulated the response of HCT-116 cell line to 5-FU. 50% growth inhibition of parental HCT-116 cells was achieved at concentration of 12.4 µM (95%CI: 7.5–20.6), whereas IC_{50} for HCT-116shID1 averaged 1.5 µM (95%CI: 0.8–2.7) (Fig. 4).

Examination of HT-29 cell line by flow cytometry showed that under normal culture conditions 92.53±0.78% of parental HT-29 cells were viable while the majority of dead cells was at the stage of late apoptosis (Table 2). ID1 suppression significantly increased proportions of late apoptotic and necrotic fractions resulting in diminished viability of HT-29shID1 cells (76.40±5.00%). Viability of parental HCT-116

![Figure 1. Efficiency of ID1 suppression in HT-29 and HCT-116 cells.](image)

Transduction with lentiviral pLL3.7 scrambled vector generated control (parental) cells while introduction of pLL3.7-shID1 vector encoding the ID1 silencing sequence (shID1) produced cells with suppressed ID1 expression (HT-29shID1 and HCT-116shID1). (A) Presentation of HT-29shID1 and HCT-116shID1 cells in normal light and under a fluorescent microscope (GFP). Efficient transduction resulted in GFP expression. Merged pictures are shown at the bottom. (B) Relative ID1 transcript levels determined by quantitative PCR. Results were normalized to ACTB mRNA level and are presented as fold of change between parental and shID1 cells. *Indicates significant difference compared to control cells (P<0.05). (C) Qualitative examination of protein content by Western Blot. ACTIN BETA was used as a reference protein. (D) An enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine ID1 protein level in control and pLL3.7-shID1 transduced HT-29 and HCT-116 cells. *P<0.05 vs control.
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expression in colon cancer cells cultivated under standard conditions remained at 86.97±0.64% which was significantly less in comparison with parental HT-29 cells (Table 2). Decreased content of ID1 in HCT-116shID1 cells was associated with a decrease of cell viability (65.42±2.22%). In both parental and transduced HCT-116 cells the second largest fraction beside viable cells consisted of early apoptotic cells. In the parental HCT-116 cell line early apoptotic cells averaged 9.67±1.80% and rose to 29.09±5.10% in HCT-116shID1 cells. Inclusion of 1 μM 5-FU to the cell culture resulted in a significant reduction of viable cells in each cell line and major enrichment was observed in fraction of early apoptotic cells. This was accompanied by a decreased fraction of late apoptotic and necrotic cells (Table 2).

Impact of ID1 suppression on the expression level of genes encoding enzymes metabolizing 5-FU

Activity of 5-FU depends on its enzymatic conversion to metabolically active forms. Thus, we examined the expression of genes encoding major 5-FU metabolizing enzymes in order to assess possible impact of ID1 sup-
pressed on biotransformation of the drug. We observed that the mRNA content of thymidine phosphorylase (TYMP) was significantly decreased (6,8-fold) in HT-29shID1 cells compared to parental line (Fig. 5). Some decrease in the expression level of genes encoding uridine-cytidine kinase 2 (UCK2) and dihydroprymidine dehydrogenase (DPYD) in HT-29shID1 cells was also evident. Suppression of ID1 in HT-29 cells did not affect the expression levels of genes encoding thymidine kinase 1 (TK1), thymidilate synthetase (TYMS), uridine monophosphate synthetase (UMPS), and uridine phosphorylase 1 (UPP1). The transcriptional response to ID1 knock-down in HCT-116 cells was more variable compared to HT-29 line. HCT-116shID1 cells showed significant increase in mRNA levels of TYMP (1,9-fold), TK1 (1,2-fold) and UCK2 (2,0-fold) compared to parental cells. On the other hand, decline in ID1 expression significantly reduced transcript levels of TYMS (2,8-fold) and DPYD (1,5-fold). HCT-116shID1 cells did not exhibit altered expression of UMPS and UPP1 compared to parental line (Fig. 5).

**DISCUSSION**

Resistance of colon cancer cells to 5-FU chemotherapeutic is a major obstacle in treatment of people suffering from CRC. In order to include or exclude patients for particular therapeutic regimen predictive biomarkers are used for identification of responsive and nonresponsive individuals. Here, we showed that expression of ID1 belonging to HLH protein family impacts the senescence rate of colon cancer cell lines 5-FU; thus, making it a candidate for further investigation on potential predictive value.

Knockdown of ID1 resulted in altered expression of mesenchymal and epithelial markers, however, the pattern of changes did not resemble “classical” mesenchymal-epithelial transition (MET). Decreased level of ID1 in HT-29 cells led to a significant reduction of CDH2 expression but did not affect transcription of CDH1. On the other hand, both genes were upregulated significantly after ID1 suppression in HCT-116 cells. Those observations suggest that depletion of ID1 did not induce cadherin switch, a basic hallmark of MET (Thiery et al., 2009). These results indicate that ID1 expression alone is insufficient to determine an evident shift between the mesenchymal and epithelial phenotype. In number of research devoted to cancer cells direct or indirect suppression of ID1 was shown to correlate with downregulation of EMT markers (Gumireddy et al., 2009; Tobin et al., 2011; Sánchez-Tilló et al., 2014). It was recently revealed, however, that overexpressed ID1 exerts antagonistic effects (Stankic et al., 2013). Upregulation of ID1 generates breast cancer cells with properties of stem cells capable of invasion. It is established that stem cell-like characteristic and mesenchymal phenotype closely overlap with each other (Thiery et al., 2009; Stankic et al., 2013). On the other hand, ID1 promotes MET in metastatic breast cancer cells, which have previously undergone EMT (Stankic et al., 2013). This dual mode of ID1 action depends on local expression of twist family bHLH transcription factor 1 (TWIST1) and snail family transcriptional repressor 1. Those transcriptional inducers and regulators of EMT differentially modulate ID1 function (Stankic et al., 2013). Moreover, the present knowledge on the transition between epithelial and mesenchymal phenotypes suggests that it is not a binary process and many tumor cells acquire an intermediate E/M states rather than evident shifts between two phenotypes. These transitional forms are called hybrid epithelial/mesenchymal phenotype (Jolly et al., 2015). It was also noted that stable lentiviral ID1 overexpression producing functional protein in melanoma cells failed to affect transcription/translation of the ID1 target genes suggesting that ID1 expression alone is insufficient to determine cell fate (Healey et al., 2010). Thus, it may be assumed that specific response triggered by ID1 depends on molecular context in the cancer cell or its environment. Further investigations should be made in order to reveal the role of this protein in signaling cascades and loops governing epithelial-mesenchymal plasticity.

We showed that suppression of ID1 resulted in an increased proliferation rate of both examined cell lines. Available data on the impact of ID1 on cancer cells proliferation varies depending on cancer type and experimental methodology. Stankic’s work (Stankic et al., 2013) reported that suppression of ID1 in breast cancer cells cultivated in a complete medium did not affect proliferation rate. On the contrary, it was shown that ID1 silencing in salivary gland cancer cells decreased proliferation rate irrespectively of FBS presence (Sumida et al., 2013). In our experiments we utilized MTT method which measures metabolic activity in viable cells and under certain conditions is considered as valuable tool for assessment of proliferation (Berridge et al., 1996). We also calculated
DT values of cells basing exclusively on the phases of exponential growth. Moreover, normalized data sets were used (at \( t_y = 1 \)) which allowed comparing only dynamics of growth between examined cell lines. Thus, the lack of universal approach for analysis of cellular proliferation leads to somewhat problematic comparison of different results. Under serum starvation condition ID1 silencing did not affect the proliferation, moreover, parental HT-29 cells grew faster than transduced counterparts. FBS is the source of growth factors, including fibroblast growth factor (FGF) (Brunner et al., 2010). In studies on calvaria development FGF2 was shown to promote TWIST1 expression, whereas ID1 antagonized TWIST1 action through blocking dimerization of TWIST1 and transcription factor 3 (TCF3) (Rice et al., 2000; Connerney et al., 2008). T-TCF dimers promote more aggressive phenotype (Gajula et al., 2015) and number of reports showed that TWIST1 enhances proliferation of cancer cells (Hasselblatt et al., 2009; Qian et al., 2013; Qiang et al., 2014; Zhang et al., 2015; Zhu et al., 2015; Li & Wu, 2016). Thus, the observed increase of FBS-dependent cell growth after ID1 silencing might be the result of enhanced formation of T-TCF heterodimers.

In our study we observed differential response of examined cell lines to 5-FU treatment. Silencing of ID1 in HT-29 cells did not affect the sensitivity of these cells to 5-FU, whereas HCT-116 cells were significantly sensitized to 5-FU by suppression of ID1. It was recently shown that CRC cells acquire chemoresistance through leukemia inhibitory factor (LIF)-MDM2 axis (Yu et al., 2014). LIF protein interacts with signal transducer and activator of transcription 3 and 1 and ID1 inducing MDM2 expression. In turn, MDM2 protein promotes degradation of tumor protein p53 (TP53) leading to a generation of cell which is less prone to apoptosis in response to 5-FU (Yu et al., 2014). HT-29 cell line already contains inactive TP53 protein, owing to mutated TP53 gene, which may result in nonfunctional and degradation-resistant TP53 protein uncontrolled by LIF-ID1-MDM2 axis. Such a mechanism may explain why ID1 suppression failed to enhance the sensitivity of HT-29 cells to 5-FU. Impaired TP53 could also account for differences between HT-29 and HCT-116 cell lines observed in flow cytometric analysis. Under normal conditions as well as in the presence of 5-FU parental and transduced HT-29 cells exhibited higher viability and smaller apoptotic fractions compared to HCT-116 counterparts.

Cellular conversion of 5-FU may lead, through metabolic activation or degradation, to fluoro-5-alanine (F-BAL) (Longley et al., 2003). DPYD catalyses transformation of 5-FU to 5-fluoro-5,6-dihydrouracil (5-FDHU) and this reaction is the primary rate-limiting step in degradation of 5-FU. Responsiveness of patients to 5-FU treatment was shown to depend on the drug catalobism (Etienne et al., 1995). Level of DPYD expression is related to sensitivity to 5-FU. Recent study on hepatocellular carcinoma cell lines revealed that indirect modulation of DPYD transcription correlated with 5-FU-mediated decrease in cells proliferation (Oie et al., 2007). Suppression of DPYD expression in HCT-116 cells produced stronger apoptotic effect of 5-FU (Offer et al., 2014). In our experiment depletion of ID1 resulted in a similar range of DPYD downregulation in both HT-29 and HCT-116 cells. Thus, it may be assumed that differences in the response to 5-FU of these cell lines do not depend on DPYD expression level.

In the cell, 5-FU activation proceeds either through synthesis of 5-fluorouridine-5-monophosphate (FUMP) or generation ofFdUMP (Longley et al., 2003). Production of FUMP may be catalyzed in direct manner by UMPs or sequentially by transformation of 5-FU to 5-fluorouridine (FUR) via UPP and subsequent production of FUMP catalyzed by UCK. FdUMP, on the other hand, is produced through conversion of 5-FU to 5-deoxy-5-fluorouridine (FdUr) catalyzed by TYMP and subsequent TK-dependent modification to FdUMP. Suppression of ID1 in HT-29 cells resulted in decreased expression of TYMP and did not alter transcription of TK1 and TYMS. HCT-116shID1 cells, on the contrary, exhibited increased mRNA levels of TYMP and TK1 concomitant with depletion of TYMS transcript. It may, therefore, be suspected that sensitization of HCT-116shID1 cells to 5-FU might be due to enhanced FdUMP production. However, according to current data major pathway of 5-FU anabolism does not lead through generation of FdUMP but rather through production of FUMP (Peters et al., 1986; Peters et al., 1991). ID1 suppression in both examined cell lines affected only the expression of UCK2, although differentially. UCK2 was upregulated in HCT-116shID1 which suggests that increased response to 5-FU in these cells is related, at least partially, with enhanced activation of drug through FUMP synthesis.

In conclusion, we showed here that the low level of ID1 in colon cancer cells may sensitize these cells to 5-FU cytotoxicity, although the ID1 effect is differentially modified by mutations present in cancer cells. We assume that the determination of ID1 expression level together with other markers may help more precisely identify patients which may respond positively to 5-FU treatment.

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