Retinoic acid-mediated down-regulation of *ENO1*/*MBP-1* gene products caused decreased invasiveness of the follicular thyroid carcinoma cell lines

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Abstract

Retinoic acid (RA) acts as an anti-proliferative and redifferentiation agent in the therapy of thyroid carcinoma. Our previous studies demonstrated that pretreatment of follicular thyroid carcinoma cell lines FTC-133 and FTC-238 resulted in decreased *in vitro* proliferation rates and reduced tumor cell growth of xenotransplants. In addition to the previous results, we found that RA led to decreased vitality and invasiveness of FTC-133 and FTC-238 cells as they reacted with reduction of intracellular ATP levels and number of migrated cells respectively. However, the molecular mechanisms by which RA mediates these effects are not well understood. Two-dimensional (2D) screening of the proteins related to ATP metabolism and western blot analysis revealed α -enolase (ENO1) to be down-regulated in FTC-133 and FTC-238 cells after RA treatment. 2D gel detection and mass spectrometric analysis revealed that ENO1 existed as three separate protein spots of distinct pIs (ENO1-A1–A3). Comparative 2D difference gel electrophoresis analysis of fluorescently labeled protein samples of RA-treated and untreated FTC-133 demonstrated a selective down-regulation of ENO1-A1 which we identified as a phosphoprotein. RA caused the dephosphorylation of ENO1-A1. Both, RA-mediated and specific knock-down of ENO1/MBP-1 resulted in the reduction of MYC oncoprotein, and simultaneously decreased proliferation rates of FTC-133 and FTC-238 cell lines. In summary, the RA-mediated down-regulation of the *ENO1* gene products and MYC oncoprotein provides a novel molecular mechanism facilitating the anti-proliferative effect of RA in human thyroid carcinoma cells and suggests new pathways for supportive RA therapies.

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Introduction

The vitamin A (retinol)-derived retinoic acids (RA) are important regulators of a diverse spectrum of physiological processes, including cell proliferation, differentiation, morphogenesis, angiogenesis, and apoptosis (Maden 2000, Biesalski & Nohr 2003, Buletic *et al.* 2006). The pleiotropic effects of retinoids are mediated by a nuclear heterodimeric pair of retinoid receptors (RAR/RXR). Retinoid-activated RAR/RXR heterodimers mediate the transcription of specific gene networks, by binding to specific DNA response elements and recruiting cofactor complexes which cause the local chromatin structure to alter and engage the basal transcription machinery. RARs

and RXRs also integrate a variety of signaling pathways through phosphorylation events which cooperate with the ligand for the control of retinoid-target genes transcription. Signaling cascades involve FOS, MAPK, PI3 kinase, AKT, cyclins, cyclin-dependent kinases and their inhibitors, Bcl proteins and caspases, all of which are involved in the control of cell growth, differentiation, and apoptosis (Niles 2000). RA was successfully used for the treatment of hematological as well as therapy and chemoprevention of solid cancers (Hansen *et al.* 2000, Lengfelder *et al.* 2005) including thyroid carcinomas (Simon *et al.* 2002). Cell culture experiments in thyroid carcinoma cell lines showed that RA treatment affects thyroid-specific functions, cell–cell or cell–matrix

interaction, differentiation markers, growth, and tumorigenicity (Schmutzler & Kohrle 2000). RA has an anti-proliferative effect on the follicular thyroid carcinoma cell lines FTC-133 and FTC-238. Furthermore, pretreatment of these cell lines with RA results in decreased *in vitro* proliferation rates and reduced tumor cell growth of xenotransplants (Schmutzler *et al.* 2004).

Enolases are glycolytic enzymes responsible for the ATP-generating conversion of 2-phosphoglycerate to phosphoenolpyruvate. In vertebrates, there are three different tissue-specific isoenzymes: the α -enolase, ENO1, is expressed in a wide variety of tissues, whereas β -enolase is localized mainly in muscle and γ -enolase is specific for neuronal tissues (Merkulova *et al.* 2000). The ENO1 transcript encodes two structurally and functionally distinct proteins, a 48 kDa ENO1 enzyme and the 37 kDa MYC promotor-binding protein MBP-1. Enzymatic activity resides within the N-terminal portion, which is unique to ENO1 and absent in MBP-1 (Feo *et al.* 2000, Subramanian & Miller 2000, Pancholi 2001). Increased ENO1 gene activity and protein production have been detected in several carcinomas, including neuroendocrine tumors, neuroblastoma, lung cancer, hepatocellular carcinoma, and breast cancer cells, and suggest an involvement of ENO1 in tumor progression (Royds *et al.* 1982, Kato *et al.* 1983, Kaiser *et al.* 1989, Ledermann 1994, Niklinski & Furman 1995, Redlitz *et al.* 1995, Ebert *et al.* 1996, Joseph *et al.* 1996, Chang *et al.* 2003, 2006, Takashima *et al.* 2005, Zhang *et al.* 2005, Huang *et al.* 2006, Yoon *et al.* 2006). The up-regulation of ENO1, glucose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in thyroid oncocyoma tumors identifies ENO1 as a member of a metabolic signature in thyroid tumors (Baris *et al.* 2004). The increased presence of ENO1 could result in accelerated ATP production and, thus, ENO1 may act as a metabolic tumor promoter conferring a selective growth advantage onto ENO1 overexpressing tumor cells.

Here we show that RA treatment of the human follicular thyroid carcinoma cell lines FTC-133 and FTC-238 causes the down-regulation of ENO1 and MBP-1 and this correlated with both reduction of cell invasiveness and the down-regulation of MYC oncoprotein. Moreover, we provide first evidence by two-dimensional (2D) gel electrophoresis and mass spectrometric analysis of an RA-induced alteration in ENO1 phosphorylation.

Materials and methods

Cell culture, RA stimulation, and RNAi

The human follicular thyroid carcinoma cell lines FTC-133 and FTC-238, and undifferentiated thyroid

carcinoma cell lines Hth74, C-643, 8505C, SW1736 were cultured in DMEM/F12 medium, supplemented with 1.125 g/l sodium carbonate and 10% FCS. For treatment with RA, 8×10^5 cells were plated in 75 cm² flasks and cultured to 80% confluency. The day before treatment, growth medium was replaced with serum-free medium. After 24 h, the cells were treated with 1 μ M RA dissolved in ethanol for 24, 48, and 72 h in a standard humidified incubator (37 °C, 5% CO₂). Untreated controls cells were cultured in medium with the same concentration of ethanol but without RA. Medium was replaced daily. For RNAi analysis, 200 nM siRNA targeting ENO1 was applied (5'-AAC-CAG-CTC-CTC-AGA-ATT-GAA-3', Qiagen). Non-silencing, randomized sequence 5'-AAT-TCT-CCG-AAC-GTG-TCA-CGT-3' not matching any known human gene served as a control. Transfection was done in the presence of OptiMem (Invitrogen) medium and analyses were performed after 72 h. All experiments were repeated at least three times.

Motility assay

Motility of FTC-133 and FTC-238 cells pretreated with RA was evaluated in 24-well Transwell chambers (Costar, Bodenheim, Germany). The upper and lower culture compartments were separated by polycarbonate filters with 8 μ m pore size. To investigate the effect of RA on the motility of differentiated thyroid carcinoma cells, FTC-133 or FTC-238 were pretreated with 1 μ M RA (dissolved in ethanol) for 72 h and then plated at 1×10^4 cells/well in DMEM/F12 medium without FCS. Control cells were pretreated with medium containing the same concentration of ethanol but without RA. The cells migrated from upper to lower compartment for 24 h in a 5% CO₂ atmosphere at 37 °C. After a 24-h motility period, cells remaining on top of the filter were wiped off with cotton swabs and those cells that had traversed the membrane pores to the lower surface of the membrane were washed with chilled PBS, incubated for 5 min in 1:1 PBS/methanol (Merck) and 15 min in methanol before staining with 0.1% toluidine blue (Merck) in 2.5% sodium carbonate (Roth, Karlsruhe, Germany). Migrated cells were counted by light microscopy (Zeiss, Jena, Germany) in four separate high-power fields per filter.

Western blot

About 20 μ g total protein lysate from RA-stimulated and untreated FTC-133 and FTC-238 cells, and 200 μ g (for 2D western blot) of total protein from wild-type FTC-133, were separated on 10% polyacrylamide gels and blotted on a PVDF membrane (Amersham Biosciences). Blocking was performed in 5% non-fat milk

powder/1×TBST (Tris buffered saline/0.05% Tween20). After thrice washing with 1×TBST, membranes were incubated overnight with enolase C-19 polyclonal goat antiserum (1:10 000), MYC (1:1000) (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin (1:10 000, Sigma) in 1×TBST. Secondary anti-goat (1:50 000) and anti-mouse (1:20 000) antibodies were used (both Santa Cruz Biotechnology). Immunoreactive bands for ENO1, MBP-1, MYC, and β -actin were visualized using the ECL Detection Kit (Amersham Biosciences) and Kodak Image System 440cf (Eastman Kodak, Rochester, NY, USA).

Protein extraction and purification for 2D gel electrophoresis

FTC-133 cells were washed twice with PBS prior to the protein extraction. Proteins were extracted using two different lysis buffers, a) a standard 2D lysis buffer was used for silver staining (8 M Urea, 4% CHAPS, 1% dithiothreitol (DTT), 0.8% Pharmalyte) and b) a 2D extraction buffer for use with fluorescent-labeled dyes (7 M Urea, 2 M Thiourea, 4% CHAPS, 2% Pharmalyte, 2% DTT). Cells were incubated with lysis buffers for 30 min at room temperature (RT) and further purified with the trichloroacetic acid (TCA)-based 2D Clean Up Kit (Amersham Biosciences). Purified proteins were dissolved in rehydration solution for use in silver-stained gels (8 M Urea, 2% CHAPS, 0.5% Pharmalyte, 40 mM DTT) or fluorescent-labeled gels (8 M Urea, 1% CHAPS, 0.4% DTT, 0.4% Pharmalyte) and stored at -80°C until use.

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF, first dimension) was carried out on an IPGphor (Amersham Biosciences). Total protein (30 or 150 μg) was loaded onto nonlinear, 18 cm (pH 3–10) immobilized pH gradient (IPG) strips and rehydrated under low voltage conditions (30 V) for 12 h. For 2D fluorescence difference gel electrophoresis (DIGE), 30 μg total protein from RA-stimulated and untreated FTC-133 control were labeled with dyes Cy2 (blue, control) and Cy3 (green, RA-treated; Amersham Biosciences) respectively. Labeling reactions were incubated for 30 min on ice in the dark and stopped for 10 min with 10 mM lysine. Labeled proteins were combined at a 1:1 ratio, dissolved in rehydration solution, and IEF was performed at 8000 V for 9 h. IPG strips were equilibrated first in 10 ml equilibration solution (6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 30% glycerol) with 100 mg DTT (Roth) for 15 min and in 10 ml equilibration solution with 250 mg iodoacetamide (IAA, Sigma) for

another 15 min. Then IPG strips were arrested on a 12.5% polyacrylamide gel (37.5:1 Rothphorese Gel 30, 10% SDS, 1.5 M Tris-HCl (pH 8.8), 10% APS, TEMED) using 0.5% agarose.

Second dimensional electrophoresis was performed in an Ettan Dalt Unit (Amersham Biosciences) using SDS electrophoresis buffer (Tris-base 25 mM, glycine 192 mM, SDS 0.1%) at 2.5W/gel for 30 min and at 5W/gel for the next 5–6 h. For silver staining, gels were fixed for 1 h in 40% ethanol, 10% acetic acid, washed 3×20 min in 30% ethanol, sensitized in 0.02% sodium thiosulfate, washed 3×20 s in distilled water (H_2Odest). Staining was performed for 20 min with 0.25% silver nitrate, 0.00925% formaldehyde. Gels were washed 3×20 s in H_2Odest , developed in 3% sodium carbonate, 0.0185% formaldehyde, washed 20 s in H_2Odest , and the silver staining reaction was stopped after exactly 10 min in 5% acetic acid and 3×10 s washing in H_2Odest . For semi-quantitative protein spot evaluation, silver stained gels were scanned using a visual light scanner Hewlett Packard scanjet 7400C and analyzed with Phoretix 2D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Fluorescently labeled protein gels were analyzed using the Typhoon scanner and the DeCyder pro software (Amersham Biosciences). Up-regulated proteins isolated from untreated FTC-133 and labeled with Cy2 appeared as blue spots. Up-regulated proteins from RA-treated FTC-133 labeled with Cy3 appeared as green spots. White, overlapped protein spots indicate no difference between control and RA-treated cells.

Protein preparation for mass spectrometry

Spots of interest (molecular range between 30 and 60 kDa) were excised from gels, chopped into cubes ($\sim 1 \text{ mm}^3$), and dried in a vacuum concentrator. For MALDI-ToF MS analysis, spots were destained with 100 mM potassium ferricyanide/30 mM sodium thiosulfate, washed with water (HPLC grade, Roth), shrunk with acetonitrile (ACN), and dried in a vacuum concentrator. The dried gel pieces were rehydrated with 30–50 μl cold trypsin solution (15 $\mu\text{g}/\text{ml}$). Digestion was performed for 16–24 h at 37°C . Peptides were extracted twice with 50 ACN/5% trifluoroacetic acid (TFA) and dried. For Q-ToF-MS/MS analysis, protein spots were washed with water (HPLC grade, Roth), shrunk with acetonitrile, and dried. Proteins were reduced (100 mM DTT in 100 mM NH_4HCO_3), alkylated (55 mM IAA in 100 mM NH_4HCO_3), and digested with 12.5 ng/ μl trypsin dissolved in 5 mM CaCl_2 /50 mM NH_4HCO_3 . Peptides were extracted with ACN/5% formic acid and dried in a vacuum concentrator. Desalting was performed with ZipTip (Millipore Corporation, Billerica, MA, USA) containing C_{18}

reverse-phase medium. Peptides desalted for MALDI-ToF MS were dissolved in 50% ACN/0.1% TFA and for Q-ToF MS/MS in 70% methanol/1% formic acid.

Mass spectrometric analysis

MALDI-ToF MS identification of peptide mixtures was performed on a VoyagerDE Pro mass spectrometer (Applied Biosystems, Forester City, CA, USA). Dissolved peptides (50% ACN/0.1% TFA) were combined with matrix (α -cyano-4-hydroxy-trans-cinnamic acid) in a 1:1 ratio (vol:vol) and spotted onto the sample plate. Mass spectra were externally calibrated with Sequazyme Protein Digest Standards Kit (Applied Biosystems) containing des-Arg¹-bradykinin, angiotensin I, Glu¹-fibrinopeptide-B, neurotensin, β -galactosidase, and glycogen phosphorylase. Protein identification was performed using Mascot DataBase, where peptide mass tolerance was set to 100 ppm. One possible missed cleavage for trypsin digestion was allowed. Spectra were reconstructed with DataExplorer and analyzed with Mascot data bank.

MS/MS protein analyses were performed using the QSTAR Q-ToF mass spectrometer (Applied Biosystems) equipped with a nanospray source. External mass calibration in ToF mode was performed using synthetic sex determining octapeptide (Bachem AG, Bubendorf, Switzerland) combined with cesium iodide (Sigma) dissolved in a mixture of water/methanol/formic acid (49.5/49.5/1). For mass calibration in MS/MS mode, we used Glu-fibrinopeptide-B (Sigma) diluted in 30% methanol/1% formic acid. Protein identification, sequencing, and modification mapping were performed using BioAnalyst software and PepSea server (Applied Biosystems). Mass tolerance was set to 50 and 100 ppm and no missed cleavages for trypsin were allowed.

MTT and ATP assays

In 96-well plates, 5×10^3 FTC-133 and FTC-238 cells were seeded and cultured with DMEM-F12 medium without FCS. After 24 h, RA was added and cells were incubated for additional 24, 48, and 72 h. For MTT assay, cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 4 h at 37 °C. Colorimetric measurements were done with a Tecan Elisa Reader (Tecan, Grödig, Austria). For ATP assays, substrate (100 μ l) was added to each well (CellTiter-Glo Luminescent, Promega) and incubated with the cells on a shaker and on the bench top for 2 min and 10 min respectively. Luminescence was measured with a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). All experiments were done in triplicates.

Apoptosis assay

Control and RA-treated FTC-133 and FTC-238 cells were incubated with Annexin-V-Fluos staining kit (Roche). Annexin V is a Ca^{2+} -dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). Since both apoptotic and necrotic cells expose PS, simultaneous staining with propidium iodide was employed in order to discriminate between red necrotic and green labeled apoptotic cells. Evaluations were performed using Axioplan 2 fluorescent microscope and Axiovision software (Zeiss). Additionally expression of annexin V was evaluated by DIGE and mass spectrometric analysis.

Results

RA-induced decreased proliferation and motility of follicular thyroid carcinoma cell lines, but not apoptosis

In order to investigate whether different thyroid carcinoma cell lines respond to RA treatment with altered proliferation, follicular thyroid carcinoma cells FTC-133 and FTC-238, and 8505C, C-643, Hth74, SW1736 representing undifferentiated thyroid carcinoma, were subjected to MTT assay. As shown in Fig. 1A, out of six cell lines analyzed, only FTC-133 and FTC-238 responded to RA treatment with significantly reduced proliferation rates. Other cell lines revealed only slightly changed proliferation profiles; however, the differences were not significant. Our further investigations were focused on differentiated thyroid cell lines FTC-133 and FTC-238 only. Previous experiments demonstrated decreased metabolic activity of both cell lines. To further verify whether RA actions are connected with reduced vitality and energy production, we measured intracellular ATP levels in these cell lines. Figure 1B demonstrates ATP assay performed by the evaluation of luminescence after 24, 48, and 72 h of RA treatment. Similar to MTT assay, also in this case, investigated cell lines responded to RA with significantly decreased vitality, especially visible after 72 h. To examine the effects of RA on invasiveness of FTC-133 and FTC-238, the cells pretreated with RA for 72 h were subjected to motility assays. As demonstrated in Fig. 2A, migrated control and RA-treated cells were stained and photographed using light microscopy (lower panel). Decreased number of migrated cells is visible on both RA-pretreated filters as compared with controls. Also cell counting revealed significantly reduced motility rates for both cell lines after RA pretreatment (upper panel). In order to examine whether observed RA effects could be due to apoptosis induction, control and RA-treated cells were stained with the apoptosis marker

Figure 1 Proliferation and vitality of thyroid carcinoma cell lines upon RA treatment. (A) Differentiated (FTC-133, FTC-238) and undifferentiated (8505C, C-643, HTH74, SW1736) thyroid carcinoma cell lines were subjected to MTT assay. Significant response to RA treatment is visible for FTC-133 and FTC-238 cell lines only. (B) Vitality assay performed on FTC-133 and FTC-238 cells. Both cell lines responded with significantly decreased intracellular ATP levels.

Figure 2 Invasiveness and apoptosis of thyroid carcinoma cell lines upon RA treatment. (A) Control and RA-pretreated FTC-133 and FTC-238 cells were subjected to motility assay. Filters with migrated cells were stained with 0.1% toluidine blue and photographed under light microscope. As demonstrated in lower panel, RA pretreatment led to visible reduction of migrated cells. Cell counting presented in upper panel revealed significantly decreased number of migrated cells upon RA treatment. (B) Apoptosis assay revealed the presence of annexin V (green staining) both in control and RA-treated cells; however, no difference is visible. Red staining with propidium iodide indicate the presence of necrotic cells.

annexin V (Fig. 2B). However, investigations performed by employing fluorescent microscopy revealed no noticeable differences in annexin V and propidium iodide staining. Both control and RA-treated cells revealed the presence of apoptotic and necrotic cells. Additionally,

our observations were further supported by DIGE and mass spectrometric analysis. As shown in Fig. 3A, identification of annexin V revealed no significant differences between its expression in control and RA-treated cells, as it appeared as a white protein spot.

Two-dimensional analyses revealed RA involvement in alteration of FTC-133 proteome

To clarify previously observed effects of RA on differentiated thyroid carcinoma cell lines and possible molecular mechanisms, control and RA-treated FTC-133 cells were subjected to DIGE (Fig. 3A). Automatic spot detection using DeCyder software was followed by filtering of spots in each gel. We found that out of 669 protein spots detected, RA treatment led to up-regulation of 1 and down-regulation of 8, while 660 protein spots remained unchanged. Mass spectrometric analysis revealed that out of eight protein spots down-regulated, two of them, ENO1 and GAPDH, are related to ATP metabolism and energy production, and were previously reported to be up-regulated in thyroid oncocy-tomas (Baris *et al.* 2004). We found that both proteins exist as isoforms with differential pI values designated in case of ENO1 as A1, A2, and A3. The smaller 37 kDa MBP-1 protein is labeled as B1. In fluorescent staining experiments, MBP-1 production and ENO1 spots A2 and A3 showed no significant differences between RA-treated and control samples. However, RA caused a down-regulation of the ENO1 spot corresponding to A1, which is demonstrated by its increased blue fluorescence in control sample. We could also demonstrate that RA led to decrease in GAPDH production (spot G1). It is worth to notice that besides ENO1, also GAPDH belongs to the glycolytic pathway.

Figure 3 (A) Two-dimensional fluorescence difference gel electrophoresis (DIGE) with Cy2-labeled and Cy3-labeled protein extracts derived from untreated and RA-treated FTC-133, respectively. Proteins up-regulated in RA-treated FTC-133 appear in green color, proteins with a stronger expression in the untreated control appear as blue, and equal amounts of overlaying proteins appear in white color. Experiments were done in triplicates and positions of ENO1 (A1, A2, A3, and B1) and GAPDH (G1) spots are labeled with arrows. The number of protein spots regulated upon RA treatment is presented on the diagram. Annexin V appears as a white spot and indicates no induction of apoptosis upon RA treatment. (B) Two-dimensional gel electrophoresis of RA-treated FTC-133. Protein spots were visualized by silver staining. Three different protein spots corresponding to ENO1 protein with different pIs (A1, A2, A3, and B1) were identified (see arrows in the enlargement) and further analyzed by mass spectrometry. Two-dimensional western blot detection verified the identity of the silver-stained protein spots A1–A3 and B1. Two-dimensional western blots were performed with 200 µg total cell lysate obtained from FTC-133. Experiments were repeated at least three times.

RA-mediated dephosphorylation of ENO1 protein

DIGE analysis revealed the presence of three distinct immunoreactive ENO1 proteins (A1, A2, and A3), which differed in their pI values. Additionally, RA treatment led to decreased expression of ENO1-A1 protein. To answer the question whether the presence of differential ENO1 isoforms and decreased expression of ENO1-A1 protein are associated with post-translational modification, control and RA-treated silver-stained gels were analyzed by mass spectrometry. Excised *ENO1*-related protein spots were identified using MALDI-ToF and verified by MS/MS sequencing. Employing a specific antiserum used to detect ENO1 and MBP-1, ENO1-A1–3 and MBP-1-B1 protein spots were confirmed by 2D-western blot analysis (Fig. 3B). MS/MS analysis and phosphorylation mapping demonstrated the RA-mediated dephosphorylation of ENO1-A1 protein spot. ENO-A1 was first identified in the ToF MS spectrum (Fig. 4A). The ESI-MS/MS spectrum (Fig. 4B) corresponded to the ENO1-A1 peptide Yp⁴⁰⁷NQLLR after trypsin digest and was obtained from untreated FTC-133 cells, thus, indicating a phospho-Tyr⁴⁰⁷ in the ENO1-A1 peptide sequence. When FTC-133 were treated with RA, dephosphorylation of the ENO1-A1 tyrosine residue 407 was detected as demonstrated by the appearance of an immonium ion at m/z 136·0791 typical for unphosphorylated tyrosine (Fig. 4C). Other mass differences visible by comparison of both MS/MS spectra did not match any known modifications.

RA led to simultaneous down-regulation of ENO1, MYC, and MBP-1

Previous reports demonstrated that both ENO1 and MBP-1 proteins could act as transcriptional repressors of MYC (Feo *et al.* 2000), and through the ability to regulate a significant number of genes, MYC itself is a key regulator of cell behavior. Additionally, many of MYC targeting pathways are deregulated in cancer cells and contribute to its enhanced expression (Vervoots *et al.* 2006). In order to clarify the relationship between RA-mediated down-regulation of *ENO1* gene products and reduced proliferation rates of FTC-133 and FTC-238 cells, we performed western blotting. Incubation of total protein extracts obtained from both cell lines with specific ENO1 and MYC antibodies revealed that RA led

to simultaneous down-regulation of all examined proteins, especially visible after 72 h of RA treatment (Fig. 5A and B).

Similar effects could be demonstrated after specific knock-down of ENO1. We found that employing siRNA targeting both ENO1 gene products led to decreased MYC levels accompanied by reduced proliferation rates of FTC-133 and FTC-238 cell lines (Fig. 6A and B).

Discussion

The present study identified ENO1 and MBP-1 as a novel target molecules of RA action and demonstrated a direct involvement of ENO1 in the invasiveness of human thyroid carcinoma cells. Furthermore, we demonstrated for the first time three different ENO1 (A1–A3) isoforms and showed the selective dephosphorylation of ENO1-A1 residue Tyr⁴⁰⁷ as a result of RA treatment in FTC-133 cells. RA caused a decrease in both ENO1 and MBP-1 total protein and this coincided with decreased MYC levels and reduced proliferation and motility of follicular thyroid carcinoma cell lines. ENO1 and MBP-1 are transcribed from a single transcript and share a common C-terminal MYC promotor-binding domain, but only ENO1 contains an enzymatically active N-terminal domain and, thus, displays enolase activity (Feo *et al.* 2000). The up-regulation of glycolytic enzymes, including ENO1 and GAPDH, appears to be a common strategy in carcinoma of diverse origin, including thyroid oncocytoma (Baris *et al.* 2004), suggesting a direct involvement of both enzymes in tumor growth. The impaired energy metabolism observed in FTC-133 and FTC-238 upon RA treatment likely reflected a reduction in GAPDH and α -enolase activity and impaired glycolytic activity. A detailed comparative analysis of the effect of RA treatment on different GAPDH and ENO1 isoforms revealed a selective down-regulation of GAPDH-G1 and ENO1-A1 as determined by fluorescent DIGE, implicating both proteins as executioners for the RA-induced suppressive metabolic effects in FTC-133. We found that RA-mediated decrease in the metabolic activity and proliferation of follicular thyroid carcinoma cell lines likely involved additional mechanisms. One such cellular event elicited by RA in FTC-133 and FTC-238 was the down-regulation of the common MYC promotor-binding domain present in both ENO1 and MBP-1 (Subramanian & Miller 2000). This

Figure 4 Mass spectrometric analyses of protein spot ENO1-A1. (A) ToF-MS spectrum of silver-stained A1-spot identified as ENO1 protein. (B) MS/MS spectrum of peptide pYNQLLR of ENO1-A1 obtained from untreated FTC-133. The characteristic immonium ion of phosphorylated tyrosine at m/z 216·0322 is present. (C) MS/MS spectrum of peptide YNQLLR in ENO1-A1 obtained from RA-stimulated FTC-133. The disappearance of the immonium ion at m/z 216·0322 and intensification of the immonium ion at m/z 136·0791 is typical for unmodified tyrosine. Analyses were done in triplicates.

Figure 5 Western blot detection of ENO1 and MYC proteins in (A) FTC-133 and (B) FTC-238 before and after RA treatment performed with 20 μ g total cell lysate. ENO1 (recognizing both 48 and 37 kDa proteins) and MYC antisera were used. β -actin served as control for equal protein loading. RA treatment resulted in the down-regulation of MYC and both *ENO1* gene products.

down-regulation of ENO1 and MBP-1 coincided with a decrease of MYC suggesting an oncogene suppressive effect by which RA can impair thyroid carcinoma growth. The same effects were observed after employing specific siRNA targeting ENO1 and MBP-1. Silencing of the *MYC* promoter-binding domain in both proteins also led to MYC down-regulation and reduced proliferation rates of FTC-133 and FTC-238 cell lines. However, MBP-1 has been largely described to act as a repressor of the MYC, but it seems that in follicular thyroid carcinoma, the bifunctional role of enolase is diminished and ENO1 possess the enzymatic activity only. Although there is no direct evidence of the regulation of α -enolase function by MYC, it is possible that α -enolase gene could be affected through its two perfect CACGTG MYC-MAX binding motifs. Previous studies demonstrated that up-regulation of glycolytic enzymes as well as glycolysis occurred as a consequence of MYC overexpression. It is suggested that increased energy production was due to the interaction of MYC with two imperfect CACGTG motifs in carbohydrate response element of pyruvate kinase. The promoter of α -enolase gene also contains CACGTC motifs and may act as MYC binding partner (Giallongo *et al.* 1990, Towle 1995).

Figure 6 (A) FTC-133 and FTC-238 cells were treated with *ENO1*-derived siRNA (targeting common MYC binding domain) or with a scrambled, non-silencing siRNA control used at 200 nM each. Western blot confirmed simultaneous knock-down of ENO1, MBP-1 and MYC. β -actin was used to ensure equal protein loading. (B) FTC-133 and FTC-238 treated with 200 nM ENO1 targeting siRNA displayed 44 and 42% reductions in growth respectively (* $P < 0.05$). Graphs summarize the results of three independent experiments.

The RA-induced down-regulation of MYC and resulting decrease in tumor cell proliferation is a common phenomenon described previously in lung cancer and myeloid cell lines (Kalemkerian *et al.* 1994, Akie *et al.* 2000, Dimberg *et al.* 2002, Vervoorts *et al.* 2006). However, information on the potential role of ENO1 and MBP-1 in these carcinoma entities is lacking.

The effect of RA on ENO1 was not restricted only to the down-regulation of the ENO1 protein, but also involved the RA-induced dephosphorylation of phospho-Tyr⁴⁰⁷ residue in ENO1-A1 as demonstrated by mass spectrometry. Single dephosphorylation events have been shown to affect protein function and reduce cell migration and angiogenesis (Moriyama *et al.* 1996, Urbich *et al.* 2002) or increased apoptosis (Matsuoka *et al.* 2003). However, as demonstrated by our experiments, RA-mediated dephosphorylation of ENO1 was not associated with induction of apoptosis. RA can act post-transcriptionally to regulate the half-life

of cell cycle proteins (Sueoka *et al.* 1999) and has been known to modify the activity of proteins by affecting their phosphorylation status as demonstrated for the RARG (Gianni *et al.* 2002). Although the functional consequences on the enzymatic or MYC promotor-binding activity of this single dephosphorylation event in ENO1-A1 remain elusive at present, our data suggested that dephosphorylated ENO1 was unable to compensate for the metabolic shortfall caused by RA.

RA therapy administrated for differentiated thyroid carcinoma is tolerated well with few side effects. We found that 1 μ M (300 ng/l) RA used in our study led to reduced invasiveness of two follicular carcinoma cell lines, accompanied by a decrease in ENO1 and MYC production. This could be partially explained by reduced glucose metabolism and tumor size, previously observed as a favorable response to RA therapy (Simon *et al.* 1998). Basal levels of natural RA (all trans) in human serum are in the range of 2–10 nM (Hong & Itri 1994). Administration of synthetic or therapeutic levels of RA (e.g. 13 cis RA) frequently used in cancer such as leukemia (Huang *et al.* 1988, Castaigne *et al.* 1990) or dermatology therapy (Verma 1987) easily reach the low micromolar levels (0.5–5 μ M) depending on time, duration, and dose of treatment. In addition, various retinoid metabolites are formed in the low to high nanomolar range. Therefore, as demonstrated before (Fex *et al.* 1996, Chanson *et al.* 2008), 1 μ M concentrations of RA in human serum are easy to reach in therapeutic protocols.

In summary, we identified a 48 kDa translation product of ENO1 gene, as a novel target and executioner of the action of RA reducing the invasiveness of the human follicular thyroid carcinoma cell lines FTC-133 and FTC-238. RA-induced reduction of both the key glycolytic enzyme and proliferation-promoting MYC may serve as an additional predictive parameter of successful redifferentiation and anti-tumor therapy.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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