

Original Paper

Functional Consequences of Intracellular Proline Levels Manipulation Affecting PRODH/POX-Dependent Pro-Apoptotic Pathways in a Novel *in Vitro* Cell Culture Model

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Key Words

Apoptosis • Collagen biosynthesis • MCF-7 breast cancer cells • Proline • Proline dehydrogenase/proline oxidase.

Abstract

Background/Aims: The effect of impaired intracellular proline availability for proline dehydrogenase/proline oxidase (PRODH/POX)-dependent apoptosis was studied. **Methods:** We generated a constitutively knocked-down PRODH/POX MCF-7 breast cancer cell line (MCF-7^{shPRODH/POX}) as a model to analyze the functional consequences of impaired intracellular proline levels. We have used inhibitor of proline utilization in collagen biosynthesis, 2-metoxystriol (MOE), inhibitor of proline oxidase that generate proline, rapamycin (Rap) and glycyl-proline (GlyPro), substrate for proline oxidase. Collagen and DNA biosynthesis were evaluated by radiometric assays. Cell viability was determined using Nucleo-Counter NC-3000. The activity of proline oxidase was determined by colorimetric assay. Expression of proteins was assessed by Western blot and immunofluorescence bioimaging. Concentration of proline was analyzed by liquid chromatography with mass spectrometry. **Results:** PRODH/POX knockdown decreased DNA and collagen biosynthesis, whereas increased proline oxidase activity and intracellular proline level in MCF-7^{shPRODH/POX} cells. All studied compounds decreased cell viability in MCF-7 and MCF-7^{shPRODH/POX} cells. DNA biosynthesis was similarly inhibited by Rap and MOE in both cell lines, but GlyPro inhibited the process only in MCF-7^{shPRODH/POX} and MOE+GlyPro only in MCF-7 cells. All the compounds inhibited collagen biosynthesis, increased proline oxidase activity and cytoplasmic proline level in MCF-7^{shPRODH/POX} cells and contributed to the induction of pro-survival mode only in MCF-7^{shPRODH/POX} cells. In contrast, all studied compounds upregulated expression of pro-apoptotic protein only in MCF-7 cells. **Conclusion:** PRODH/POX was confirmed as a driver of apoptosis and proved the eligibility of MCF-7^{shPRODH/POX} cell line as a highly effective model to elucidate the different mechanisms underlying proline utilization or generation in PRODH/POX-dependent pro-apoptotic pathways.

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Introduction

An increased proline biosynthesis and accumulation in neoplastic cells has been shown earlier [1, 2], however the mechanism of this process and its metabolic consequences on the cell functions are not fully understood. Important source of proline are extracellular collagen degradation products undergoing internalization and further degradation to free amino acids in lysosomes or cytoplasm [3]. Essential group of collagen degradation products are iminodipeptides (e.g. glycyl-proline) that are degraded to amino acids in cytoplasm by specific iminodipeptidase, prolidase [E.C.3.4.13.9]. Cytoplasmic prolidase activity is important in regulation of proline accumulation and proline-dependent metabolic responses in the cell [4, 5].

Proline play an important role in inhibition of degradation of hypoxia-inducible factor 1-alpha (HIF-1 α) a transcription factor that activates several pro-angiogenic and neoplastic genes such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF) or tumor necrosis factor (TNF) [4, 6, 7]. The mechanisms that utilize proline are collagen biosynthesis and proline conversion into pyrroline-5-carboxylic acid (P5C) [4, 8-10]. The second process is catalyzed by mitochondrial proline dehydrogenase/proline oxidase (PRODH/POX). It has been proposed that cytoplasmic proline that enters mitochondria is a sensor of cellular energy status [11]. This usually happens under low glucose stress, when PRODH/POX activation induces ATP production for energy supply and cell survival [12-14]. On the other hand, conversion of mitochondrial proline into P5C by PRODH/POX generates superoxide anion that may contribute to ROS-dependent intrinsic and extrinsic apoptotic pathways [11, 12, 15-18]. In the presence of proline, overexpression of PRODH/POX causes cytochrome c release from mitochondria to cytosol and activation of caspase-9 and caspase-3 [16]. Moreover, it was showed that up-regulation of PRODH/POX contributes to activation also caspase-8 in the extrinsic apoptotic pathway mainly because of its ability to stimulate expression of TNF-related apoptosis inducing ligand (TRAIL) and death receptor 5 (DR5) [11, 16]. Therefore, PRODH/POX may play dual role, but the mechanism that switches PRODH/POX from tumor growth inhibiting to growth stimulating factor is unknown. One of the major limiting factors in this research aspect was lack of an *in vitro* model that would facilitate the intracellular proline manipulation and could be helpful in showing the mechanistic aspects of PRODH/POX roles as tumor inhibiting or stimulating factors.

In view of an important role of availability of proline in the regulation of apoptosis/survival, our goal was to generate a constitutively stable PRODH/POX knocked-down cell line (MCF-7^{shPRODH/POX}) to inhibit proline utilization into P5C. Accordingly, PRODH/POX knock-down contributed to decrease in collagen biosynthesis and increase in prolidase activity in MCF-7 cells. We further used different treatments and methods to increase the free pool of cytoplasmic level of proline. Finally, we measured cell viability, collagen biosynthesis, prolidase activity, DNA biosynthesis and tested the induction of apoptosis by its markers – all to establish the functionality and eligibility of MCF-7^{shPRODH/POX} cell line as an *in vitro* model to study proline utilization or stimulation in PRODH/POX-dependent pro-apoptotic pathways.

Materials and Methods

Design of experiment

To determine the role of proline metabolism in apoptosis in MCF-7^{shPRODH/POX} and wild-type MCF-7 control breast cancer cells, we designed several experimental conditions that limit utilization of proline for HIF-1 α transcriptional activity, collagen biosynthesis and prevent proline generation by prolidase activity. 2-metoxystriadiol (MOE) was used as an inhibitor of HIF-1 α and collagen biosynthesis while rapamycin (Rap) to inhibit mTOR and prolidase activity. In order to increase cytoplasmic level of proline, glycyl-proline (GlyPro) was used as a substrate for prolidase.

Cell lines and culture

Brest cancer cell line MCF-7 was obtained from ATCC (HTB-22, ATCC, Manassas, VA, USA). The MCF-7 and MCF-7^{shPROD/POX} cells were maintained in DMEM and 5% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 50 IU/ml penicillin (Gibco), and 50 µg/ml streptomycin (Gibco) at 37 °C in a humidified atmosphere in the presence of 5% CO₂. MCF-7 and MCF-7^{shPROD/POX} cells were cultured in glutamine-free DMEM (Gibco) and treated for 24 h with rapamycin (Rap, 22, 85 µg/ml), 2-metoxystriadiol (MOE, 22, 65 µg/ml), glycyl-proline (GlyPro, 17, 22 µg/ml) or the combination of MOE and GlyPro (22, 65 µg/ml and 17, 22 µg/ml, respectively).

shRNA-based PRODH knock-down

To knock-down expression of *PRODH*, 3 pairs of short herpins complement single-stranded DNA oligonucleotides were designed using AsiDesigner online tool (<http://sysbio.kribb.re.kr:8080/AsiDesigner/menuDesigner.jsf>) (Table 1). HPLC purified oligonucleotides were ordered from Genomed (Warsaw, Poland). Annealed double-stranded oligonucleotides were cloned into pSuper.puro expression vector (Oligo-Engine, Seattle, WA, USA) via BglII and HindIII restriction sites using T4 DNA ligase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Positive colonies of heat-shock transformed NEB 10-beta competent *E. coli* bacteria (Bio Labs, Ipswich, United Kingdom), were verified by PCR, restriction enzyme BglII and HindIII digestion (Thermo Fisher Scientific,) and gel electrophoresis. Amplified plasmid DNA was extracted and purified by NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany). Oligonucleotide sequences (shRNA oligos) of *PRODH* are shown in Table 1.

MCF-7 cells were cultured in 6-well plate (1x10⁵ cells per well in 2 ml culture media). Twenty-four hours later, cells were transfected with three different shRNA expressing vectors or shRNA non-targeting control using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific) in OptiMem media (Gibco) according to the manufacturer's protocol. *PRODH* shRNA-expressing cells were selected using puromycin antibiotic (Invitrogen) with previously optimized concentration of 1 µg/ml. The MCF-7^{shPROD/POX} stable transfected cells were generated from selecting the single puromycin-resistant „ best clone (highest knocked down *PRODH/POX*) “ and was further characterized and used in this study.

Western blot

Cell lysates of *PRODH/POX* shRNA stably transfected MCF-7 cell clones and MCF-7 cell controls were harvested and subjected to SDS-PAGE in 10% polyacrylamide gel electrophoresis [1h, 125 V, room temperature (RT)]. The protein was transferred to 0.2 µm pore-sized nitrocellulose (wet transfer, 1 h, 100 mA, RT).

Table 1. Oligonucleotide sequences (shRNA oligos) of *PRODH* used in the study are in bold (tv - transcript variant)

Name	Sequences (5'- 3')	Target
PRODH	F: gatccccCTAGGACAGAGGCTATTCaagagaGTTGAATAGCCTCTGTCTAGtttta	PRODH tv. 1 502-
	R: agcttaaaaaCTAGGACAGAGGCTATTCaagagaGTTGAATAGCCTCTGTCTAGggg	522
shRNA 2	F: gatccccGCATGTGTGACCAGATCAGCTtcaagagaAGCTGATCTGGTCACACATGctttt	PRODH tv. 2 87-107
	R: agcttaaaaaGCATGTGTGACCAGATCAGCTtctcttgaagAGCTGATCTGGTCACACATGCggg	PRODH tv. 1 1790-1810
shRNA 1	F: gatccccGTGTACAAGTACGTGCCCTATtcaagagaATAGGGCAGCTACTTGTACACtttta	PRODH tv. 2 1374-1394
	R: agcttaaaaaGTGTACAAGTACGTGCCCTATtctcttgaagTAGGGCAGCTACTTGTACACggg	PRODH tv. 1 1837-1857
shRNA 3	F: gatccccGTGTACAAGTACGTGCCCTATtcaagagaATAGGGCAGCTACTTGTACACtttta	PRODH tv. 2 1422-1442
	R: agcttaaaaaGTGTACAAGTACGTGCCCTATtctcttgaagTAGGGCAGCTACTTGTACACggg	

After the transfer membranes were blocked with 5% non-fat dry milk (BioRad, Hercules, Kalifornia, USA) in TBS-T (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.05% Tween 20, pH 7.4) and incubated with following antibodies: goat monoclonal anti-PRODH/POX [EB11136, Everest Biotech, Upper Heyford, UK, 1:1000], purified mouse anti-human p53 [554169, Becton, Dickinson and Company (B&D), New Jersey, USA, 1:1000], purified mouse anti-human HIF-1 α [610959, B&D, 1:1000], rabbit anti-caspase-3 [#9665, Cell Signaling (CS), Danvers, USA, 1:1000], rabbit anti-cleaved-caspase-3 [#9664, CS, 1:1000], rabbit anti-caspase-9 [#9508, CS, 1:1000], mouse anti-cleaved-caspase-9 [#7237, CS, 1:1000], rabbit anti-PARP [#9542, CS, 1:1000], rabbit anti-cleaved-PARP [#5625, CS, 1:1000], rabbit anti-Atg7 [#8558, CS, 1:1000], rabbit anti-Beclin-1 [#3495, CS, 1:1000], mouse anti-NF- κ B [610868, B&D, 1:1000] and monoclonal anti- β -actin antibody produced in mouse [A2228, Sigma-Aldrich, Saint Louis, Missouri, USA] in blocking buffer. Then membranes were washed in TBS with 0.05% Tween (TBST) 3 x 15 min and incubated with anti-goat, anti-rabbit or anti-mouse HRP-linked secondary antibody at concentration 1:7500 (Sigma-Aldrich) for 60 min at RT with gentle agitation. After washing in TBS-T (5 x 5 min) membranes were incubated with Amersham ECL Western Blotting Detection Reagent, (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Pictures were taken using BioSpectrum Imaging System UVP (Ultra-Violet Products Ltd, Cambridge, UK). Densitometry of western blot bands was analyzed using ImageJ software [19].

Cell viability assay

Cytotoxicity of tested substances was determined using Nucleo Counter NC-3000 (ChemoMetec, Copenhagen, Denmark). Prior the experiment MCF-7 and MCF-7^{shPRODH/POX} cells were cultured in six-well plates at 1×10^5 cells/well with 2 ml of growth medium. After 24 h incubation of the cells with Rap, MOE, GlyPro and MOE+GlyPro, glutamine-free medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). Then the cells were harvested, washed and stained with VitaBright-48 (VB-48) (ChemoMetec), acridine orange (AO) (ChemoMetec), propidium iodide (PI) (ChemoMetec) and analyzed using NC-3000 cell counter. Viability of the cells treated with above compounds was calculated as a percent of control.

Proliferation assay

Proliferation of MCF-7 and MCF-7^{shPRODH/POX} cells was measured by [methyl-³H]thymidine (Hartman Analytic GmbH, Braunschweig, Germany) incorporation into DNA. Prior the experiment MCF-7 and MCF-7^{shPRODH/POX} cells were cultured in 24-well plate at 1×10^4 cells/well with 1 ml of growth medium. After 48 h the cells were incubated in glutamine-free DMEM (Gibco) with Rap, MOE, GlyPro and MOE+GlyPro for 24 h and next with 0.5 μ Ci/ml of [methyl-³H]thymidine for 4 h. PBS-rinsed cells were solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS and 5 ml of scintillation fluid Ultima Gold XR (Perkin Elmer, Waltham, USA). Incorporation of the tracer into DNA was measured by Liquid Scintillation Analyzer Tri-Carb 2810 TR (Perkin Elmer) and calculated using Quanto Smart TM software (Perkin Elmer).

Collagen biosynthesis

MCF-7 and MCF-7^{shPRODH/POX} cells were cultured in 24-well plate at 1×10^4 cells/well with 1 ml of growth medium. After 48 h, the cells were incubated in glutamine-free DMEM (Gibco) with Rap, MOE, GlyPro or MOE+GlyPro and 5 μ Ci/ml of 5[³H]-proline (Hartman Analytic GmbH, Braunschweig, Germany) for 24 h. Incorporation of tracer-labeled proline into collagen was measured in collagenase-digested (Clostridium histolyticum collagenase, Sigma-Aldrich) protein homogenate by Liquid Scintillation Analyzer Tri-Carb 2810 TR (Perkin Elmer) according to the method of Peterkofsky et al [20]. Results are shown as combined values of cellular and medium fractions.

Determination of prolidase activity

MCF-7 and MCF-7^{shPRODH/POX} cells were incubated 24 h in glutamine-free DMEM media (Gibco) with Rap, MOE, GlyPro or MOE+GlyPro. Protein concentration was measured by the method of Lowry et al [21]. The activity of prolidase was determined according to the method of Myara et al [22]. Amount of released proline from synthetic substrate (GlyPro) by prolidase from cell homogenate was measured by using colorimetric method. Enzyme activity was reported as nanomoles of proline released from GlyPro, during 1 min per milligram of supernatant protein of cell homogenate.

Immunofluorescence staining

Cells grown on a coverslip were fixed with 3.7% paraformaldehyde and permeabilized with 0.01% TritonX100. After blocking with 3% FBS (30 min, RT), cells were incubated (1 h, RT) with following antibodies anti: purified mouse anti-human HIF-1 α [610959, B&D, 1:1000], rabbit anti-caspase-3 [#9665, CS, 1:1000], rabbit anti-cleaved-caspase-3 [#9664, CS, 1:1000], rabbit anti-caspase-9 [#9508, CS, 1:1000], mouse anti-cleaved-caspase-9 [#7237, CS, 1:1000], rabbit anti-PARP [#9542, CS, 1:1000], rabbit anti-cleaved-PARP [#5625, CS, 1:1000], rabbit anti-Becn1 [#3495, CS, 1:1000]. Next, cells were washed with PBS (3 x 3 min) and incubated with FITC goat anti-mouse IgG [554001, B&D, 1:1000] and anti-rabbit IgG [554020, B&D, 1:1000] for 1h in the dark at RT. Cells were washed with PBS (3 x 3 min) and incubated with Hoechst 33342 for 15 min. Samples were visualized with confocal laser scanning microscope BD Pathway 855 Bioimager (B&D) using AttoVision software (B&D).

Concentration of proline

Samples were analyzed by an HPLC system (1260 Infinity series, Agilent Technologies, Waldbronn, Germany) consisting of a degasser, binary pump, and thermostated autosampler maintained at 4°C connected to an Agilent Technologies QTOF (6530) mass spectrometry detector. Electrospray ionization (ESI) was used as an ion source in positive ionisation mode. Samples (2 μ L) were injected onto a HILIC column (Luna HILIC, 100x2.0mm; 3 μ m; Phenomenex) thermostated at 40 °C. The system was operated in positive and negative mode at flow rate 1 mL/min with solvent A - water with 10mM ammonium formate (70221, Sigma-Aldrich) and solvent B - acetonitrile/ water (9:1, v:v) with 10mM ammonium formate. Mobile phase was 100% B during 1.5min in isocratic mode. The gradient started in 1.5 min from 100% B to 70% B in 5.5min, then 40% B in 6.0min, maintained 40% B during 1 min and returned to starting conditions in 0.5 min, keeping the re-equilibration until 10 min. The detector operated in full scan mode from 50 to 1000 m/z with a scan rate of 1 scan per second. Accurate mass measurements were obtained by online mass correction to reference masses delivered continuously during analyses. Reference masses at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis (1H,1H,3Htetrafluoroproxy) phosphazine or HP-921]. The capillary voltage was set to 3000V, the gas temperature was 330°C, the nebulizer gas flow rate was 10, 5 L/min. MS TOF parameters were as follows: fragmentor was set to 140V, skimmer 65 V.

Quantitative analysis was performed monitoring monoisotopic ions of proline and the internal standard (Proline-d3) (791261, Sigma-Aldrich). Ions from isotopic distribution pattern were used as qualifiers. Calibration curve of proline was registered in concentration range: 6.32-101.12 [μ M]. Samples were analysed in triplicates and average value of signal was used for calculations. Calculated concentrations were normalized first to internal standard to correct ion suppression and then to the amount of protein in a sample.

Statistical analysis

Results are presented as mean values of six independent experiments \pm s.e.m. The statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison test with 95% confidence interval in GraphPad PRISM v. 5 (GraphPad Software, Inc., California, USA). Results were considered significant at * P<0.01, ** P<0, 001.

Results

Design and efficacy of shRNA-based PRODH/POX knock-down in MCF-7 cells

Proline dehydrogenase/proline oxidase (PRODH/POX) is encoded by the *PRODH* gene located on chromosome 22q11.21 and possesses two transcription variants. Designed shRNA constructs targeted exons 3 and 2 (PRODH shRNA1), exons 14 and 13 (PRODH shRNA2) and exons 15 and 14 (PRODH shRNA3) of transcription variant 1 and 2, respectively. Western blot protein analysis showed that the PRODH shRNA 2 construct was the most effective one in silencing PRODH/POX, decreasing its expression over 40% of control MCF-7 cells (Fig. 1). This cell clone was named MCF-7^{shPRODH/POX} and selected for further *in vitro* analysis.

Fig. 1. Expression of PRODH/POX and β -actin in MCF-7 and MCF-7^{shPRODH/POX} cells. Transfection of the MCF-7 cells with different PRODH/POX shRNA constructs (clone 1-3) were done. Fibroblasts were used as a negative control and colorectal cancer cells DLD-1, as positive control for the expression of PRODH/POX.

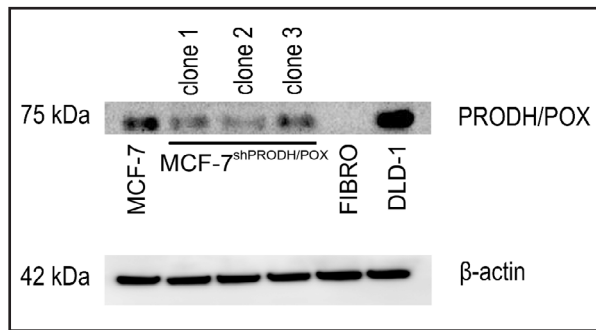
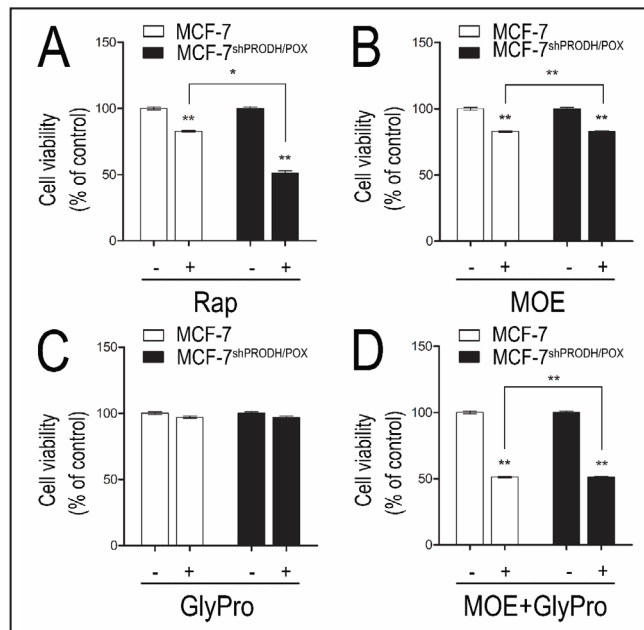


Fig. 2. Cell viability of MCF-7^{shPRODH/POX} versus MCF-7 cells, and the effect of A. rapamycin (Rap), B. 2-metoxiestradiol (MOE), C. glycyl-proline (GlyPro) and D. the combination of 2-metoxiestradiol and glycyl-proline (MOE+GlyPro) on the process. The mean values \pm SEM from 3 experiments done in duplicates are presented. Asterisks indicate differences between studied cells compared to control untreated MCF-7 cells as well as between treated MCF-7 and MCF-7^{shPRODH/POX} cells at * $P < 0.01$; ** $P < 0.001$.



Effect of inhibition of proline utilization on cell viability, DNA and collagen synthesis and prolidase activity in MCF-7^{shPRODH/POX} cells

There was no significant difference in cell viability between MCF-7^{shPRODH/POX} and MCF-7 control cells (Fig. 2). Rap, MOE or combination of MOE+GlyPro decreased viability of MCF-7 and MCF-7^{shPRODH/POX} cells compared to untreated control MCF-7 cells (Fig. 2A, B, D), while GlyPro alone had no effect on cell viability (Fig. 2C). We also confirmed cell viability by MTT Cell Proliferation Assay (data not shown).

Basal DNA biosynthesis was decreased in MCF-7^{shPRODH/POX} compared to MCF-7 cells (Fig. 3). DNA biosynthesis was further decreased by Rap, MOE and combination of MOE+GlyPro stimulation in both cell lines (Fig. 3A, B, D), but GlyPro did not affect the process in MCF-7 cells and decreased it only in MCF-7^{shPRODH/POX} cells (Fig. 3C). The data is supported by the growth cells curve and viability (data not shown).

PRODH/POX silencing significantly decreased collagen biosynthesis in MCF-7 cells (Fig. 4). Rap, MOE or combination of MOE+GlyPro decreased collagen biosynthesis in both cell lines (Fig 4A, B, D). However, GlyPro alone increased collagen biosynthesis in MCF-7 cells, while in MCF-7^{shPRODH/POX} cells it decreased the process compared to control cells (Fig. 4C).

Prolidase activity was increased in both stimulated and non-stimulated MCF-7^{shPRODH/POX} cells, compared to MCF-7 cells. However, all studied compounds, except MOE+GlyPro inhibited prolidase activity in MCF-7^{shPRODH/POX} cells compared to untreated MCF-7^{shPRODH/POX} cells (Fig. 5A-D). In MCF-7 cells, the compounds differently affected prolidase activity; Rap inhibited, while GlyPro and MOE+GlyPro stimulated the enzyme activity (Fig. 5).

Fig. 3. Proliferation of MCF-7^{shPROD_H/POX} versus MCF-7 cells, and the effect of A. rapamycin (Rap), B. 2-metoxiestradiol (MOE), C. glycyl-proline (GlyPro) and D. the combination of 2-metoxiestradiol and glycyl-proline (MOE+GlyPro) on the process. The mean values ± SEM from 3 experiments done in duplicates are presented. Asterisks indicate differences between studied cells compared to control untreated MCF-7 cells as well as between treated MCF-7 and MCF-7^{shPROD_H/POX} cells at * P<0.01; ** P<0.001.

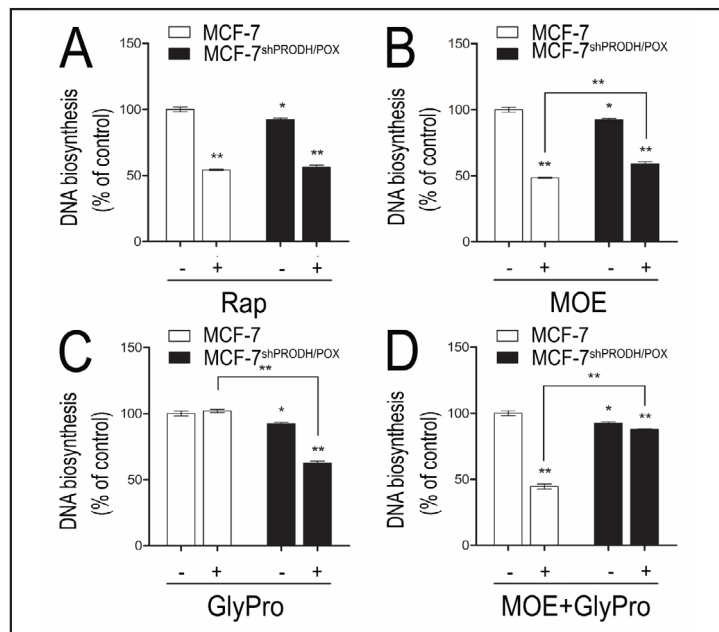
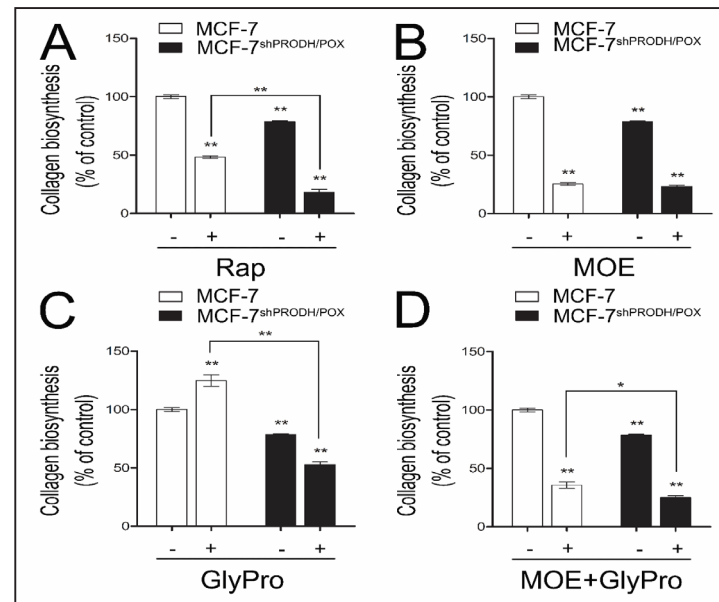


Fig. 4. Collagen biosynthesis in MCF-7^{shPROD_H/POX} versus MCF-7 cells, and the effect of A. rapamycin (Rap), B. 2-metoxiestradiol (MOE), C. glycyl-proline (GlyPro) and D. the combination of 2-metoxiestradiol and glycyl-proline (MOE+GlyPro) on the process. The mean values ± SEM from 3 experiments done in duplicates are presented. Asterisks indicate differences between studied cells compared to control untreated MCF-7 cells as well as between treated MCF-7 and MCF-7^{shPROD_H/POX} cells at * P<0.01; ** P<0.001.



Inhibition of proline utilization induces PROD_H/POX-dependent apoptosis

To evaluate effectiveness of PROD_H/POX silencing we analyzed the enzyme expression in designed experimental conditions. Treatment of MCF-7 cells with Rap, MOE, GlyPro or combination of MOE+GlyPro upregulated expression of PROD_H/POX, while in MCF-7^{shPROD_H/POX} cells the enzyme expression was non-detectable (Fig. 6).

To test the role of proline in regulation of PROD_H/POX-dependent apoptosis or survival we analyzed the effect of Rap, MOE, GlyPro and MOE+GlyPro on expression of p53, cleaved and non-cleaved caspase-3, caspase-9 and PARP, beclin-1, atg7, HIF-1α and NF-κB in MCF-7 and MCF-7^{shPROD_H/POX} cells by Western blotting. Expression of beclin-1, atg7 and HIF-1α proteins were upregulated in MCF-7^{shPROD_H/POX} compared to MCF-7 cells (Fig. 6). In cells with lower expression of PROD_H/POX the expression of beclin-1 was higher than in MCF-7 cells. However, in MCF-7 cells, MOE decreased the level of beclin-1 expression. Expression of atg7 was increased in treated and untreated MCF-7^{shPROD_H/POX} cells, while in MCF-7 cells only

Fig. 5. Prolidase activity in MCF-7^{shPRODH/POX} versus MCF-7 cells, and the effect of A. rapamycin (Rap), B. 2-metoxiestradiol (MOE), C. glycyL-proline (GlyPro) and D. the combination of 2-metoxiestradiol and glycyL-proline (MOE+GlyPro) on the process. The mean values ± SEM from 3 experiments done in duplicates are presented. Asterisks indicate differences between studied cells compared to control untreated MCF-7 cells as well as between treated MCF-7 and MCF-7^{shPRODH/POX} cells at * P<0.01; ** P<0.001.

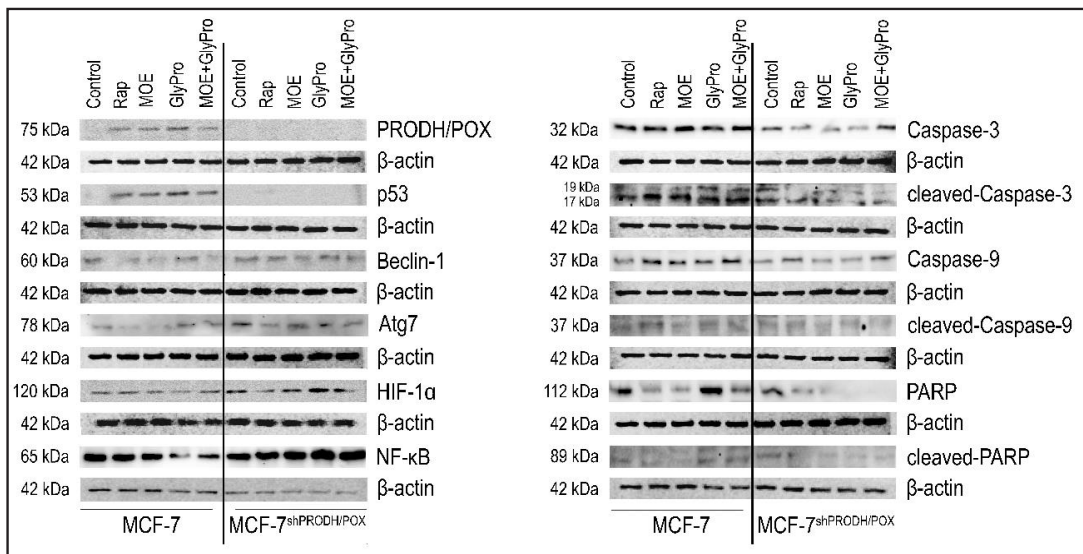
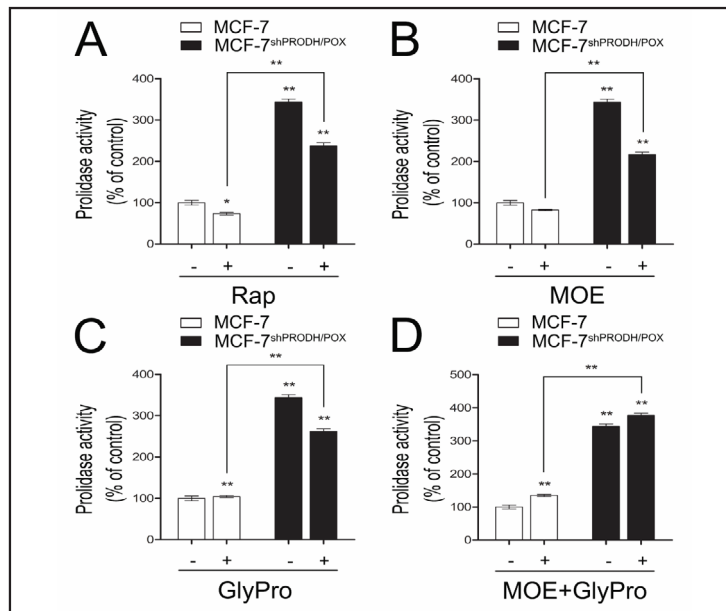


Fig. 6. Expression of PRODHD/POX, p53, un-cleaved caspase-3, cleaved-caspase-3, un-cleaved caspase-9, cleaved-caspase-9, un-cleaved PARP, cleaved-PARP, beclin-1, atg7, HIF-1α, NF-κB and β-actin in MCF-7^{shPRODH/POX} versus MCF-7 cells and the effect of rapamycin (Rap), 2-metoxiestradiol (MOE), glycyL-proline (GlyPro) and the combination of 2-metoxiestradiol and glycyL-proline (MOE+GlyPro) on the process. The mean values of 6 pooled cell homogenates extracts from 3 independent experiments are presented. β-actin was used as a control.

GlyPro and combination MOE+GlyPro increased expression of this protein. In MCF-7 cells MOE and GlyPro decreased expression of HIF-1α, while Rap and MOE+GlyPro had no effect on the process (Fig. 6). In MCF-7^{shPRODH/POX} cells MOE, GlyPro and combination of MOE+GlyPro upregulated HIF-1α expression, while Rap down-regulated the process, compared to control (Fig. 6).

In MCF-7 cells treated with Rap, MOE, GlyPro and MOE+GlyPro expression of NF-κB was decreased, while in MCF-7^{shPRODH/POX} cells the compounds increased the expression of this transcription factor.

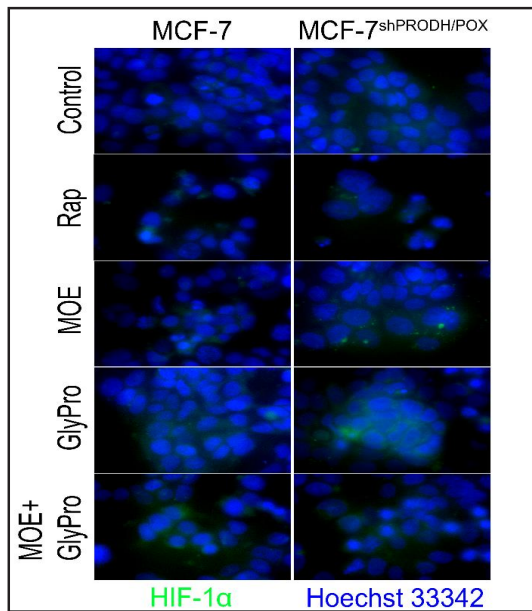


Fig. 7. Immunofluorescence analysis of HIF-1 α in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxiestradiol (MOE), glycyL-proline (GlyPro) and the combination of 2-metoxiestradiol and glycyL-proline (MOE+GlyPro).

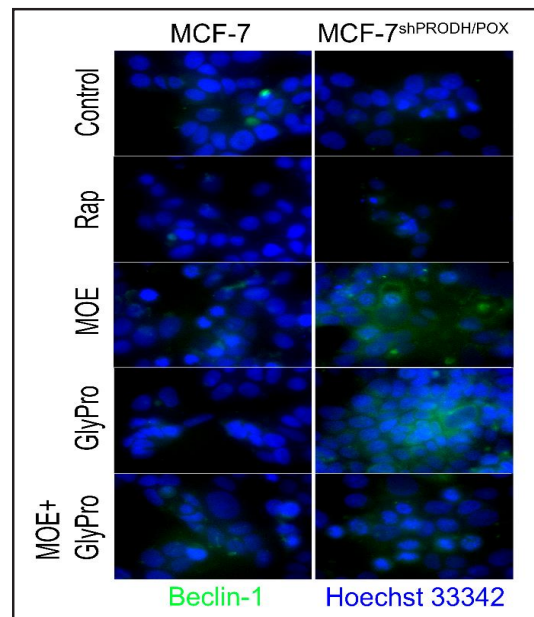


Fig. 8. Immunofluorescence analysis of beclin-1 in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxiestradiol (MOE), glycyL-proline (GlyPro) and the combination of 2-metoxiestradiol and glycyL-proline (MOE+GlyPro).

Expression of p53, cleaved and un-cleaved caspase-3, caspase-9 and PARP were increased in treated MCF-7 cells, compared to MCF-7^{shPRODH/POX} cells (Fig 6).

We further confirmed our data by immunofluorescence bio-imaging (Fig. 7, 8 and 9). HIF-1 α , beclin-1, cleaved and un-cleaved caspase-3 and caspase-9 were detected by FITC-labelled specific antibodies. The cells were stained with Hoechst 33321 to visualize cell nucleus. Expression of HIF-1 α and beclin-1 were upregulated in MCF-7^{shPRODH/POX} cells, especially when treated with MOE, GlyPro and combination of MOE+GlyPro, compared to MCF-7 cells (Fig. 7; Fig. 8). Expression of un-cleaved caspase-3 were upregulated in treated and untreated MCF-7 and MCF-7^{shPRODH/POX} cells (Fig. 9), while expression of cleaved-caspase-3 was up-regulated only in MCF-7 cells (Fig. 10). Expression of un-cleaved caspase-9 was upregulated in MCF-7 cells treated with MOE and GlyPro and in treated and untreated MCF-7^{shPRODH/POX} cells (Fig. 11), while the expression of cleaved-caspase-9 was up-regulated in treated MCF-7 and only in Rap treated MCF-7^{shPRODH/POX} cells (Fig. 12).

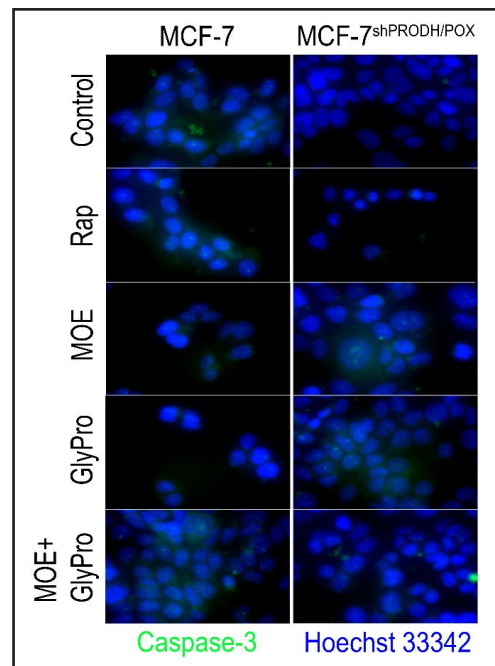


Fig. 9. Immunofluorescence analysis of un-cleaved caspase-3 in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxiestradiol (MOE), and glycyL-proline (GlyPro) and the combination of 2-metoxiestradiol and glycyL-proline (MOE+GlyPro).

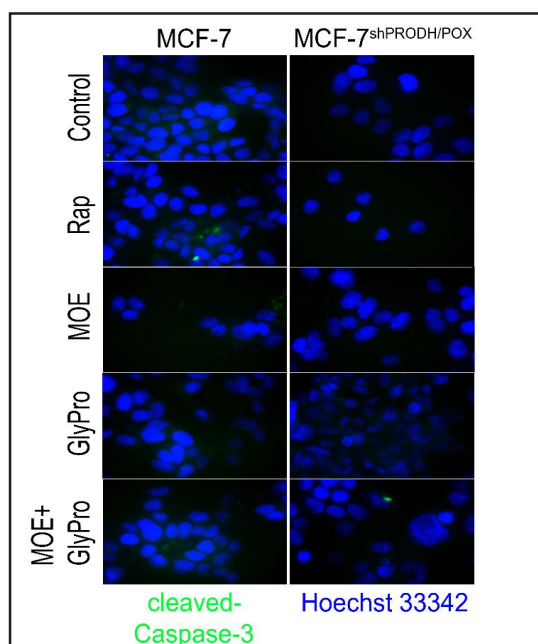


Fig. 10. Immunofluorescence analysis of cleaved-caspase-3 in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxysteadiol (MOE), and glycyL-proline (GlyPro) and the combination of 2-metoxysteadiol and glycyL-proline (MOE+GlyPro).

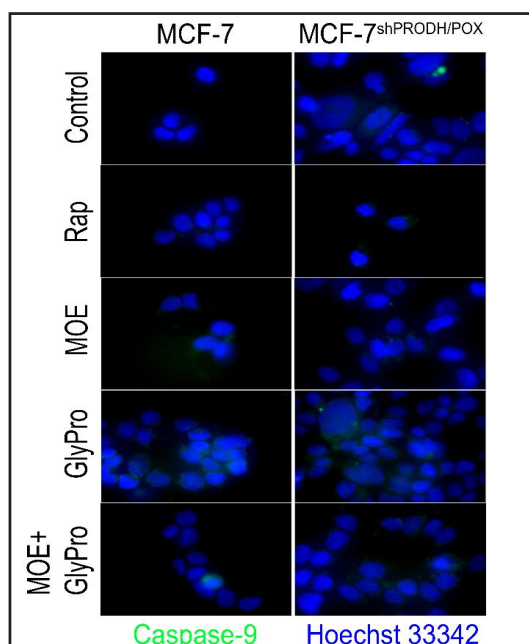


Fig. 11. Immunofluorescence analysis of un-cleaved caspase-9 in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxysteadiol (MOE), and glycyL-proline (GlyPro) and the combination of 2-metoxysteadiol and glycyL-proline (MOE+GlyPro).

Effect of inhibition of proline utilization on proline concentration in MCF-7^{shPRODH/POX} cells

There was a significant increase in proline concentration in MCF-7^{shPRODH/POX} compared to MCF-7 control cells (Fig. 13). Proline concentration further increased in both cell lines after stimulation by MOE, GlyPro and combination of MOE+GlyPro, compared to untreated MCF-7 cells (Fig. 13).

Discussion

Although the functional significance of proline catabolism by PRODH/POX is well recognized [13, 15, 23, 24] the mechanism of PRODH/POX-dependent apoptosis and autophagy is poorly understood. Here we provide evidence that PRODH/POX silencing contributes to induction of pro-survival pathways in MCF-7 cells. We knocked-down significantly PRODH/POX expression and created constitutively silenced cell line on the basis of MCF-7 cells, which induced functional phenotypic changes. We were not able

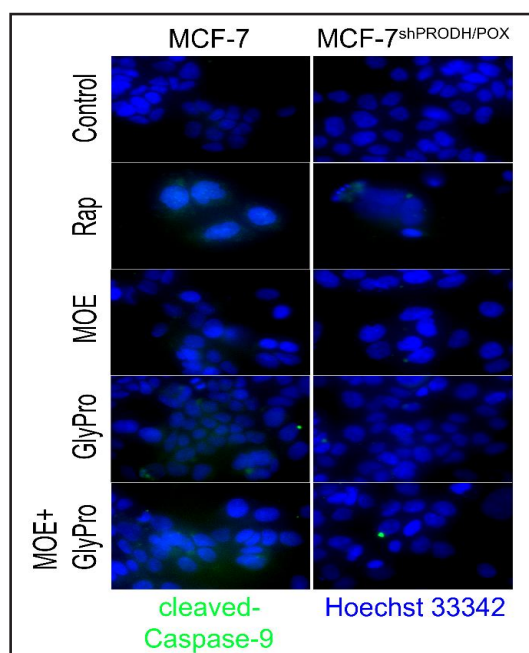


Fig. 12. Immunofluorescence analysis of cleaved-caspase-9 in MCF-7 and MCF-7^{shPRODH/POX} cells treated with rapamycin (Rap), 2-metoxysteadiol (MOE), and glycyL-proline (GlyPro) and the combination of 2-metoxysteadiol and glycyL-proline (MOE+GlyPro).

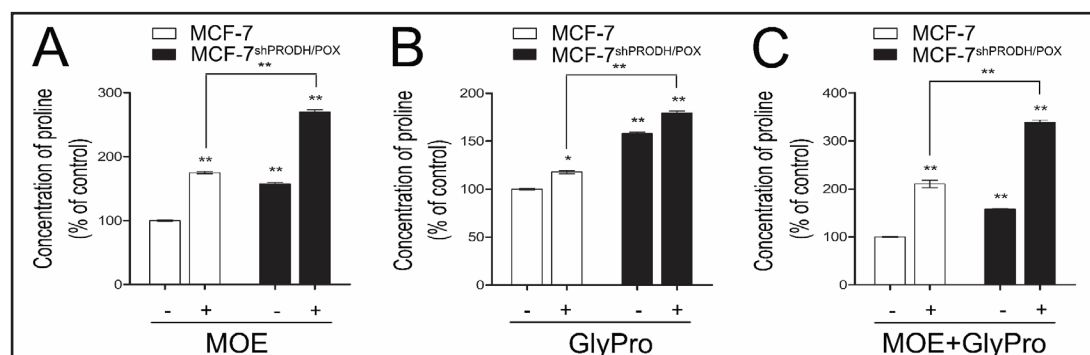


Fig. 13. Proline concentration in MCF-7^{shPRODH/POX} versus MCF-7 cells, and the effect of A. 2-metoxiestradiol (MOE), B. glycyl-proline (GlyPro) and C. the combination of 2-metoxiestradiol and glycyl-proline (MOE+GlyPro) treatment on intracellular proline concentration. The mean values \pm SEM from 3 experiments done in duplicates are presented. Asterisks indicate differences between studied cells compared to control untreated MCF-7 cells as well as between treated MCF-7 and MCF-7^{shPRODH/POX} cells at * $P < 0.01$; ** $P < 0.001$.

to achieve a complete (< 90%) knock-down of PRODH/POX in MCF-7 cells due to a technical aspect of the experiment. However, both MCF-7^{shPRODH/POX} and MCF-7 cells transfected with empty vector evoked similar growth curve to wild-type of MCF-7 cells during time course of experiment (data not shown). We also confirmed that PRODH/POX silencing does not affect cell viability and cell proliferation (data not shown).

PRODH/POX silencing in MCF-7 cells decreased DNA and collagen biosynthesis and increased prolidase activity. A plausible explanation of this phenomenon could be that the increase in the proline level in cytoplasm occurred as a result of decreased PRODH/POX expression. Proline and its metabolism play an important role in the proper functioning of the cell [4]. Proline is known to act as a stress signalling molecule in mitochondria [11, 22, 25] and cytoplasm [4, 7, 23, 24]. Proline bearing reducing potential must be converted in mitochondria to P5C by PRODH/POX for regeneration of oxidizing potential. On the other hand, P5C is converted in cytoplasm to proline by P5C reductase. The interconversion is known as a "proline cycle" (conversion of proline-P5C in mitochondria by PRODH/POX and P5C-proline in cytosol by P5C-reductase) that transfers reducing and oxidizing potential between mitochondria and cytosol using NADPH/NADP⁺ [23, 24]. This shuttle is coupled to glycolysis and pentose phosphate pathway that supports biosynthesis of pyridine nucleotides [10]. Therefore, down-regulation of PRODH/POX may affect DNA biosynthesis and cell proliferation.

Inhibition of collagen biosynthesis in MCF-7^{shPRODH/POX} cells may result from proline-dependent inhibition of prolyl hydroxylase [4]. Prolyl hydroxylase is a key enzyme, required for triple helical structure of collagen during its biosynthesis [23]. Free proline has been shown to inhibit prolyl hydroxylase [4], suggesting a mechanism for proline-dependent attenuation of collagen biosynthesis. Although, we do not know what could be the mechanism of increased prolidase activity in MCF-7^{shPRODH/POX} compared to MCF-7 cells, this process could lead to proline accumulation in MCF-7^{shPRODH/POX} cells. We hypothesized that proline availability for PRODH/POX-dependent functions is regulated by prolidase activity, while utilization of proline by collagen biosynthesis. Therefore, we used several compounds that affect cytoplasmic pool of proline. We used 2-metoxiestradiol (MOE) as an inhibitor of HIF-1 α and collagen biosynthesis while rapamycin (Rap) to inhibit mTOR and prolidase activity. In order to increase cytoplasmic level of proline, glycyl-proline (GlyPro) was used as a substrate for prolidase. Although the mechanism by which the studied compounds upregulate PRODH/POX is unknown, the possible explanation comes from studies of Krishnan et al., showing that there is a tight correlation between cytoplasmic proline level and mitochondrial PRODH/POX activity [26].

Rap (inhibitor of prolidase activity and mTOR signaling) treatment of MCF-7^{shPRODH/POX} cells further decreased cell viability, DNA and collagen biosynthesis, while prolidase activity (despite Rap-dependent inhibition) was still very high, compared to MCF-7 cells. Since Rap inhibits proliferation of normal [27] and cancer [28] cells, the growth-inhibiting activity of Rap in MCF-7^{shPRODH/POX} cells may suggest an important link between mTOR and PRODH/POX in regulation of cell growth. In fact, Rap was found to activate PRODH/POX [29] resulting in an inhibition of cell proliferation. Regarding collagen biosynthesis, Rap was found to inhibit collagen production [30].

MOE (an inhibitor of collagen biosynthesis and HIF-1 α transcriptional activity) decreased cell viability, DNA and collagen biosynthesis similarly in MCF-7 and MCF-7^{shPRODH/POX} cells. However, prolidase activity (despite MOE-dependent inhibition) was maintained very high in MCF-7^{shPRODH/POX} compared to MCF-7 cells. Increase in prolidase activity in these cells was accompanied by an increase in proline levels in cytoplasm. It is well recognized that MOE induces mitotic arrest and apoptosis in carcinoma cells [31]. However, in ovarian carcinoma cells lacking PRODH/POX [32], on the contrary, MOE stimulated cell proliferation [33]. The discrepancy may suggest that the underlying mechanism of this phenomenon could involve other factors than the proline availability and PRODH/POX activity. The inhibition of cell proliferation in MCF-7^{shPRODH/POX} is likely to be associated with modulation of cell signaling pathways and cell cycle regulatory processes, because it was found modulation of EGFR, COX-2 and MAPK signaling Wnt/ β -catenin pathway by PRODH/POX [34, 35].

GlyPro, a substrate for prolidase further decreased DNA and collagen biosynthesis, however, prolidase activity (despite GlyPro-dependent inhibition) was still very high in MCF-7^{shPRODH/POX} cells, compared to MCF-7 cells. Considering that GlyPro provides substrate for proline generation, the decrease in above mentioned processes is most likely a consequence of proline accumulation in the cell. In fact, in GlyPro-treated cells, intracellular proline was significantly increased. High level of proline due to GlyPro stimulation and its impaired conversion into P5C in MCF-7^{shPRODH/POX} cells could impair the regeneration of oxidizing potential, resulting in an attenuation of DNA and protein synthesis [36, 37]. The collagen biosynthesis inhibition by GlyPro in MCF-7^{shPRODH/POX} may result from stimulation of expression of NF- κ B – inhibitor of collagen gene expression [38]. Inhibition of prolidase activity by GlyPro in MCF-7^{shPRODH/POX} cells could result from the substrate inhibition [39].

Although cell viability was similar in MCF-7 and MCF-7^{shPRODH/POX} cells, MOE+GlyPro treatment significantly decreased cell viability in both cell lines. In respect to DNA biosynthesis, MOE+GlyPro significantly inhibited this process only in MCF-7 cells. It shows that simultaneous inhibition of proline utilization for collagen biosynthesis and providing substrate for proline generation may attenuate proliferation only in MCF-7 cells, expressing PRODH/POX. The functional significance of simultaneous inhibition of proline utilization and stimulation of proline generation was shown by Western blot and confocal microscopy bioimaging for cleaved and un-cleaved caspase-3 and -9.

Our data suggest that proline availability and PRODH/POX expression are required for induction of apoptosis as detected by expression of cleaved caspases-3, caspase-9 and PARP in MCF-7 cells. Several environmental conditions, such as hypoxia and nutrient stress are known to upregulate PRODH/POX expression [13, 40]. Interestingly, in response to stress signals, PRODH/POX expression is up-regulated by tumor suppressor p53 [41]. In fact, we found that p53 was expressed only in PRODH/POX expressing MCF-7 cells treated with MOE and GlyPro that increased cytoplasmic level of proline.

mTOR pathway is dependent on the availability of nutrients [24, 42]. Inhibition of mTOR by Rap and/or by glucose deprivation upregulates PRODH/POX expression [13, 24, 29]. Although, many factors may play a role in the regulation of PRODH/POX and apoptosis/survival [1, 43, 44], data presented in this report suggest that the key role in switching into apoptotic mode may play massive proline utilization in mitochondria by PRODH/POX. Such a situation may take place when cytoplasmic level of proline is high. The main factor responsible for increase in proline concentration is prolidase [45, 46]. However, proline is quickly utilized in the process of collagen biosynthesis in order to remove the reducing potential of proline

from metabolic pool and maintain redox balance [46]. Therefore, when collagen biosynthesis is blocked and prolidase is active, proline accumulate in cytoplasm, enters mitochondria and is converted into P5C, yielding ROS-induced apoptosis. Such a hypothesis is supported by our data showing that in MCF-7 cells MOE as well as MOE+GlyPro induced expression of cleaved caspases-3 and -9 more effectively than in MCF-7^{shPRODH/POX} cells.

On the other hand, autophagy markers (e.g. beclin-1, Atg7, HIF-1 α) were up-regulated in treated MCF-7^{shPRODH/POX} cells. Although, the mechanism of proline-dependent up-regulation of proteins reflecting autophagy is unknown, the mechanism of proline-dependent up-regulation of HIF-1 α transcriptional activity is well recognized.

Proline, by inhibition of HIF prolyl hydroxylase activity [4] suppresses proteosomal degradation of HIF-1 α and increases HIF-1 α transcriptional activity [4, 7, 47]. Therefore, increase in cytoplasmic proline concentration, which mostly depends on prolidase activity, contributes to increase in HIF-1 α transcriptional activity [4]. HIF-1 α by targeting pro-inflammatory and pro-angiogenic genes (e.g. COX-2, VEGF, TNF- α , IL-1, NF- κ B) promote pro-survival signalling pathways [48]. Interestingly, HIF-1 α was also found to play a regulatory role in hydroxylation and secretion of collagen [49] suggesting another modulatory mechanism of collagen production. We provided evidence that expression of HIF-1 α was more pronounced in MCF-7^{shPRODH/POX} cells than in MCF-7 cells, suggesting pro-survival mode of the cells.

In this study, we found that the inhibition of proline utilization in collagen biosynthesis or stimulation of its accumulation enhanced PRODH/POX-dependent pro-apoptotic pathways in MCF-7 human breast cancer cells. However, the role of specific metabolites of proline interconversion in the mechanism that leads to apoptosis or autophagy remains unknown. Large amount of proline is also derived from glutamine that is linked to the tumor metabolism [10, 12, 50, 51]. Therefore, the identification of specific pathway of proline metabolism that is involved in regulation of apoptosis/survival is of particular importance and requires further studies. Furthermore, we had been able to establish and characterize a constitutively downregulated PRODH/POX MCF-7 cell line (MCF-7^{shPRODH/POX}) that may serve as a model for studying proline utilization or generation in PRODH/POX-dependent pro-apoptotic pathway.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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