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CoAsy knockdown in TNBC cell lines resulted in no overt effect on cell proliferation in vitro

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Abstract

Triple-negative breast cancer (TNBC) remains the most challenging breast cancer subtype to treat. CoA synthase (CoAsy) is a bifunctional enzyme, encoded by the COASY gene, which catalyzes the last two steps of CoA biosynthesis. COASY has been reported as a hit in several large RNAi library screens for cancer. Therefore, we sought to investigate the dependency of TNBC cell line proliferation on CoAsy expression. Initially, knockdown of CoAsy expression was achieved by RNAi and reduced proliferation was observed in two TNBC cell lines, HCC1806 and MDA-MB-231. To further investigate the role of CoAsy, we established stable inducible shRNA cell lines from the same TNBC cell lines as well as the normal-like breast cell line MCF10A. Three separate cell lines, each expressing one of three different shRNA constructs targeting COASY, and a non-targeted shRNA control cell line were generated from each parent cell line. The induction of COASY shRNA for 4 days resulted in >99% knockdown of CoAsy for all three COASY shRNA constructs. However, this robust knockdown of CoAsy protein expression had no detectable impact on cell growth with 4-day induction times. Even 8-day induction times resulted in no apparent impact on cell growth. There was also no effect of CoAsy knockdown on the rate of cell migration. Measurement of CoA levels in cell lysates indicated that CoAsy knockdown reduced CoA to approximately half the normal level. Thus, CoAsy knockdown showed no detectable effect on the in vitro proliferation and migration of these cell lines possibly due to the cell's ability to maintain adequate levels of CoA through some unknown mechanism.

Keywords

Cancer; COASY; CoA synthase; CoA

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Introduction

Breast cancer is the most common type of cancer in women. There are at least 5 subtypes of breast cancer based on gene expression [1-3]. However, common clinical classification is still based on the expression of three markers - estrogen receptor (ER), progesterone receptor (PR) and Epidermal Growth Factor Receptor 2 (Her2). One of the most serious subtypes of breast cancers is termed triple-negative breast cancer (TNBC), which overlaps significantly, but not completely, with the basal type molecular subtype of breast cancer. The definition of TNBC is that it lacks the expression of ER, PR and HER2 [4-6]. It is estimated that about 10-15% of all breast cancers have a TNBC phenotype. TNBC tends to be more aggressive than the other subtypes of cancer with a propensity for early relapses and metastasis with lower five year survival rates [1,6,7].

Coenzyme A (CoA) is a cofactor for many cellular enzymes and is involved in about 4% of the enzymatic activities in cells [8,9]. CoA functions to accept and donate hydrocarbon moieties, such the acetyl and fatty acyl groups, in enzymatic reactions. CoA is synthesized starting from pantothenate (vitamin B5), a vitamin that humans cannot synthesize and must be obtained from the diet or bacterial sources [8]. The final two steps of the *de novo* synthesis of CoA is carried out by coenzyme A synthase (CoAsy, encoded by the COASY gene), a bifunctional mitochondrial enzyme that has two catalytic domains, phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK) [9]. Mutations in COASY appear to be one of the genetic causes of the neurodegenerative diseases with brain iron accumulation (NBIA) [10].

Several reports have proposed an association between COASY and cancer [11-15]. More recently, in vitro and in vivo enhanced radiosensitivity has been reported for rectal cancer cell lines expressing low CoAsy [11]. In addition, CoAsy was reported as a hit in several large RNAi library screens for cancer or cell viability [13,14,16], but the activity was not confirmed nor studied further. Since the role of CoAsy in TNBC is unknown, we sought to investigate the dependency of TNBC cell line proliferation on CoAsy expression.

Materials and Methods

Materials

General reagents and plasticware were obtained from Fisher Scientific. Cell culture media components were obtained from ThermoFisher (Waltham, MA) and Hyclone (GE Healthcare, Chicago, IL).

Cell culture, COASY Knockdown and Cell Proliferation/viability Assays Methods

Cell lines were obtained from ATTC (Manassas, VA) and cultured under standard conditions as outlined in Supplementary material. COASY RNAi and inducible shRNA knockdown and cell proliferation/viability assay methods are provided in Supplementary material.

Western blot

Western blot analysis was performed by standard methods using a fluorescence detection system (Licor, Lincoln, NE) with a brief outline provided in Supplementary material.

CoA Determination

CoA was measured in cell lysates using a fluorescence-based kit (cat# ab138889, Abcam) according to manufacturer's instructions. Relative fluorescence values were obtained using a PHERAstar FSX reader.

Scratch Assay

Cells were treated with doxycycline for 96 hours in culture flasks, then harvested and plated in 96-well plates (+dox) and allowed to reach 90% confluency. Scratches were created in the cell monolayer and monitored every two hr for cell density (by phase contrast) in a real-time microscopic imaging system (Incucyte, Essen BioScience, Ann Arbor, MI). Data was analyzed by the instrument software.

Results

CoAsy knockdown using RNAi inhibited cell proliferation

We sought to investigate the role of COASY in the growth of TNBC cell lines in vitro. We used the TNBC cell lines HCC1806 and MDA-MB-231 as the primary model cell lines. We used four different RNAi sequences to knockdown COASY plus a non-targeted RNAi (NT) as a control RNAi. Four days after RNAi transfection, we validated the knockdown of CoAsy protein by western blot (Fig. 1A, 1B). Quantitative analysis of the western blot for both cell lines showed a >99% reduction in CoAsy protein levels in cells transfected with all four COASY RNAi that target four different regions of the COASY mRNA, while no loss of CoAsy was seen in cells transfected with the control non-targeted RNAi (Fig.1C, 1D). We then tested the effect of CoAsy knockdown on cell proliferation/viability at the same time point. The COASY RNAi was associated with significant reduction in the cell proliferation of both cell lines (Fig. 1E, 1F). This reduced proliferation appeared to be specific with COASY knockdown because it was observed after transfection with four different COASY RNAis, but not with NT RNAi. This experiment appeared to be consistent with literature reports that knockdown of CoAsy by RNAi had an effect on cell health [12-14,16] and thus, we sought to study this effect further using stable cell lines.

Induction of stable inducible COASY shRNA cell lines resulted in knockdown of CoAsy protein

We established stable inducible cell lines using the TNBC cell lines HCC1806 and MDAMB-231 along with the normal-like breast cell line MCF10A. We generated four sublines of each cell line using lentiviral transduction using three different inducible lentiviral COASY shRNA vectors and a non-targeted shRNA vector as a control. These inducible constructs express the shRNA upon treatment of cells with doxycycline. Initially, we sought to determine the rate of reduction in CoAsy protein after dox exposure for the HCC1806 cell line. CoAsy levels were determined every day of dox treatment for 4 days via western blot (Fig. 2A,B). Quantification of the western blot data indicated that COASY levels (compared to 0 time) were reduced by 44%, 89.7%, >99% (below detection level) and >99% (below detection level) at 24, 48, 72 and 96 hr, respectively. Thus, we chose to use the dox induction time of 96 hr as our standard induction time. Stable shRNA sublines derived

from all three parental cell lines were treated with dox for 96 hours and then cells harvested for western blot analysis for CoAsy levels (Fig. 2C). Dox induction resulted >99% reduction in CoAsy for all three COASY shRNAs for all cell lines, while no significant reduction in CoAsy was observed with dox for NT cell lines and without dox for COASY cell lines (Fig. 2D).

CoAsy knockdown using shRNA had no effect on cell proliferation

Stable shRNA cell lines were treated with dox for 96 hours, and then relative viable cell number was determined by an ATP detection assay to assess cell proliferation (Fig. 3A-C). The results indicated that there was no significant difference in the rate of cell proliferation/ cell viability for cells that had the COASY or NT shRNA, with or without dox. Longer term knockdown was performed by treating HCC1806 and MCF10A cells with dox in flasks for 4 days, then these cells were seeded into 96-well plates and, still under dox treatment, incubated another 4 days (for a total of 8 days induction), before cell proliferation was determined as before (Fig. S1). These studies also indicated that the cells grew at a normal rate with CoAsy knockdown. Thus, CoAsy knockdown had no effect on in vitro cell proliferation for two TNBC cell lines and a normal-like breast cell line.

CoAsy knockdown had no effect on cell migration.

In order to evaluate whether CoAsy is involved in cell migration, we treated the four HCC1806 stable cell lines (3 COASY shRNA and NT shRNA lines) with dox for 96 hours in flasks then harvested and seeded them into 96-well plates (with dox). After an overnight incubation, a scratch wound was created when the cells were 90% confluent. The scratched cell monolayers were imaged every two hours for 16 hours to determine the rate at which the cells filled the gap (Fig. 3D). Analysis of this kinetic scratch assay data indicated that there was no significant difference in the rate of wound healing (cell migration) between NT and COASY shRNA cell lines. Thus, HCC1806 cells, cultured under standard conditions, migrated normally even with a robust knockdown of CoAsy.

Cellular CoA levels were only modestly impacted by CoAsy knockdown

The reduction of CoAsy levels in the cells was expected to impact the levels of *de novo* CoA synthesis. Therefore, we sought to determine the effect of CoAsy knockdown on relative cellular CoA levels in HCC1806. Using a fluorescent CoA assay kit, we measured the level of CoA at three different dox induction time points, 24, 48 and 72 hours, for the NT shRNA and all the COASY shRNA cell lines (Fig. 3E). At 24 hr dox induction, CoA remained at 100% of the NT shRNA cells. At 48 hr dox induction time, the CoA levels were reduced to almost 50% of the NT shRNA induced cells. Moreover, no significant further reduction in CoA levels was observed, comparing 48 to 72 hour induction times. Thus, CoAsy knockdown only modestly suppressed CoA levels to about half the normal level, despite the greatly reduced CoAsy expression. This level of cellular CoA may be sufficient to allow normal cell proliferation and migration in vitro and may explain the lack of phenotype observed in our experiments despite a robust knockdown of CoAsy.

Incubation in dialyzed serum did not yield significant differences in cell survival with CoAsy knockdown

We attempted to use dialyzed serum to remove a potential source of CoA from the growth media. We sought to determine if COASY knockdown cell line growth would be differentially effected by the lack of exogenous CoA. However, even short term culture of HCC1806 in media with dialyzed FBS resulted in cell death for NT shRNA cells (Fig. S2 and data not shown). In an attempt to reduce this effect, we slowly adapted the cell lines to growth in 7.5% dialyzed serum and 2.5% normal serum. We then shifted the cells to media +dox and 10% dialyzed serum (no normal serum) and monitored confluency over time. Although growth of COASY shRNA cell lines under these conditions resulted in a trend for more rapid loss of confluency over time compared to NT cells, the differences were not statistically significant (Fig. S2). Thus, these results were inconclusive possibly due to the minimal amount of proliferation in dialyzed serum and thus cellular CoA levels may remain adequate and also complicated by the lack of other low molecular weight factors that were critical to support even short term cell survival.

Discussion

Several published large RNAi-based screening studies have identified the COASY gene as a hit in several types of cancer cell lines, where knockdown of COASY resulted in cell growth inhibition [12-14,16]. Based on these studies and our own preliminary data, we sought to determine the importance of CoAsy for the *in vitro* growth of TNBC cell lines. Our initial studies with RNAi-mediated knockdown of COASY resulted in inhibited cell proliferation, in agreement with published reports [12-14]. This effect appeared to be specific since cell growth inhibition was observed with 4 different RNAi sequences, but not with the non-targeted RNAi. This RNAi-mediated effect was also in general agreement with a recent report that used one of the same cell lines, MDA-MB-231 [12]. In that report, knockdown of COASY by RNAi in MDA-MB-231 resulted in inhibited proliferation, prolonged mitosis and multi-nucleation [12]. Therefore, we extended our investigation into COASY and TNBC by establishing stable inducible cell lines where the COASY shRNA was induced by dox. We were able to achieve >99% knockdown of CoAsy, below the detection limit of the western blot, after 72 hr of induction. Surprisingly, this shRNA-mediated knockdown, using 3 difference shRNA sequences, had no impact on cell viability or growth of the TNBC cell lines HCC1806 and MDA-MB-231, even for longer term 8 day inductions. We also examined a normal-like breast cell line MCF10A and found the same result. This data directly contradicted our own initial RNAi-mediated experiments as well as those in the COASY RNAi literature. We believe that the RNAi results were an experimental artifact, perhaps due to off-target effects. It also suggests that the reports of COASY as an RNAi screening hit for other cell types should be confirmed with other techniques such as shRNA or CRISPR. Other studies indicated knockdown of CoAsy affected cell migration in culture [17]. However, our data with the shRNA stable cell lines indicated no effect of COASY knockdown on cell migration in the scratch assay. Some adult patients with NBIA caused by mutations in COASY have no detectable CoAsy protein as a result of apparently destabilizing mutations in CoAsy [10,18]. Although these patients have degenerative neurological disease, they survive through adulthood with no or little functional CoAsy. The

growth of cells with COASY knockdown is also consistent with survival in this human genetic disease.

Given the surprising viability of the stable cell lines after induction, we determined the levels of intracellular CoA. CoA levels dropped to nearly half the control level from 24 to 48 hr of shRNA induction, but not significantly more by 72 hr, indicating the effect of COASY knockdown on CoA levels was near maximal. We speculate that this remaining half-normal level of CoA after CoAsy knockdown was sufficient for the cells to proliferate and migrate in culture and explains the lack of overt growth phenotype in the tested cell lines. This data raised the question of how there can be a significant amount of CoA in the cells when CoAsy was knocked down to below detection. One formal possibility is that CoA is known to recycle from its various derivatives [19]. However, cell proliferation, especially in the long term studies, would divide the CoA (and CoA derivatives) concentration in half with each doubling time and so CoA would be rapidly depleted by cell division. One possibility is that the <1% of remaining COASY enzyme was sufficient to generate the observed CoA. However, the kinetics of cellular CoA decline (rapidly decreasing from 24 to 48 hr, then not decreasing much more after 72 hr), suggested that the remaining CoA was not due to residual CoAsy activity. Another formal possibility is that there is an alternate enzyme(s) capable of substituting for CoAsy. However, there has been no published data for such an alternate pathway for CoA biosynthesis. Thus, it seems most likely that the cells were able to source enough CoA to survive from an unidentified exogenous source. One likely source of the CoA is the bovine serum used in the growth media. An alternate CoA biosynthetic pathway has been demonstrated where exogenous addition of CoA to cells in culture resulted in the generation of intracellular CoA despite lack of pantothenate in the media [20]. However, this alternate pathway still required CoAsy to complete the final two steps of CoA biosynthesis. Therefore, this alternate CoA pathway lies “upstream” of CoAsy and would not explain our data. One other possible pathway would be for the cells to take up CoA derivatives from the bovine serum in the media. A search of the human Serum Metabolome Database [21] for “CoA” did not reveal detectable CoA in serum, but resulted in the identification of one major detectable CoA derivative, 3-trans,5-cis-Octadienoyl-CoA, which is a metabolite from beta-oxidation of unsaturated fatty acids. It was found in human serum at the average concentration of 37.5 nM. Other CoA derivatives are likely present in serum at much lower concentrations, but together could provide a significant supply of CoA to the cells. Thus, we propose that cells are capable of actively transporting the hydrophobic CoA derivative 3-trans,5- cis-Octadienoyl-CoA, and/or other CoA derivatives, present in bovine serum through some unknown uptake mechanism, which are then hydrolyzed inside the cell to CoA, thus bypassing the requirement for CoAsy. This hypothesis is also consistent with the survival of COASY mutant NBIA patients whose cells may be taking up dietary CoA derivatives as the sole or primary source of CoA [22].

Currently the role of CoAsy in cancer is still unclear and it has only recently gained some attention. A recent study described the knockdown of COASY using stable constitutive expression of shRNA in two colorectal cancer cell lines [11]. These cell lines displayed normal cell growth in vitro and normal tumor growth in vivo. Thus, our in vitro viability data with COASY knockdown in TNBC cell lines was consistent with this study. Interestingly, knockdown of COASY made the cells more sensitive to radiation both in vitro,

causing enhanced cell death, and in vivo, where radiation treatment dramatically reduced tumor growth[11]. Whether TNBC cell lines lacking CoAsy are also sensitized to radiation remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations used:

TNBC	triple-negative breast cancer
CoA	coenzyme A
CoAsy	coenzyme A synthase
ER	estrogen receptor
PR	progesterone receptor
Her2	epidermal growth factor receptor 2
PPAT	phosphopantetheine adenylyltransferase
DPCK	dephospho-CoA kinase
NBIA	neurodegenerative diseases with brain iron accumulation
NT	non-targeted
UT	untreated
dox	doxycycline

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Highlights

- RNAi-mediated knockdown of CoA synthase (CoAsy) inhibited TNBC cell proliferation
- Stable, inducible COASY shRNA cell lines were generated for three cell lines
- Induction of COASY shRNA resulted in CoAsy protein knockdown to below detection
- Induction of CoAsy knockdown by shRNA reduced CoA levels to half of control
- CoAsy knockdown by shRNA had no effect on in vitro cell proliferation and migration

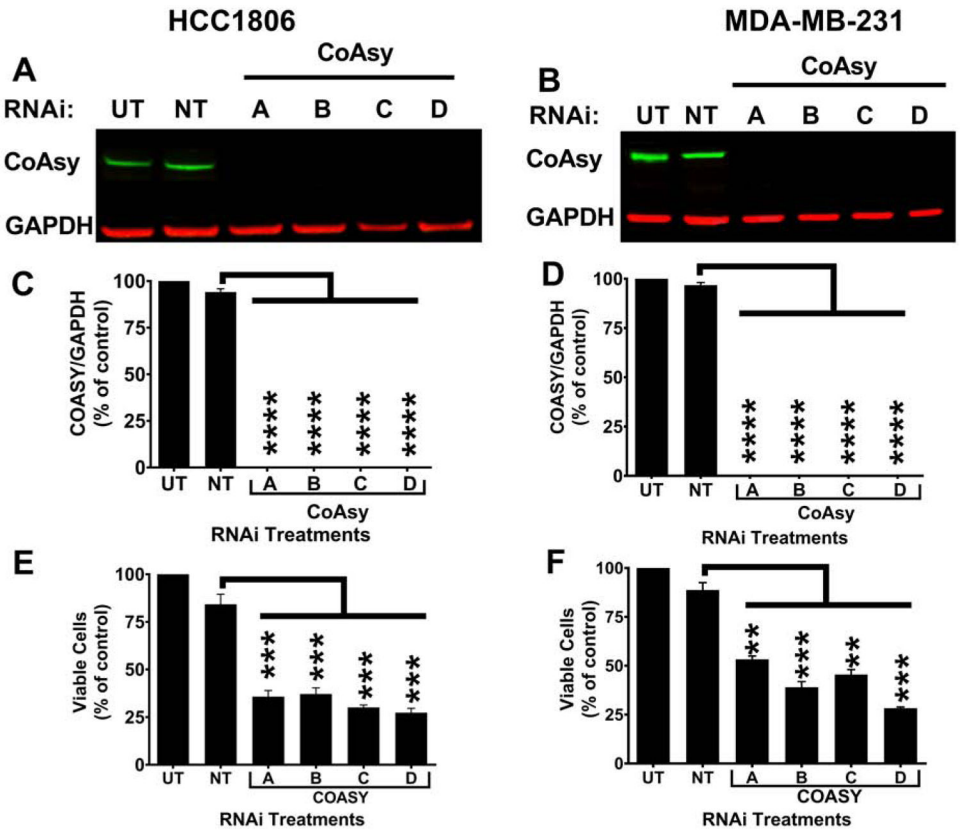

Fig. 1. COASY knockdown using RNAi reduced cell proliferation.

HCC1806 and MDA-MB-231 cells were transfected with 4 different RNAi targeting COASY (RNAi labeled A-D), a non-targeted RNAi (NT) and no RNAi, an untreated (UT) control. After 96 hr, analysis for CoAsy expression and cell proliferation was performed. Western blot analysis was performed to detect CoAsy and the housekeeper GAPDH for HCC1806 (A) and MDA-MB-231 (B). Quantitative analysis of the western blot was performed for HCC1806 (C) and MDA-MB-231 (D) using band intensities of the CoAsy signal normalized by the corresponding GAPDH signal. Cell proliferation for HCC1806 (E) and MDA-MB-231 (F) was measured using ATP detection and the assay signal normalized to controls. All graphs represent the mean ± SD of aggregated data from replicate (n=3) independent experiments. Western blot images are representative of n=3 experiments. A two-tailed unpaired t-test was used to determine the statistical significance of differences between the indicated groups with resulting p values indicated by the following: ns, not significant ($p>0.05$); *, $p<0.05$; **, $p\leq0.01$; ***, $p\leq0.001$; ****, $p\leq 0.0001$.

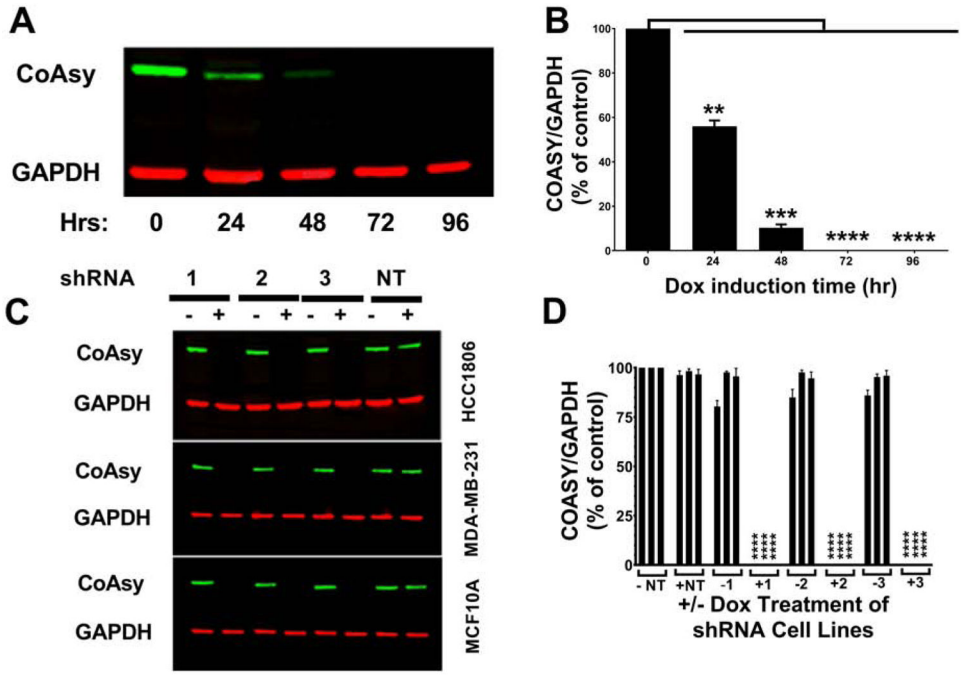

Fig. 2. Inducible COASY knockdown achieved using shRNA in three cell lines.

Stable inducible shRNA expressing HCC1806, MDA-MB-231 and MCF10A cell lines were generated using three different shRNA sequences (shRNA1, shRNA2, shRNA3) targeting COASY and a control cell line expressing a non-targeted shRNA (NT). (A) CoAsy KD time course was performed using HCC1806 by treating the cells with dox for the indicated times followed by western blot analysis. Western blots were performed to detect CoAsy and the housekeeper GAPDH. (B) Quantitative analysis of the western blots was performed using band intensities of the CoAsy signal normalized by the corresponding GAPDH signal. (C) All cell lines were treated with or without dox for 96 hr followed by western blot analysis. (D) Quantitative analysis of the western blots was performed using band intensities of the CoAsy signal normalized by the corresponding GAPDH signal. The order of the graph bars for each experimental group (left to right) is HCC1806, MDA-MB-231 and MCF10A. Statistical significance comparison is with the +dox NT group. All graphs represent the mean ± SD of aggregated data from replicate (n=3) independent experiments. Western blot images are representative of n=3 experiments. A two-tailed unpaired t-test was used to determine the statistical significance of differences between the indicated groups with resulting p values indicated by the following: ns, not significant ($p>0.05$); *, $p<0.05$; **, $p\leq0.01$; ***, $p\leq0.001$; ****, $p\leq 0.0001$.

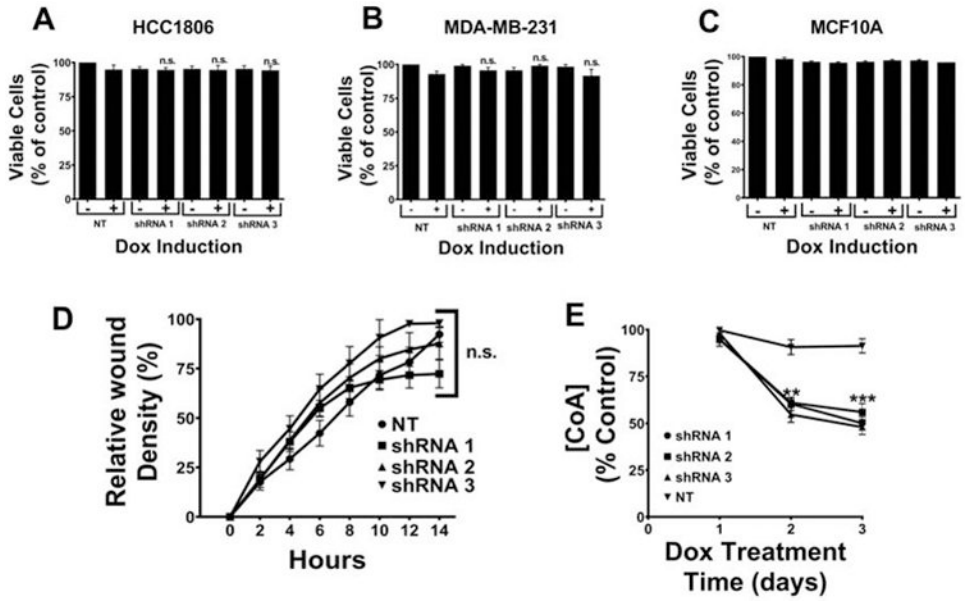

Fig. 3. Inducible CoAsy knockdown moderately reduced CoA levels, but had no effect on cell proliferation and migration.

(A-C) HCC1806, MDA-MB-231 and MCF10A stable inducible shRNA cell lines (shRNA1, shRNA2, shRNA3 and NT) were treated with (+) or without (−) dox for 96 hour. Subsequently, cell viability/proliferation was measured using ATP detection and the assay signal normalized to no dox, NT control. Statistical significance comparison was with the +dox NT group. (D) After 96 hr of dox treatment, HCC1806 stable cell lines were plated in a 96- well plate (+dox) and allowed to reach 90% confluency. A scratch was created and the rate of wound closure was plotted over time. (E) Relative CoA concentration in HCC1806 cell lysates were determined at the indicated times and the assay signal normalized to NT cells. Statistical significance comparison is with the +dox NT group at the same time point. All graphs represent the mean ± SD of aggregated data from replicate (n=3) independent experiments, except D and E (n=2). A two-tailed unpaired t-test was used to determine the statistical significance of differences between the indicated groups with resulting p values indicated by the following: ns, not significant ($p>0.05$); *, $p<0.05$; **, $p\leq0.01$; ***, $p\leq0.001$; ****, $p\leq 0.0001$.