

A Phenotypic Switch of Differentiated Glial Cells to Dedifferentiated Cells Is Regulated by Folate Receptor α

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ABSTRACT

In a previous study, we showed that folate receptor- α (FR α) translocates to the nucleus where it acts as a transcription factor and upregulates *Hes1*, *Oct4*, *Sox2*, and *Klf4* genes responsible for pluripotency. Here, we show that acetylation and phosphorylation of FR α favor its nuclear translocation in the presence of folate and can cause a phenotypic switch from differentiated glial cells to dedifferentiated cells. *shRNA-FR α* mediated knockdown of FR α was used to confirm the role of FR α in dedifferentiation. *Ocimum sanctum* hydrophilic fraction-1 treatment not only blocks the folate mediated dedifferentiation of glial cells but also promotes redifferentiation of dedifferentiated glial cells, possibly by reducing the nuclear translocation of ~38 kDa FR α and subsequent interaction with chromatin assembly factor-1. *STEM CELLS* 2019;37:1441–1454

SIGNIFICANCE STATEMENT

The clinical significance of this study is best understood in the context of a near-dramatic increase in glial cells derived tumors of supratentorial pediatric brain following folic acid fortification in the U.S. in the year 1996. This work offers a plausible mechanism of how high folate via folate receptor- α may trigger dedifferentiation of glial cells and could possibly induce tumorigenesis in the cranial neural crest derived brain cells in the pediatric population. This study also shows that the folate mediated dedifferentiation can be blocked by hydrophilic fraction-1 of *Ocimum sanctum* hydrophilic fraction-1 and cells made to redifferentiate to glial cell phenotype.

INTRODUCTION

Folate receptor- α (FR α) is a cell surface glycosylphosphatidylinositol-anchored glycoprotein that is highly expressed on many types of cancer cells including ovary, lung, breast, kidney, brain, endometrium, and colon cancer, but undetectable on normal cells [1], except in mid-brain dopamine neural progenitors and nascent dopamine neurons [2]. There are several reports describing the use of antibodies for characterization of FR α and folate receptor- β (FR β). Using monoclonal antibodies specific to FR α (mAb343) and FR β (m909). Shen et al. [3] showed that the molecular weight of FR α is ~48 kDa and FR β ~40 kDa, whereas O'Shannessy et al. [4] reported 38 kDa for FR α and 34 kDa for FR β . Although FR α is expressed in many types of cancers, its role in cancer and tumorigenesis is not fully understood. Our lab has shown that FR α is a transcription factor [5]. FR α transcriptionally

activates the pluripotent stem cell genes, *Oct4*, *Sox2*, and *Klf4* [4], *Hes1*, and *Fgfr4* [6].

Post-translational modification of transcription factors orchestrates multiple cellular processes [7, 8]. Many transcription factor functions are controlled by acetylation [9, 10]. For example, GATA-1 activity is regulated by p300-mediated acetylation [11]. Other well-characterized targets of nonhistone protein acetylation include p53, nuclear factor- κ B, p65, cyclic-AMP response element binding protein (CBP), p300, signal transducer and activator of transcription 3 (STAT3), tubulin, positive cofactor 4 (PC4), nuclear receptors, c-Myc, HIF1 α , FoxO1, heat-shock protein, fms like tyrosine kinase 3 (FLT3) kinase, and c-Raf kinase [12, 13]. Acetylated Pax3 regulates *Hes1* and *Neurog2*, such that *Hes1* levels decrease and *Neurog2* levels increase when neurogenesis begins [14]. Similarly, phosphorylation of transcription factors has been linked to nuclear translocation and signaling [15–17]. In this study, we hypothesized

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that post-translational modification of FR α , such as lysine acetylation and serine phosphorylation, regulate its nuclear translocation.

FR α ranges between 38 and 45 kDa and has a high affinity for folate ($K_D < 10^{-9} \sim 10^{-10}$) [18, 19]. Using subcellular fractionation, we reported earlier that the nuclear localized FR α is ~ 38 kDa [5]. If FR α regulates transcription and increases the expression of downstream target genes, it is hypothesized that the ~ 38 kDa nuclear FR α would be increased in cancers that show increased FR α expression. If that is the case, it is further hypothesized that a differentiated cell, for example a glial cell, can be made to dedifferentiate under high folate conditions to cancer-like cells. Therefore, preventing the FR α from translocating to the nucleus is of paramount importance in preventing pediatric brain cancer.

Several lines of evidence suggest that the extract of *Ocimum sanctum* leaves can induce apoptosis, deplete intracellular glutathione (GSH), and increase levels of lipid peroxidation products [20]. Several extraction procedures using *O. sanctum* have been reported [20–23] highlighting the benefits such as antiproliferative and antimigratory effects and drawbacks such as hepatotoxicity of each extraction methods. We understood that there is a pressing need to devise an extraction method which does not have any untoward effects on normal cells and yet it has beneficial effects of blocking cell proliferation and tumorigenesis. We have isolated a hydrophilic fraction of methanolic extracts of *O. sanctum* hydrophilic fraction-1 (OSHP-1), which has ascorbic acid (AA), rosmarinic acid (RA), and caffeoylquinic acid (CA) active ingredients as confirmed by high performance liquid chromatography (HPLC)-mass spectrometric analysis. We hypothesized that OSHP-1 has ingredient(s) that can block the nuclear translocation of ~ 38 kDa FR α and can redifferentiate the dedifferentiated glial cells.

Here, we show that (a) nuclear FR α can cause a phenotypic switch from differentiated glial cells to dedifferentiated cells; (b) OSHP-1 treatment blocks the 5-methyl tetra hydro folate (MTHF) mediated dedifferentiation of glial cells; (c) OSHP-1 treatment reduces the nuclear ~ 38 kDa FR α levels; (d) acetylation and phosphorylation of FR α favors its nuclear translocation; and (e) nuclear FR α binds to chromatin assembly factor-1 (CAF-1) in MTHF induced dedifferentiated cells and not in differentiated or re-differentiated glial cells.

MATERIALS AND METHODS

SJ-GBM2 cell line was obtained from Children's Oncology Group, Texas Tech University Health Sciences Center. Primers and probes used in this study were designed using Primer Express software (PerkinElmer Life Sciences, Naperville, IL, <http://www.perkinelmer.com/corporate/what-we-do/markets/life-sciences>). Primers were synthesized by Eurofins (Louisville, KY, <http://www.eurofinsgenomics.com/en/products/dnarna-synthesis/oligo-optimations.aspx>).

Antibodies and other reagents used in this study, gateway cloning method for generating lysine and serine mutants of FR α -V5tag, HPLC-mass spectrometry analysis of OSHP-1, primer sequence information and nuclear/cytoplasmic extraction methods used for coimmunoprecipitation studies are described in the Supporting Information section.

Cranial Neural Crest Cells

Cranial neural crest cell (CNCC) cell line O9-1 obtained from Wnt1-Cre: R26R-GFP from E8.5 mouse embryos were kindly provided by Dr. Robert E. Maxson (Department of Biochemistry

and Molecular Biology, USC/Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA). Basal medium for NC culture was prepared as described by Ishii et al. [24].

Glial Differentiation of O9-1 Cells

To induce glial differentiation, O9-1 cells were cultured for 10 days in: Dulbecco's modified Eagle's medium/F12 (1:1), B27 (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 ng/ml bone morphogenetic protein 2 (BMP2), 50 ng/ml LIF (Millipore, Burlington, MA), 1% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Statistical Analysis

The graph shows an average of six biological replicates \pm SEM. Values given are means \pm SEM. Probabilities (p) were calculated with Student's unpaired t test using GraphPad Prism version 6 (San Diego, CA). p values of $< .05$ were considered to be statistically significant. For multiple comparisons between data, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests were used. All data are expressed as mean \pm SEM. Results were assessed by using two-tailed unpaired Student's t test or two-way ANOVA (GraphPad Prism 6). A p value less than $.05$ was considered significant.

RESULTS

Folate/FR α Causes a Phenotypic Switch from Differentiated Glial Cells to Dedifferentiated Cells

To test the hypothesis that folate/FR α causes a phenotypic switch from differentiated to dedifferentiated cells, we subjected O9-1 cells (a murine E8.5 CNCC line), to glial differentiation medium for 10 days [24]. Figure 1A shows the progression of undifferentiated CNCCs (cell markers such as Sox9, A2B5), to glial differentiation (cell markers such as glial fibrillary acidic protein [GFAP], O4). We observe that in undifferentiated CNCCs, Sox9, and A2B5 are highly expressed, whereas Oct4, Sox2, and Klf4 are expressed at very low levels. When these cells are glial differentiated, they express GFAP and O4. The undifferentiated CNCCs when trypsinized and grown in NeuroBasal medium (supplemented with epidermal growth factor [EGF] and basic fibroblast growth factor [bFGF]) for 4 days in the absence or presence of 100 ng/ml MTHF (Fig. 1B) on low attachment 6-well plates, they form colonies or spheres both with and without the presence of MTHF. It must be noted, however, that the differentiated CNCCs when treated with MTHF show re-expression of A2B5, but not Sox9. Additionally, they express Oct4, Sox2, and Klf4 but not GFAP. The MTHF nontreated glial cells however express GFAP (Fig. 1A, 1C). A2B5 is expressed in MTHF induced dedifferentiated cells, but to a far lesser extent as compared with the undifferentiated CNCCs. Oct4, Sox2, and Klf4 expressions are more in the MTHF-induced dedifferentiated cells as compared with the undifferentiated CNCCs. Additionally, Sox9 expression which is the hallmark of undifferentiated CNCCs is diminished in MTHF-induced dedifferentiated cells. These observations suggest that MTHF drive the dedifferentiation past the CNCC stage.

It was noted that the differentiated cells only form spheres in the presence of MTHF, but not in the absence of MTHF. These spheres, also called "crestospheres" are in vitro maintained primary cultures of premigratory neural crest cells that maintain a

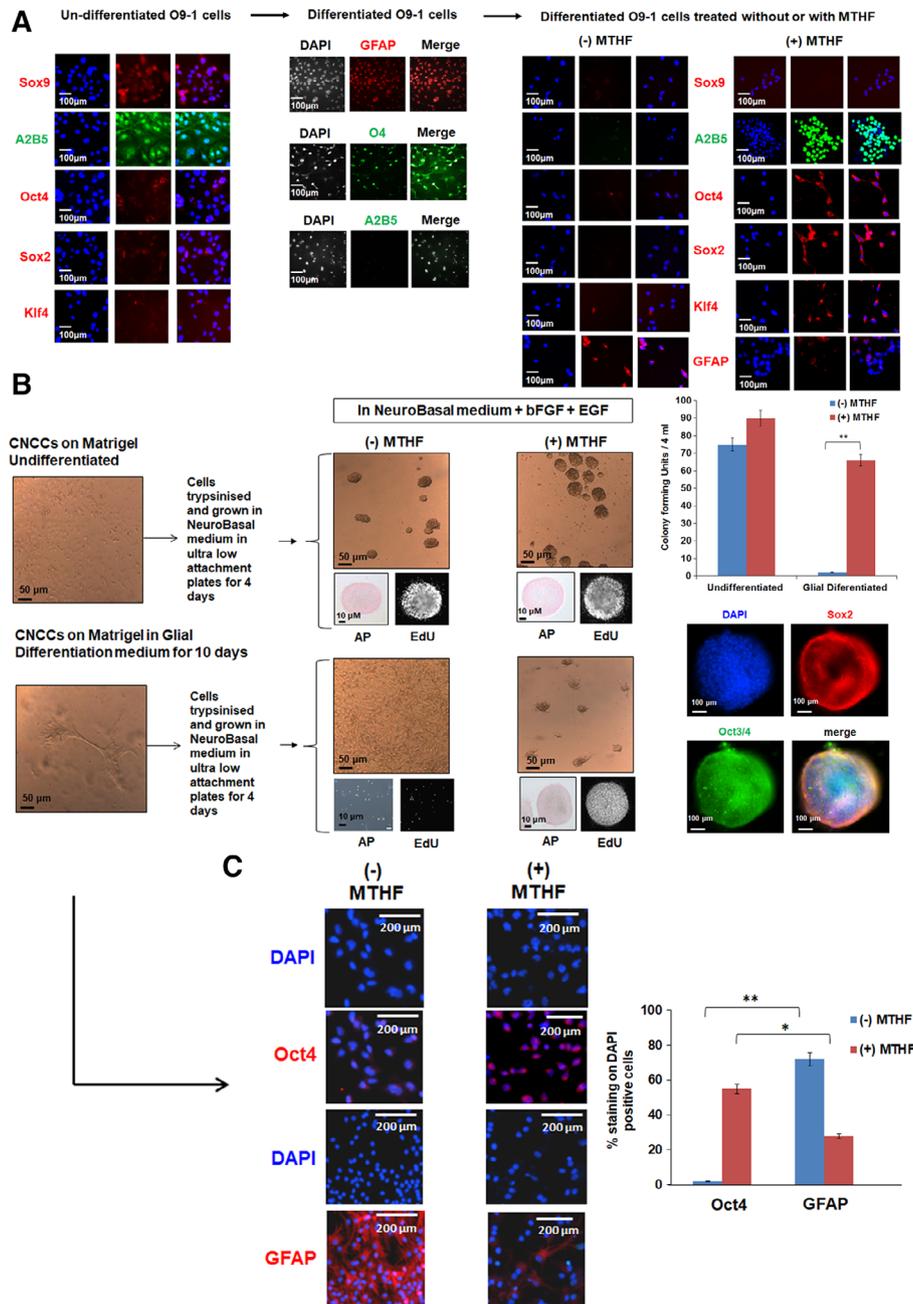


Figure 1. (Continued on next page).

mixture of neural crest stem and progenitor cells for weeks without spontaneous differentiation [24, 25]. These crestspheres stain positive for alkaline phosphatase (AP), and 5-ethynyl-2'-deoxyuridine (EdU) as well as Oct4 and Sox2, but not for GFAP. The glial differentiated cells, however, showed GFAP positive Oct4 negative staining in the absence of MTHF (Fig. 1C), suggesting that folate/FR α may be responsible for these phenotypic changes observed above.

To determine whether these phenotypic changes are due to FR α , the receptor was knocked down by *shRNA-FR α* as described in our previous study [6]. The glial differentiated cells were grown in low attachment plates in NeuroBasal media supplemented with EGF and bFGF for 4 days in the

presence of MTHF. The results (Fig. 1D) show that the *shRNA-FR α* knocked down cells did not form crestspheres, but the scrambled control *shRNA (scr-sh-RNA)* treated cells formed AP and EdU positive crestspheres. Overall, these data suggest that MTHF mediated phenotypic switch from differentiated glial cells to undifferentiated cells may be caused by FR α .

OSHP-1 Blocks the MTHF Mediated Dedifferentiation of Glial Cells and Promotes Redifferentiation

It was hypothesized that if folate/nuclear FR α could cause a phenotypic switch from differentiated glial cells to dedifferentiated Oct4 and Sox2 positive cells, blocking FR α translocation to the nucleus as reported earlier [3, 4], could prevent folate mediated

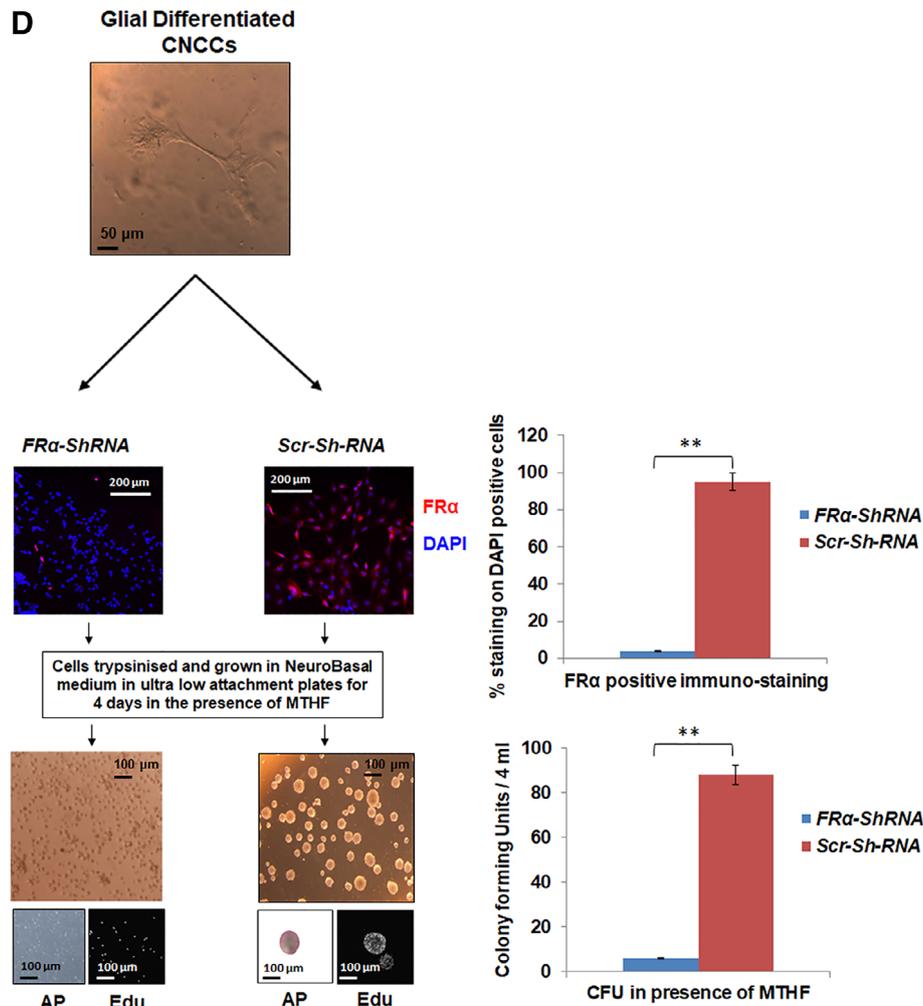


Figure 1. Role of high folate levels and folate receptor- α (FR α) in dedifferentiating the differentiated glial cells. **(A):** Undifferentiated O9-1 cells immunostained for Sox9, A2B5, Oct4, Sox2, and Klf4 and counterstained with 4',6-diamidino-2-phenylindole (DAPI). O9-1 cells were subjected to differentiation into glial cell lineage. O9-1 cells were grown in glial differentiation medium (Dulbecco's modified Eagle's medium/F12, 1-B27 [Invitrogen], 2 mM L-glutamine, 50 ng/ml BMP2, 50 ng/ml LIF [Millipore], 1% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin) for 10 days and then fixed in 4% paraformaldehyde and immunostained for glial fibrillary acidic protein (GFAP), O4, and A2B5 antibodies. After 10 days in glial differentiation medium, the cells were trypsinized and grown in NeuroBasal medium (supplemented with basic fibroblast growth factor (bFGF) [20 ng/ml; R&D Systems] and epidermal growth factor (EGF) [20 ng/ml; Sigma] in ultra-low attachment plates for 4 days in the absence or presence of 100 ng/ml methyl tetra hydro folate (MTHF). The cells were then trypsinized and grown on low growth factor matrigel for 24 hours and then immunostained for Sox9, A2B5, Oct4, Sox2, and Klf4 and counterstained for DAPI. **(B):** O9-1 undifferentiated cells and O9-1 cells subjected to glial differentiation medium. After 10 days in glial differentiation medium, the cells were trypsinized and grown in NeuroBasal medium (supplemented with bFGF [20 ng/ml; R&D Systems] and EGF [20 ng/ml; Sigma] in ultra-low attachment plates for 4 days in the absence or presence of 100 ng/ml MTHF. The crestspheres >50 μ m in size were counted and stained for alkaline phosphatase, 5-ethynyl-2'-deoxyuridine (EdU), Sox2, and Oct4. **(C):** Crestospheres were trypsinized and grown on laminin coated slides overnight, fixed in 4% PFA and stained with Oct4 (for stem cell phenotype) and GFAP (for glial differentiated cell phenotype) and counterstained for nuclei with DAPI. Percentage DAPI positive Oct4 and GFAP cells were counted in each slide using eight visual fields and the average \pm SEM was used to calculate the significance using Student's *t* test. *, $p < .05$; **, $p < .001$. **(D):** The role of FR α in redifferentiation of glial differentiated cells by folate was confirmed by *scrambled shRNA* and *shRNA-FR α* knockdown of FR α . FR α knockdown cells did not form crestspheres in the presence of MTHF but *scrambled shRNA* controls formed.

dedifferentiation. There is no known FR α nuclear translocation blocker reported yet. Since *O. sanctum* extracts have been shown to possess anticancer properties [23,26–28], we used OSHP-1 (hydrophilic fraction-1 of aqueous extracts of *O. sanctum* leaves subjected to 80% methanolic fractionation) to test the hypothesis whether it can (a) block the folate mediated dedifferentiation, (b) reverse the dedifferentiation to redifferentiation, and (c) block FR α nuclear translocation.

Extraction strategy of OSHP-1 is shown in Supporting Information Figure S1. To test which fraction of the extract has the

property of blocking MTHF mediated dedifferentiation, the differentiated glial cells were tested with different fractions of *O. sanctum* leaf extracts for their ability to block the MTHF mediated dedifferentiation of glial cells. Of all the fractions OSHP-1 alone abolished the MTHF mediated dedifferentiation even in the presence of MTHF (Fig. 2A). The O9-1 cells form crestspheres in the absence of MTHF because of the NeuroBasal media which contains bFGF and EGF help in sphere maintenance and formation. The presence of MTHF caused a further increase (30%–40% more) in crestspheres formation.

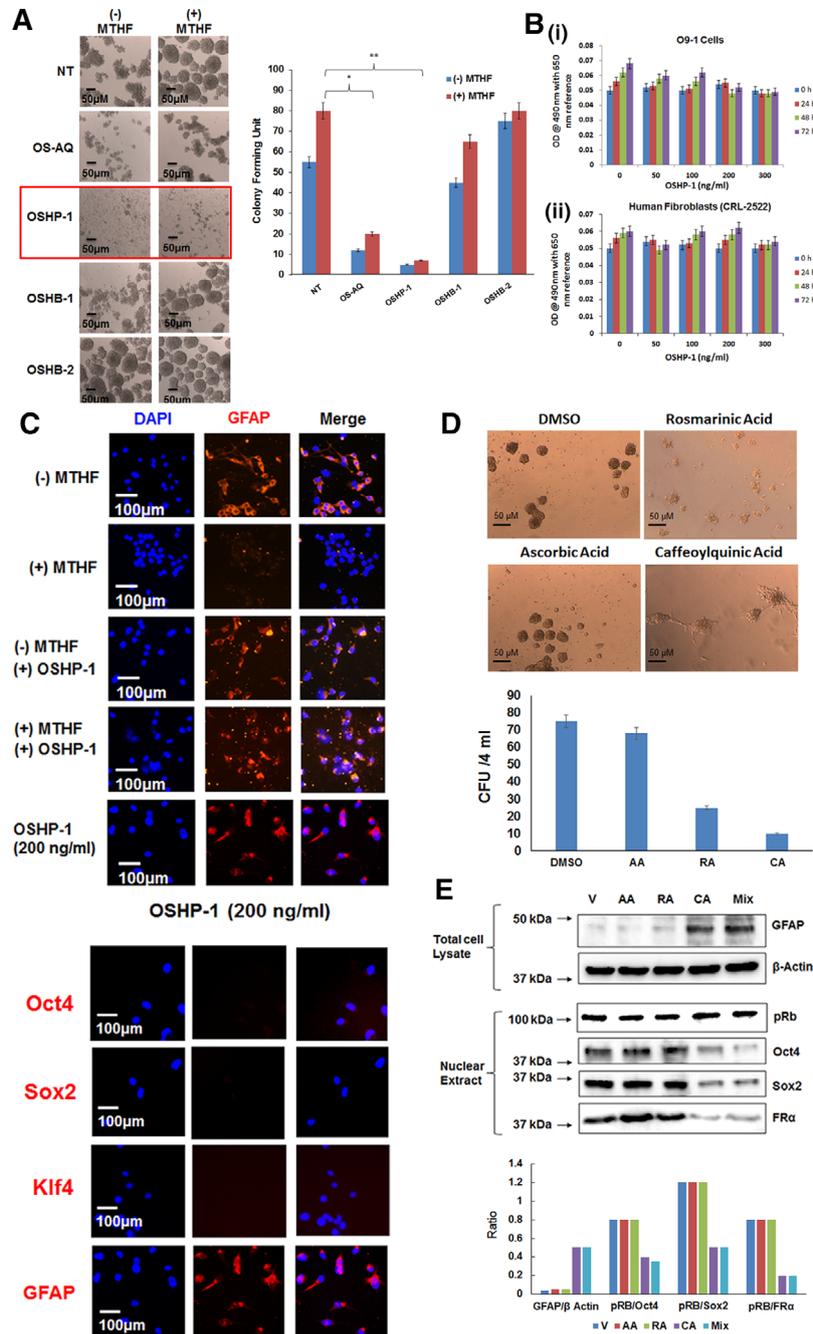


Figure 2. *Ocimum sanctum* hydrophilic fraction-1 (OSHP-1) blocks the methyl tetra hydro folate (MTHF)-mediated dedifferentiation of glial cells and promotes redifferentiation. **(A):** O9-1 cells were first differentiated to glial cells for 10 days. Then they were subjected to (+) or (-) MTHF mediated dedifferentiation in the presence of 200 ng/ml OS-AQ, OSHP-1, OSHB-1, and OSHB-2. The crestospheres >50 μm size were counted ($n = 6$). *, $p < .001$; **, $p < .0001$, Student's t test. **(B):** Cell viability/cell proliferation assays were performed using MTS Assay Kit (ab197010). Two cell lines were taken: (i) O9-1 cells and (ii) human fibroblast CRL-2522 (ATCC). Cells were treated with vehicle or OSHP-1 (50–300 ng/ml) for 24, 48, and 72 hours. After that the cells were washed with PBS three times and assayed for cell viability/proliferation as per the manufacturer's instructions ($n = 4$, each experiment done in triplicate). **(C):** The MTHF mediated dedifferentiated glial cells treated with OSHP-1 in the presence or absence of MTHF or after washing away MTHF were triturated and plated on laminin coated glass slides and immunostained for GFAP and counterstained with DAPI. OSHP-1 caused redifferentiation of spheres obtained from MTHF mediated dedifferentiated glial cells. The MTHF mediated spheres were cultured in the absence of glial differentiation media but in the presence of OSHP-1 (200 ng/ml) for 4 days and then the cells were immunostained for GFAP, Oct4, Sox2, Klf4, and counterstained with DAPI. **(D):** Effect of active components of OSHP-1, AA (AA, 250 μM), rosmarinic acid (RA, 12.5 μg/ml), and caffeoylquinic acid (CA, 125 μM) on MTHF-mediated dedifferentiated cells to form crestospheres. **(E):** MTHF-mediated dedifferentiated O9-1 cells were treated with AA (250 μM), RA (12.5 μg/ml), CA 125 μM, and mixture of AA + RA + CA for 72 hours Western blots of the total cell lysate (RIPA lysates) and nuclear fractions. Fifteen micrograms protein per well was immunoblotted using GFAP, β-actin (positive control), pRb (as nuclear marker control), Sox2, Oct4, and folate receptor-α. Semiquantitative blot quantization was done by the software provided in Bio-Rad ChemiDoc^{MP} system.

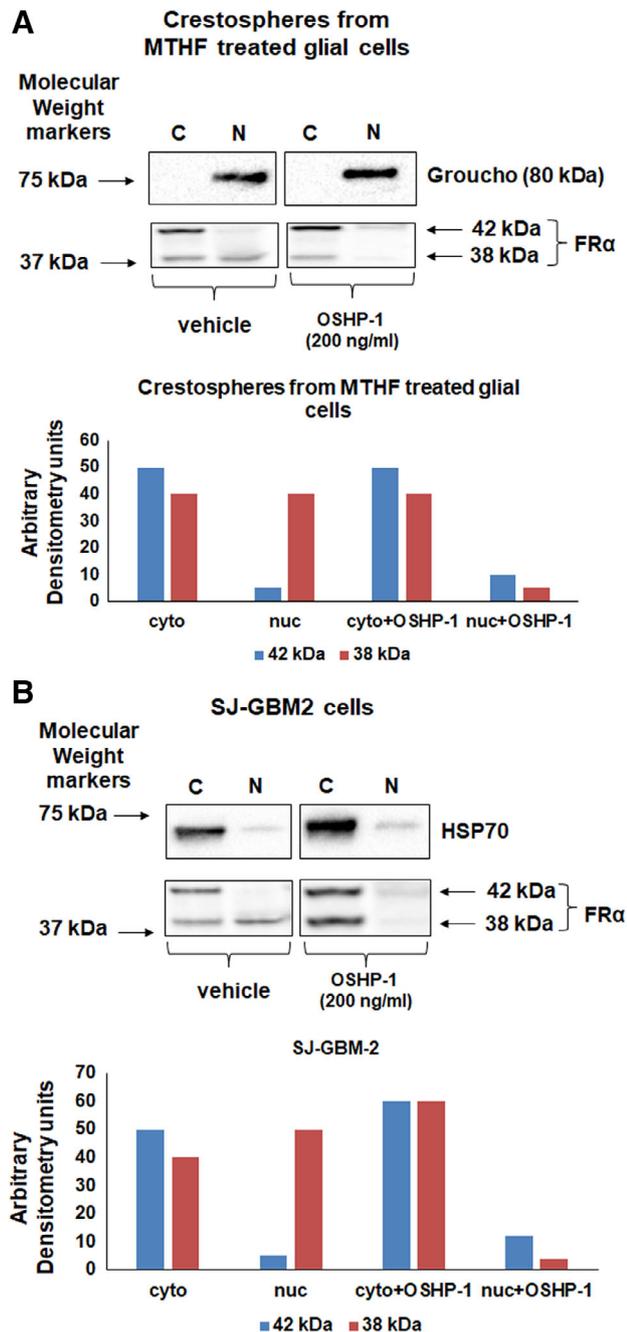


Figure 3. *Ocimum sanctum* hydrophilic fraction-1 (OSHP-1) treatment reduces the nuclear folate receptor- α (FR α) \sim 38 kDa levels. **(A):** Cytoplasmic (C) and nuclear (N) extracts were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) from spheres obtained from MTHF treated glial cells and immunoblotted using FR α antibody. The immunoblot data suggest that after 72 hours of vehicle or OSHP-1 (200 ng/ml) treatment, the \sim 38 kDa FR α level is reduced. **(B):** SJ-GBM2 cells were treated with vehicle or OSHP-1 (200 ng/ml) for 72 hours and then subjected to fractionation into cytoplasmic and nuclear fractions. The purity of cytoplasmic fraction was ascertained by heat shock protein 70 (HSP70). FR α immunoblot using cytoplasmic and nuclear fractions of SJ-GBM2 cells show that the nuclear \sim 38 kDa FR α levels after 72 hours of treatment of OSHP-1 are reduced (Fig. 3B). These observations suggest that the nuclear translocation of the \sim 38 kDa FR α is blocked by OSHP-1 treatment, further strengthening the premise that nuclear FR α in the glial cells may be responsible for dedifferentiation. (Figure legend continues on next page.)

Therefore, we hypothesized that this additional increase in crestospheres formation maybe due to FR α translocating to the nucleus [5,6]. It should be noted that the lack of crestospheres formation by OSHP-1 does not reflect death of the cells. MTS assays demonstrate their viability (Fig. 2B). To rule out toxicity and undue side effects of OSHP-1 we

performed MTS assays on O9-1 cells and human fibroblast cells (CRL-2522 from ATCC) by treating the cells with different concentrations (50–300 ng/ml) of OSHP-1 for different time periods. The data presented in Figure 2B showed that there were no toxicity or undue side effects on these cells at the concentrations studied. Experiment in Figure 2C shows the effect of OSHP-1 in the absence or presence of MTHF, and OSHP-1 alone on MTHF-induced dedifferentiated glial cells. The results show that when the MTHF induced dedifferentiated glial cells were treated with OSHP-1 they showed increased GFAP immunostaining, suggesting that OSHP-1 not only blocked the dedifferentiation of glial cells in culture but also caused the MTHF-induced dedifferentiated cells to redifferentiate (Fig. 2C). To further confirm whether the MTHF induced crestospheres can be made to redifferentiate in the presence of OSHP-1, we cultured them in the absence of glial differentiation media but in the presence of OSHP-1 to test the hypothesis that OSHP-1 can redifferentiate the already dedifferentiated cells, as evidenced by GFAP positive and Oct4, Sox2, and Klf4 negative immunostaining (Fig. 2C, lower panel).

The active fractions of the OSHP-1 were characterized by HPLC fractionation and mass spectrometric analysis and are presented in the supplemental information section (Supporting Information Figs. S2–S7). The HPLC fractionation (Supporting Information Fig. S2) and mass spectrometric analysis (Supporting Information Figs. S3–S7) revealed that the mixture contains three important constituents: AA, RA, and CA. IC₅₀ for AA, RA, and CA on O9-1 cells was 250 μ M, 12.5 μ g/ml, and 125 μ M, respectively. Glial differentiated O9-1 cells were dedifferentiated using MTHF and then treated with AA (250 μ M), RA (12.5 μ g/ml), and CA (125 μ M) constituent of OSHP-1. The AA component of OSHP-1 did not decrease the crestospheres formation, whereas RA decreased it and CA not only decreased the crestospheres formation but also promoted differentiation of the dedifferentiated glial cells (Fig. 2D). Additionally, CA caused a decrease in the \sim 38 kDa FR α , Oct4, and Sox2 in the nucleus, and an increase in the expression of GFAP (Fig. 2E).

To further confirm that OSHP-1 blocks the nuclear translocation of FR α , we used the crestospheres obtained from MTHF treated glial cells and subjected them to subcellular fractionation using the cytoplasmic and nuclear extraction kit from Fisher Scientific. The cytoplasmic and nuclear fractions were immunoblotted using FR α antibody. The purity of nuclear fraction was ascertained by Groucho, a nuclear protein. The immunoblot data (Fig. 3A) suggest that after 72 hours of OSHP-1 (200 ng/ml) treatment, the nuclear \sim 38 kDa FR α level is reduced, thereby strengthening the argument that it may play a major part in dedifferentiation. To confirm the hypothesis that OSHP-1 could block the \sim 38 kDa FR α from translocating to the nucleus, we used SJ-GBM2, recurrent glioblastoma multiforme grade 2 brain tumors. This tumor, which originates from primitive precursors of glial cells or glioblasts [29] was treated with OSHP-1 (200 ng/ml) for 72 hours and then subjected to fractionation into cytoplasmic and nuclear fractions. The purity of cytoplasmic fraction was ascertained by heat shock protein 70 (HSP70). FR α immunoblot using cytoplasmic and nuclear fractions of SJ-GBM2 cells show that the nuclear \sim 38 kDa FR α levels after 72 hours of treatment of OSHP-1 are reduced (Fig. 3B). These observations suggest that the nuclear translocation of the \sim 38 kDa FR α is blocked by OSHP-1 treatment, further strengthening the premise that nuclear FR α in the glial cells may be responsible for dedifferentiation.

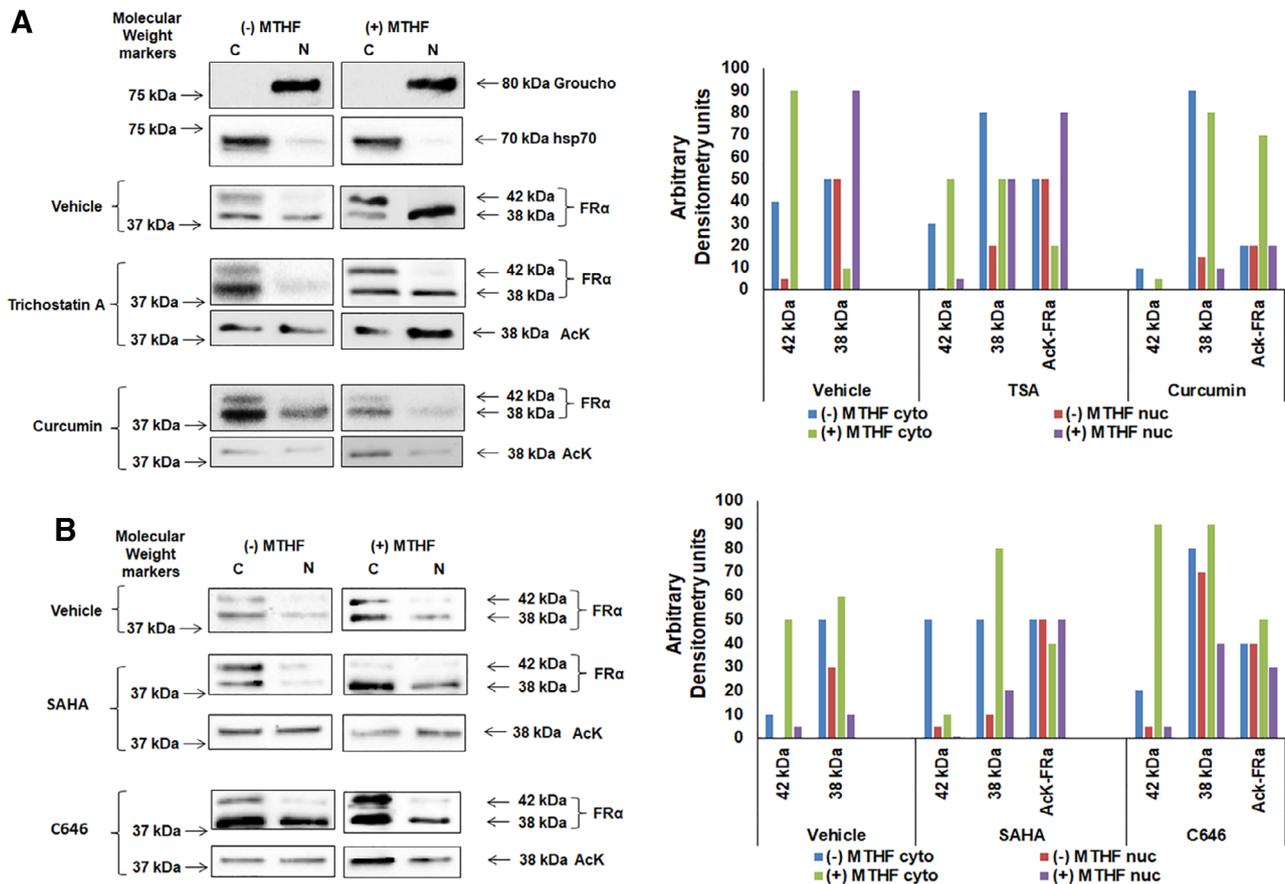


Figure 4. Acetylation of folate receptor- α (FR α) facilitates its nuclear translocation. **(A):** Murine neural crest cells were treated with TSA, a deacetylase inhibitor (10 μ M for 4 hours followed by 10 ng/ml methyl tetra hydro folate [MTHF] for 30 minutes), and curcumin, an acetyltransferase inhibitor (10 μ M for 4 hours followed by 10 ng/ml MTHF for 30 minutes). Fifteen micrograms of protein from cytosolic and nuclear fractions per lane was immunoblotted using anti-rabbit FR α antibody as well as with acetyl lysine antibody. Groucho and hsp70 antibodies were used to assess the purity of nuclear fractions. **(B):** Suberoylanilide hydroxamic acid (SAHA), a known histone deacetylase (HDAC) inhibitor was used at 10 μ M (final concentration), and C646, a known acetyl transferase p300 inhibitor was used at 20 μ M (final concentration) for 24 hours followed by 10 ng/ml MTHF for 30 minutes. Fifteen micrograms of protein from cytosolic and nuclear fractions per lane was immunoblotted using anti-rabbit FR α antibody as well as acetyl lysine antibody. Semiquantitative blot quantization was done by the software provided in Bio-Rad ChemiDoc^{MP} system.

Acetylation and Phosphorylation of FR α Favors its Nuclear Translocation

The above results suggest that folate/nuclear FR α may be responsible for dedifferentiation of differentiated glial cells. Many transcription factor functions and nuclear translocation are controlled by acetylation [9,10]. For example, GATA-1 activity is regulated by p300-mediated acetylation [11]. Other well-characterized targets of nonhistone protein acetylation include p53, nuclear factor- κ B, p65, CBP, p300, STAT3, tubulin, PC4, nuclear receptors, c-Myc, HIF1 α , FoxO1, heat-shock protein, FLT3 kinase, and c-Raf kinase [12,13]. Acetylated Pax3 regulates *Hes1* and *Neurog2*, such that *Hes1* levels decrease and *Neurog2* levels increase when neurogenesis begins [14]. Therefore, we wanted to know whether the nuclear translocation of ~38 kDa FR α is affected by acetylation.

To test this hypothesis, O9-1 cells were treated with trichostatin-A (TSA 10 μ M), a deacetylase inhibitor [30], or curcumin (10 μ M), an acetyltransferase inhibitor [31] for 4 hours, with each treatment followed or not followed with 10 ng/ml 5'-methyl-tetra-hydro-folate (MTHF) added to the media for 30 minutes. Fifteen micrograms of protein from cytosolic or nuclear fractions per

lane were immunoblotted using anti-rabbit FR α antibody. Hsp70 and Groucho served as the loading controls as well as markers for the purity of cytoplasmic and nuclear fractions, respectively. In the presence of Trichostatin A followed by MTHF treatment, the localization of the ~38 kDa FR α in the nucleus was higher than in the absence of MTHF (Fig. 4A). Trichostatin A is a general inhibitor of deacetylase hence it is expected that FR α among other proteins remains acetylated. In the absence of MTHF, the nuclear translocation of acetylated FR α as confirmed by immunoprecipitation by FR α antibody and crossblotting with acetyl-lysine antibody, is less compared with the MTHF treated samples, suggesting that MTHF favors the translocation of acetylated form of FR α . Exact opposite results were observed with curcumin, a p300 acetyltransferase inhibitor, treatment. In the presence of MTHF, there was less nuclear localization of ~38 kDa FR α than in its absence. Overall, the results suggest that acetylation of ~38 kDa FR α favors its nuclear translocation. We however observed the FR α -38 kDa in the nucleus after TSA treatment (in the absence of MTHF) to be lower as compared with vehicle. The reason why this may be so, is because with TSA treatment FR α may be acetylated, but its nuclear translocation is favored by MTHF. We do not know the reason why the nuclear

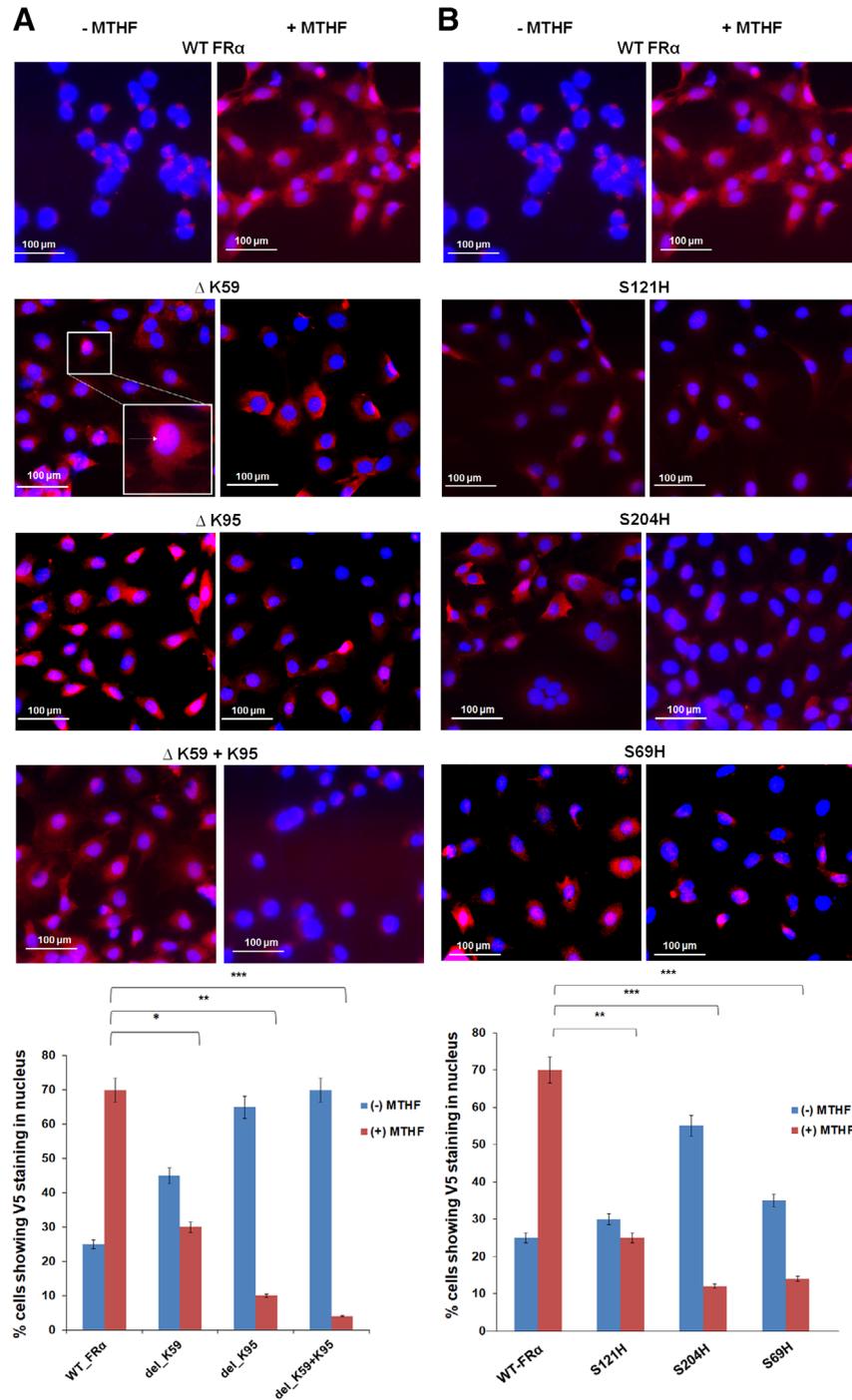


Figure 5. Lysine and serine mutants of folate receptor- α (FR α) show that acetylation and phosphorylation of FR α favor its nuclear translocation. **(A):** Lysine residues K59 and K95 play an important role in FR α nuclear localization. Using gateway cloning BP reaction protocol, deletion mutants of FR α with deleted K59 and K95 and K59 + K95 double mutants were created with V5 tag introduced after the alanine (A231) amino acid. Forty-eight hours post-transfection of these FR α lysine deletion mutants in O9-1 cells, these cells were treated with methyl tetra hydro folate (MTHF; 10 ng/ml) for 30 minutes. The cells were fixed and immune-stained with V5 antibody and counterstained with DAPI nuclear stain. $n = 6$ and each data point consisted of 10 visual fields and the average used for calculating significance between MTHF treated and nontreated cells. *, $p < .05$; **, $p < .001$; ***, $p < .0001$. **(B):** Serine residues S69, S121, and S204 play an important role in FR α nuclear localization. Serine substitution mutants of FR α : S121H, S204H, and S69H, with V5 tag introduced after the alanine (A231) amino acid. Forty-eight hours post-transfection of these FR α lysine deletion mutants in O9-1 cells, these cells were treated with MTHF (10 ng/ml) for 30 minutes. The cells were fixed and immune-stained with V5 antibody and counterstained with DAPI nuclear stain. $n = 6$ and each data point consisted of 10 visual fields and the average used for calculating significance between MTHF treated and nontreated cells. **, $p < .001$; ***, $p < .0001$. **(C, D):** V5 immunoblots of the FR α lysine deleted and serine substituted constructs. Ten micrograms of nuclear proteins per lane were used. Semiquantitative blot quantization was done by the software provided in Bio-Rad ChemiDoc^{MP} system.

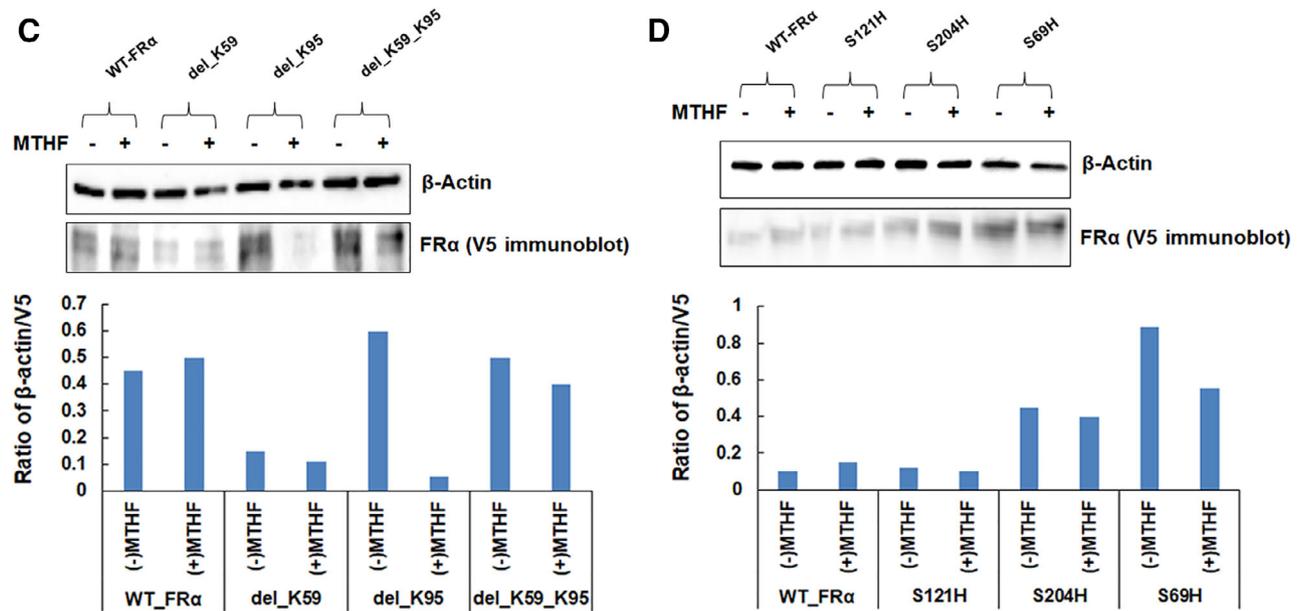


Figure 5. Continued.

localization of FRα is higher in the vehicle. At best, we could ascribe it to baseline acetylation of FRα. That is why we reconfirmed the above data with other well-known inhibitor of histone deacetylase (HDAC) and acetyl transferases, such as suberoylanilide hydroxamic acid [32] and C646 [33], respectively, and observed almost similar results (Fig. 4B).

To reconfirm the observation that acetylation of FRα favors its nuclear translocation, an online program which predicts the acetylation of internal lysine residues on FRα (<http://bdmpail.biocuckoo.org/prediction.php>) was used to determine which lysine residues have the highest predication score for becoming acetylated. Lysine 59 and lysine 95 residues were identified with this program (Supporting Information Fig. S2). We asked, what happens to the nuclear translocation of FRα in the absence of lysine residues. Using the gateway (Invitrogen) cloning BP reaction protocol, we generated lysine deletion mutants of FRα: ΔK59, ΔK95, and ΔK59 + 95 and introduced the V5 tag after the alanine 231 (A231) to detect FRα. O9-1 cells were transfected with tagged FRα lysine deletion mutants and treated with MTHF (10 ng/ml) for 30 minutes 48 hours post-transfection. The cells were fixed and immune-stained with V5 antibody and counterstained with DAPI nuclear stain. Figure 5A shows that deleting lysine residues 59 and 95 decreased FRα nuclear translocation. These observations strongly suggest that nuclear translocation is favored by acetylated FRα, with acetylation of K59 and K95 potentially playing a significant role in this process.

Phosphorylation of several transcription factors are responsible for their nuclear import; therefore, we hypothesized that serine phosphorylation may be involved in FRα nuclear translocation. We asked, what happens to the nuclear translocation of FRα if serine residues S121, S204, and S69 are substituted by a structurally similar amino acid histidine. To address this question, we generated serine substitution mutants in FRα: S121H, S204H, and S69H, and introduced the V5 tag after A231 to test this hypothesis. We chose these serine residues because they have the maximum context scores of 0.976, 0.985, and 0.984, respectively, for likely sites for potential phosphorylation (NetPhos 3.1 for prediction of serine

phosphorylation sites, <http://www.cbs.dtu.dk/services/NetPhos/>). Figure 5B shows that substituting serine residues S204 and S69 with histidine significantly decreased FRα nuclear translocation. These observations suggest that the nuclear translocation of FRα is favored by phosphorylation at S204 and S69 residues. Taken together, the above data suggest that acetylation of FRα at K95 and K59 residues and phosphorylation at S204 and S69 favors its nuclear translocation.

A representative sample of confocal images of wild-type FRα-V5, FRα-V5-deleted K59, or FRα-V5-S69H transfected cells without or with MTHF treatment O9-1 cells are presented in Supporting Information Figure S9.

To confirm the above V5 immunostaining results, we performed the V5 immunoblots of the lysine deletion mutants and serine substitution mutants and observed that the results although not quantifiable, were almost similar to the immunostaining data (Fig. 5C, 5D).

Nuclear FRα Binds to CAF-1 (p60) in MTHF Induced Dedifferentiated Cells and Not in Differentiated or Redifferentiated Glial Cells

To understand the mechanism of MTHF induced dedifferentiation and OSHP-1 mediated redifferentiation of dedifferentiated cells, initially we used coimmunoprecipitation assays, but owing to the masking effects of heavy chain, we were unable to clearly identify the p60, (60 kDa band of CAF-1), or 50 kDa band of HDAC2, so we used Amino-Link beads (Thermo-Fisher, Catalog number: 44890) on which the CAF-1 (p60) polyclonal antibody was covalently attached (immobilized) to make chromatography columns for use in affinity purification of CAF-1 (p60) associated proteins (HDAC2, FRα, and proliferating cell nuclear antigen [PCNA]) as per the manufacturer's instructions. We used three rounds of purification from nuclear extracts and eluted the CAF-1 interacting protein complex by 150 mM Glycine HCl (Ph.2.5) and neutralized by 1 M phosphate buffer pH 9.0. The eluted proteins were concentrated by Centricon-10

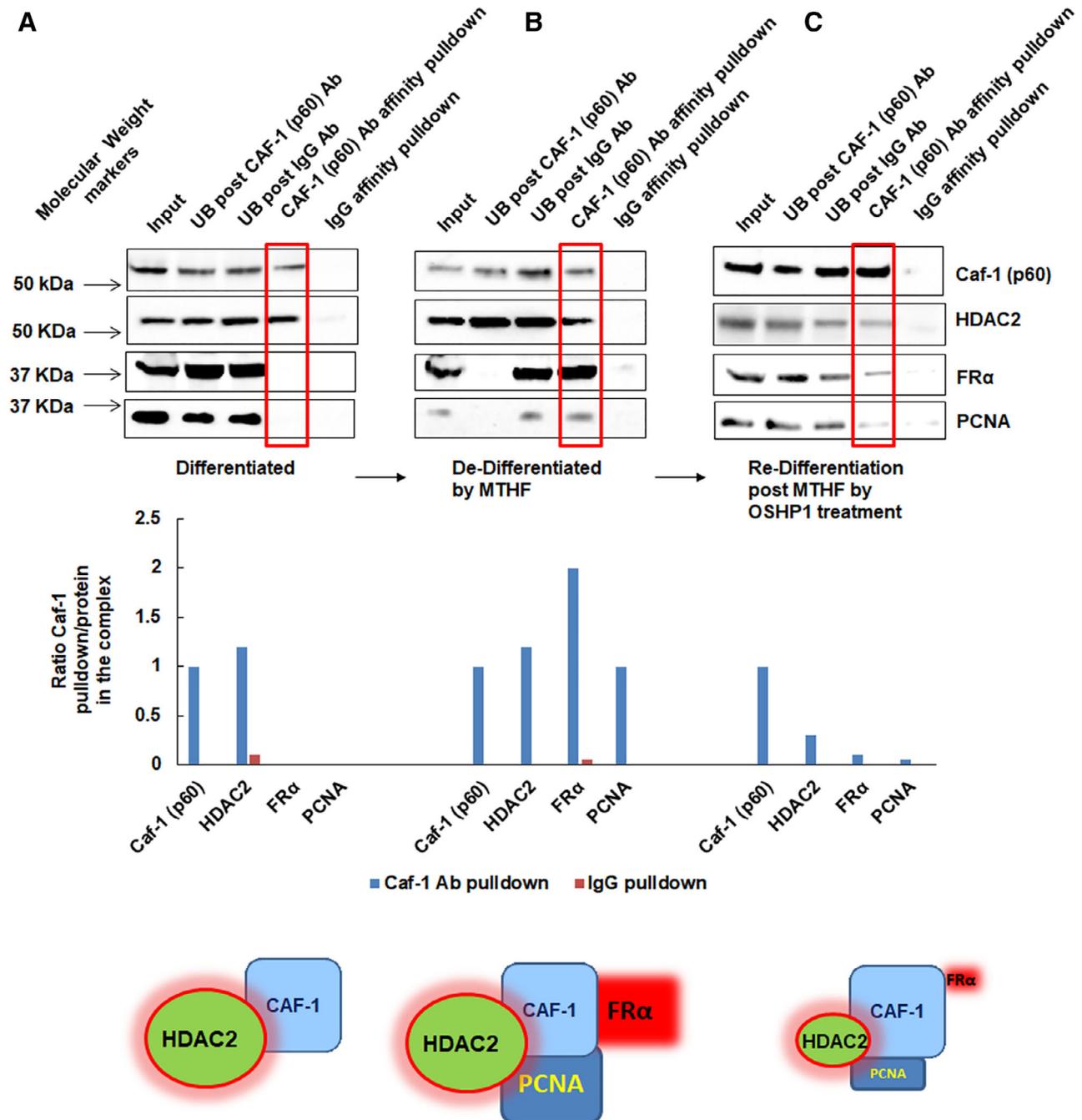


Figure 6. Nuclear folate receptor- α (FR α) binds to chromatin assembly factor-1 (CAF-1) in methyl tetra hydro folate (MTHF) induced dedifferentiated cells and not in differentiated glial cells. **(A):** Represents the experiment using glial differentiated cells obtained by treating O9-1 cells to glial differentiating conditions. **(B):** Represents the experiment using dedifferentiated cells obtained by treating the glial cells obtained from condition A with MTHF. **(C):** Represents the experiment using redifferentiated cells obtained by treating the cells from condition B with *Ocimum sanctum* hydrophilic fraction-1 (OSHP-1). A mixture of soluble nuclear proteins and soluble chromatin proteins from (A) glial differentiated cells O9-1 cells; (B) MTHF-mediated dedifferentiated spheres; (C) OSHP-1 mediated redifferentiated glial cells were subjected to copurification of the CAF-1 associated protein complex using CAF-1 mouse polyclonal antibody and the CAF-1 copurified were crossblotted with CAF-1, FR α , proliferating cell nuclear antigen (PCNA), and HDAC2 antibodies. The data suggest that in glial differentiated O9-1 cells the CAF-1 interacts with HDAC2. In the MTHF-mediated dedifferentiated spheres CAF-1 interacts with HDAC2, FR α , and PCNA. In OSHP-1 treated spheres which showed redifferentiation, the CAF-1 associates strongly with HDAC2 and not as strongly with FR α or PCNA. Semiquantitative blot quantization was done by the software provided in Bio-Rad ChemiDoc^{MP} system.

filters and immunoblotted using CAF-1 (p60), HDAC2, FR α , and PCNA antibodies.

CAF-1 suppression has been reported to enhance the reprogramming of somatic cells to induced pluripotent stem cells (iPSCs)

and is enough to restore embryonic stem cells (ESCs) to a totipotent-like state [34, 35]. Since CAF-1 interacts with PCNA and nucleosome remodeling deacetylase (NuRD) complex, we hypothesized that CAF-1 activity may be suppressed by nuclear FR α binding to the CAF-1/

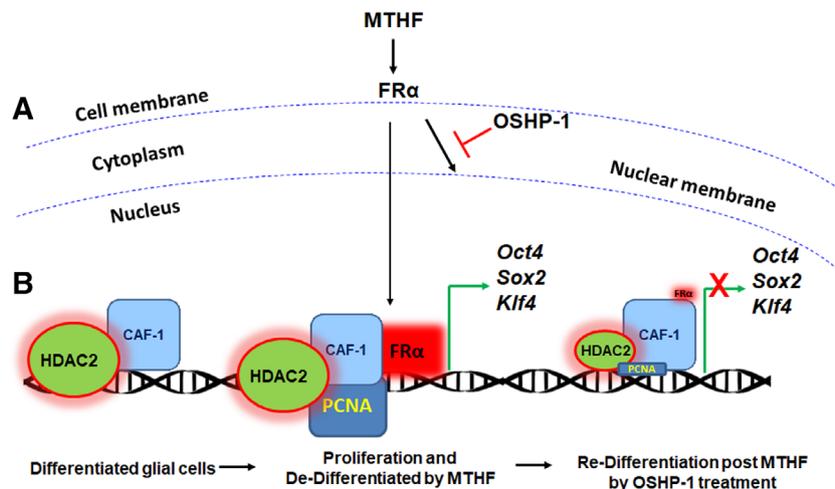


Figure 7. Hypothetical model: a possible hypothetical mechanism by which *Ocimum sanctum* hydrophilic fraction-1 (OSHP-1) prevents the formation of folate receptor- α (FR α) complex could be as follows: (A): the caffeoylquinic acid component of OSHP-1 presumably decrease acetylation of FR α by decreasing the acetyl transferase or increasing the deacetylases activity, and subsequent nuclear translocation; (B): since nuclear FR α binds to chromatin assembly factor-1 (CAF-1) in folate induced dedifferentiated cells and not in differentiated glial cells, the reduction of FR α in the nucleus by OSHP-1 may reactivate CAF-1 and favor differentiation.

NuRD/PCNA complex thereby causing the differentiated glial cells to undergo MTHF mediated dedifferentiation; and OSHP-1 treatment restores the CAF-1 function by disrupting the dedifferentiation promoting complex in the nucleus and causing redifferentiation. To test this hypothesis, we copurified using anti-CAF-1 (p60) polyclonal antibody linked to Amino-Link beads and the mixture of soluble nuclear protein and chromatin fractions obtained from the following experimental conditions: condition A, represents the experiment using glial differentiated cells obtained by treating O9-1 cells to glial differentiation; condition B, represents the experiment using dedifferentiated cells obtained by treating the glial cells obtained from condition A with MTHF; condition C, represents the experiment using redifferentiated cells obtained by treating the cells from condition B with OSHP-1.

The CAF-1 and its interacting proteins were resolved on 4%–20% SDS-PAGE and crossblotted using HDAC2, PCNA, and FR α antibodies. Figure 6A shows that CAF-1 (p60) interacts with HDAC2 component of NuRD complex and not PCNA or FR α in glial differentiated cells. Upon MTHF-mediated dedifferentiation of these glial cells, the CAF-1 (p60) is seen associated with HDAC2 component of NuRD-PCNA-FR α complex (Fig. 6B). When these dedifferentiated cells were treated with OSHP-1, they redifferentiate and only CAF-1 is seen associated with HDAC2 (Fig. 6C). Taken together, the above results may suggest the role of FR α in MTHF-mediated dedifferentiation of glial cells by blocking the ability of CAF-1 to safeguard the differentiated glial cell identity [34, 35]. OSHP-1 treatment possibly frees FR α and PCNA from the dedifferentiation complex, thereby restoring the function of CAF-1.

DISCUSSION

FR α and FR β are present on many cancers with little expression in normal tissues [36]. Because FR α expression is elevated in cancers, the use of tumor-targeted fluorescent folate dyes to illuminate undetected malignant tissue and thereby facilitate its surgical resection has shown promise for reducing morbidity

and mortality associated with unresected malignant disease [37]. Recent studies have shown FR α as potential molecular target in cancer therapies [38, 39].

The clinical significance of our study would be best understood in the context of a near dramatic increase in glial cells derived supratentorial pediatric brain tumors (<https://seer.cancer.gov/>), following folic acid fortification in the U.S. started in the year 1996. Although, the birth prevalence of neural tube defects (NTDs; proportion of babies in the population born with an NTD) decreased by 35% and more over the years in the U.S. since folic acid fortification was made mandatory in 1998, the rise of supratentorial pediatric brain tumors rose exponentially since then (Supporting Information Figs. S10, S11). The supratentorial region gives rise to ependymal type ependymomas which are of glial origin. This work offers a plausible mechanism to show how high folate via FR α may trigger tumorigenesis in the supratentorial region of the brain in the pediatric population.

Supplementation of folate combined with mandatory fortification of foods has led to high levels of folic acid and related metabolites in women of childbearing age [40]. Recent studies have reported that over-supplementation, defined as exceeding either the recommended dietary allowance or the upper limit of the daily reference intake of folic acid, may have negative effects on human health [40]. This work delineates how high folate levels could cause dedifferentiation of already differentiated glial cells. Using a cellular model of CNCCs O9-1 that can be differentiated into glial cells expressing GFAP, we tested the hypothesis that high folate can dedifferentiate these glial cells back to cells displaying proliferative stem cell characteristics expressing Sox2 and Oct4. Our data also show that the folate mediated dedifferentiation can not only be blocked by OSHP-1 but the dedifferentiated cells can be made to redifferentiate to glial cell phenotype as well. OSHP-1 treatment reduced the levels of nuclear ~38 kDa FR α in crestospheres from MTHF treated glial cells. In summary, our data show that FR α occupies the center stage of high folate mediated dedifferentiation, and OSHP-1 blocks the nuclear translocation of ~38 kDa FR α . Therefore, the possible hypothetical

mechanism(s) by which OSHP-1 prevents the formation of FR α complex could be as follows: (a) The CA component of OSHP-1 presumably decrease acetylation of FR α by decreasing the acetyl transferase or increasing the deacetylases activity, and subsequent nuclear translocation; (b) since nuclear FR α binds to CAF-1 in folate induced dedifferentiated cells and not in differentiated glial cells, the reduction of FR α in the nucleus by OSHP-1 may reactivate CAF-1 and favor differentiation (Fig. 7).

The role that lysine acetylation in the cytoplasm plays in driving and coordinating key events such as cytoskeleton dynamics, intracellular trafficking, vesicle fusion, metabolism, and stress response is beginning to be understood and appreciated [41]. Acetylation directs survivin nuclear localization to repress STAT3 oncogenic activity [42]. Similarly, acetylation of retinoblastoma protein by P300/CBP-associated factor (PCAF) is required for nuclear localization and keratinocyte differentiation [43]. Earlier, we have shown the Pax3 acetylation results in decreased Hes1 and increased Neurog2 activity, thereby promoting sensory neuron differentiation [14]. Our data suggested that decrease in acetylated FR α causes a decrease in MTHF mediated nuclear translocation. It is somewhat surprising that the absence of MTHF showed increased nuclear localization in lysine mutants (Fig. 5A). These observations could indicate that folate may also cause a decrease in proliferation in certain cancers where lysine mutations could be found on the FR α , and nuclear localization of FR α could be reduced by folate supplementation. Whole exome-sequencing could shed light on such a possibility in certain human cancers. Folic acid inhibits COLO-205 colon cancer cell proliferation through activating the FR α /c-SRC/ERK1/2/NF κ B/TP53 pathway [44]. Additionally, our data show that the nuclear translocation of FR α is favored by serine phosphorylation at S204 and S69 residues, implying that FR α -kinase specific inhibitors which may prevent phosphorylation of FR α which could block its nuclear translocation and hence can be used in the treatment of certain types of cancer. FR α -specific kinases are not yet reported, and it would be a fascinating new avenue of research. There are a couple of anomalous observations. First, in the absence of MTHF, there is a low level of nuclear localization of FR α . Why we observe this is not very clear, but this can be attributed to low-level turnover of FR α nuclear trafficking in the absence of MTHF. Second, it appears that the overall expression of mutant FR α is affected by the lysine and serine mutations. The only logical explanations can be the rapid lysosomal degradation of aberrant mutant-FR α proteins artificially created.

Of the different phytoconstituents in *O. sanctum*, only a few have antitumor properties: Flavonoids-Apigenin, Luteolin; Phenylpropanoids-RA and Eugenol; Sesquiterpenes-Taxol, Ursolic acid and Oleanolic acid; Sterols-Sitosterol. Many extraction procedures to isolate the active anticancer fraction from *O. sanctum* [23,45,46] are reported in literature, but they all contain mixtures of other active ingredients. For example, the active ingredient eugenol has anticancer [47] and inflammatory function [27] but it is also hepatotoxic [48,49]. Other extraction methods [28] reported the presence of methyl chavicol (estragole) which is a mild hepatocarcinogen [49,50] that has shown genotoxicity [51]. Our method of extraction (Supporting Information Fig. S1) is unique because it contains AA, RA, and CA which are neither hepatotoxic nor genotoxic.

AA has been shown recently to enhance the efficacy of bromodomain and extra terminal inhibitors (BETi) by decreasing

acetylation of histone H4 but not H3 [52]. Similarly, RA decreased cell proliferation in a cell viability assay, and inhibited colony formation and tumor spheroid formation, along with a decrease in HDAC2 expression [53]. 5-CA inhibits invasion of nonsmall cell lung cancer cells through the inactivation of p70^{S6K} and Akt activity [54]. Here, we show that CA can be instrumental in causing redifferentiation of rapidly dividing cells by upregulating GFAP, a marker of glial cell differentiation, and downregulating Sox2 and Oct4 expression.

Several questions remained unresolved: How CA component of OSHP-1 reduces the nuclear localization of FR α ? From the data, it appears that CA component of OSHP-1 may either be decreasing the acetyl transferase activities or increasing the deacetylases activity in the cytoplasm; or may be decreasing the FR α -specific kinase activity which phosphorylates the serine residues S204 and S69. How CA component OSHP-1 promotes redifferentiation of the dedifferentiated cancer cells is also not known. Future studies will highlight some of these questions.

In summary, our work shows that (a) nuclear FR α can cause a phenotypic switch from differentiated glial cells to dedifferentiated cells. (b) *O. sanctum* hydrophilic fraction 1 (OSHP-1) treatment both blocks the folate mediated dedifferentiation of glial cells and promotes redifferentiation of folate-mediated dedifferentiated glial cells. (c) OSHP-1 treatment reduces the nuclear FR α ~38 kDa levels; (d) acetylation at K95 and K59 residues and phosphorylation at S204 and S69 residues of FR α favors its nuclear translocation, and (e) nuclear FR α binds to CAF-1 in folate induced dedifferentiated cells and not in differentiated glial cells.

CONCLUSION

The work identifies a plausible mechanism for the role folate/FR α in dedifferentiation of glial cells by interacting with CAF-1 in folate induced dedifferentiated cells and not in differentiated cells. Hydrophilic fraction -1 of *O. sanctum* (OSHP-1) treatment reduces the FR α levels in the nucleus and also reduces the interaction with CAF-1.

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AUTHOR CONTRIBUTIONS

S.M., V.M.: conception and design, collection and/or assembly of data, final approval of manuscript; M.K.: collection/

assembly of data, final approval of manuscript; G.Y., R.K., J.C., B.R.S.: collection and/or assembly of data, final approval of manuscript; S.I., T.T.: conception and design, final approval of manuscript; G.X.: conception and design, manuscript revision/editing, final approval of manuscript; C.S.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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