

Report

# N-CoR Pathway Targeting Induces Glioblastoma Derived Cancer Stem Cell Differentiation

Deric M. Park<sup>1</sup>

Jie Li<sup>1</sup>

Hiroaki Okamoto<sup>1</sup>

Oluwaseun Akeju<sup>2</sup>

Stephanie H. Kim<sup>1</sup>

Irina Lubensky<sup>1</sup>

Alexander Vortmeyer<sup>1</sup>

James Dambrosia<sup>3</sup>

Robert J. Weil<sup>4</sup>

Edward H. Oldfield<sup>1</sup>

John K. Park<sup>2,\*</sup>

Zhengping Zhuang<sup>1,\*</sup>

<sup>1</sup>Surgical Neurology Branch; and <sup>2</sup>Surgical and Molecular Neuro-oncology Unit; National Institute of Neurological Disorders and Stroke; National Institutes of Health; Bethesda, Maryland USA

<sup>3</sup>Biostatistics Branch; National Institutes of Neurological Disorders and Stroke; National Institutes of Health; Bethesda, Maryland USA

<sup>4</sup>Brain Tumor Institute; Cleveland Clinic Foundation; Cleveland, Ohio USA

\*Correspondence to: Zhengping Zhuang; Surgical Neurology Branch; National Institute of Neurological Disorders and Stroke; National Institutes of Health; Building 10, Room 5D37; Bethesda, Maryland 20892 USA; Tel.: 301.435.8445; Fax: 301.480.1839; Email: zhuangp@ninds.nih.gov/ John K. Park; Surgical and Molecular Neuro-oncology Unit; National Institute of Neurological Disorders and Stroke; National Institutes of Health; Building 35, Room 2B-1002; Bethesda, Maryland 20892 USA; Tel.: 301.435.8445; Fax: 301.480.1839; Email: parkjk@ninds.nih.gov

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## KEY WORDS

N-CoR, glioma, cancer stem cell, differentiation, retinoic acid

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## ABSTRACT

Nuclear receptor corepressor (N-CoR) is a critical regulator of neural stem cell differentiation. Nuclear localization of N-CoR is a feature of undifferentiated neural stem cells and cytoplasmic translocation of N-CoR leads to astrocytic differentiation. Comparative proteomic analysis of microdissected glioblastoma multiforme (GBM) specimens and matched normal glial tissue reveals increased expression of N-CoR in GBM. In GBM primary cell cultures, tumor cells with nuclear localization of N-CoR demonstrate an undifferentiated phenotype, but are subject to astroglial differentiation upon exposure to agents promoting phosphorylation of N-CoR and its subsequent translocation to the cytoplasm. Treatment of glioma cell lines with a combination of retinoic acid and low-dose okadaic acid decreases the corepressor effect of N-CoR and has a striking synergistic effect on growth inhibition. The identification of N-CoR in GBM provides insights into the tumorigenesis process and supports the development of differentiation-based therapeutic strategies.

## INTRODUCTION

Patients with GBM, the most common and malignant primary brain tumor, have a median survival of one year.<sup>1</sup> This grim prognosis has remained virtually unchanged over the past several decades despite technological advances in clinical medicine and active research in GBM pathogenesis. The infiltrative growth of the tumor into surrounding parenchyma, which is a major reason for its refractory nature, has been suspected to reflect the property of the tumor cell of origin rather than a particular genetic defect.<sup>2</sup> Consonant with this idea, several groups have recently identified GBM derived cells that have a stem cell-like phenotype, are capable of self-renewal, and can recapitulate the original source tumor.<sup>3-5</sup> These findings suggest that an undifferentiated tumor cell of origin is responsible for the high proliferation index of these aggressive tumors and that a malignant stem cell compartment may serve as a rational therapeutic target. The elucidation of cancer stem cell (CSC) growth and differentiation mechanisms is therefore likely to reveal new phenotypic markers for CSC as wells as targets for pharmacologic intervention. To identify such markers and targets, we compared the proteomes of GBM and normal brain tissue, and found increased expression of the nuclear receptor corepressor (N-CoR) an essential regulator of the normal neural stem cell pool, in GBM. N-CoR is expressed in the nucleus of neural stem cells (NSC).<sup>6</sup> Following phosphatidylinositol-3-OH kinase/Akt1 kinase-dependent phosphorylation, N-CoR translocates to the cytoplasm and induces the astrocytic differentiation of NSC.<sup>6</sup> Nuclear retention of N-CoR, therefore, is necessary for the maintenance of NSC in the undifferentiated state. The importance of N-CoR in the regulation of NSC differentiation led us to investigate a possible parallel function in the differentiation of CSC. Our findings demonstrate the nuclear expression of N-CoR by undifferentiated CSC and suggest a therapeutic strategy targeting its mechanism of action.

## MATERIALS AND METHODS

**Microdissection, two-dimensional gel electrophoresis and protein identification.** Selective tissue microdissection of the seven GBM specimens and seven matched normal controls was performed as described previously.<sup>7</sup> Briefly, a 10  $\mu$ m-thick section was taken from all tissue specimens and stained with H&E for histologic evaluation. A semiquantitative cell count was obtained in tumor-rich (>90% tumor cells) regions uncompromised by necrosis, inflammation and endothelial proliferation. Corresponding areas on subsequent serial sections were then subjected to microdissection to yield approximately 50,000 cells.

Tissue microdissection was done manually on unstained sections to avoid introduction of heat-related artifacts caused by laser-assisted technology or chemical artifacts induced by tissue staining.<sup>8</sup> Protein content was obtained for all samples and found to be equivalent. Microdissected cells were placed into 50  $\mu$ L of extraction buffer II containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 0.2% (w/v) Bio-Lyte 4/7, and 2 mM tributyl phosphine (Bio-Rad, Hercules, CA), vortexed at room temperature for 10 minutes, and centrifuged in a microcentrifuge at 12,000 rpm for 10 minutes. The supernatant was combined with 200  $\mu$ L of rehydration buffer (Bio-Rad) containing 8 M urea, 2% CHAPS, 50 mM DTT, and 0.2% (w/v) Bio-Lyte 4/7 ampholytes, before isoelectric focusing.

The first dimension of two-dimensional gel electrophoresis was performed on a Protean IEF System (Bio-Rad) with ReadyStrip IPG strips (pH4/7, 11cm) (Bio-Rad) rehydrated with 185  $\mu$ L of sample for 12 hours and subsequently subjected to high voltages at 20°C for electric focusing: 250 V for 20 minutes, 8000 V for 2 hours and 30 minutes, and a final step of 2,500 V for 10 hours. IPG strips were washed in rehydration buffer I containing 6 M urea, 2% SDS, 375 mM Tris-HCl (pH 8.8), 20% glycerol, and 2.5% (w/v) iodoacetamide (Bio-Rad), for 10 minutes each. Criterion Precast Gels (8-16% Tris-HCl, 1.0 mm; Bio-Rad) were used for the second dimension of protein separation in a Criterion Dodeca cell (Bio-Rad) under a constant voltage of 200 V for 55 minutes. Silver Stain kit (Bio-Rad) was used to detect protein spots according to the manufacturer's instructions. Methanol and acetic acid needed for fixing gels were obtained from Sigma-Aldrich (St. Louis, MO). All samples were run in duplicate to confirm the gel electrophoretic patterning.

Statistically significant ( $p < 0.05$ ) protein spots of interest were excised from the gel, placed into 0.5 ml tubes, and stored at -20°C. Silver-stained spots were de-stained as previously described.<sup>9</sup> Then, each protein gel spots were subjected to reduction and alkylation, followed by in situ digestion with trypsin. The resultant peptide mixtures were recovered by sequential extraction and dried to near completion in a vacuum centrifuge, and diluted to 4 mL final volume in 2% CH<sub>3</sub>CN, 0.1% HCO<sub>2</sub>H, 0.01% trifluoroacetic acid, as previously described.<sup>10</sup> Peptides from in-gel digests were analyzed by Nano-Spray liquid chromatography tandem mass spectrometry (MS/MS) as previously described.<sup>11</sup> The unprocessed data files containing MS/MS spectra were submitted to the Mascot search engine (MatrixScience Ltd., London, UK) for database searching using the Mascot daemon. Mascot compares the mass values of observed product ions with the mass values calculated for theoretical product ions from peptide sequences present in a specified genomic database. From this comparison, a probability-based score is calculated, which reflects the statistical significance of the match between the product ion spectrum and the sequences contained in a database. The SwissProt-Trmbl database was searched using *Homo sapiens* as a taxonomic restrictor. To optimize the accuracy of protein identification, we required a minimum of two unique peptides at disparate sites within the protein for a positive identification. For instance, the protein sequence was identified only after mass spectrometric analysis of two or more unique peptides, each of whose probability scores met or exceeded the threshold ( $p < 0.05$ ) for statistical significance.

**Immunohistochemistry, immunocytochemistry and immunoblotting.** Immunohistochemistry and immunoblotting were performed as previously described.<sup>12</sup> The cytoplasmic protein fraction of the CSC was separated with the ReadyPrep protein extraction kit (Bio-Rad). For immunocytochemistry, cells were fixed for 10 minutes with 4% paraformaldehyde with 0.1% Triton X-100

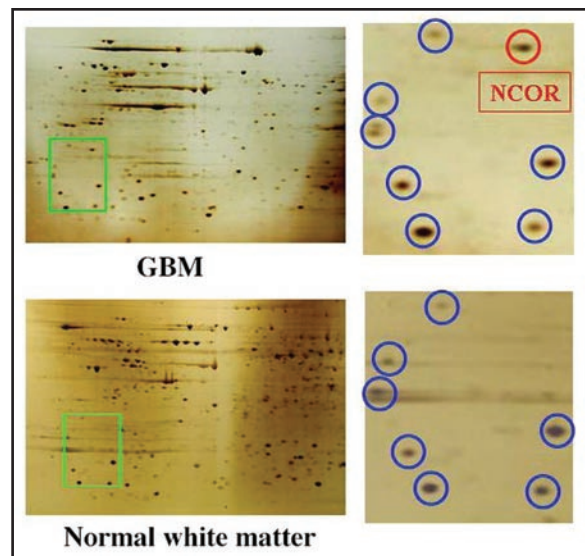


Figure 1. Differential expression of N-CoR in normal brain and GBM. Total proteomic analysis of microdissected normal glial tissue (white matter) compared with GBM was performed by two dimensional gel electrophoresis. The highlighted region in green which is magnified on the right panels shows a consistent protein pattern (blue) in normal white matter and GBM, and unique expression of N-CoR (red) in GBM. Protein identification was performed by liquid chromatography-mass spectrometry.

followed by 10 minutes of blockade using 5% serum of the species corresponding to the secondary antibody. Primary antibodies were incubated overnight at 4°C followed by wash and incubation with secondary antibodies for 30 minutes.

**Antibodies.** Rabbit anti-N-CoR (Abcam, Cambridge, MA; EMD Biosciences, San Diego, CA; Upstate, Waltham, MA; SCBT, Santa Cruz, CA; ABRAffinity BioReagents, Golden, CO), Mouse and rabbit anti-GFAP (Sigma, St. Louis, MO; DakoCytomation, Carpinteria, CA), and mouse and rabbit anti-Nestin (EMD Biosciences; R and D Systems, Minneapolis, MN) were used with secondary antibodies from various vendors (Molecular Probes, Carlsbad, CA; Vector Laboratories, Burlingame, CA).

**Cancer stem cells and cell lines.** Freshly resected surgical specimens of GBM (later confirmed by histopathology) were acutely dissociated by enzymatic digestion and automated mechanical disaggregation (MediMachine; Becton Dickinson, San Jose, CA). Cells were grown and selected on poly-d-lysine/laminin coated dishes in DMEM/F12 medium (Invitrogen, Carlsbad, CA) with N-2 supplement (0.5x; Invitrogen), B-27 supplement without vitamin A (0.5x; Invitrogen), basic fibroblast growth factor (20 ng/ml; Invitrogen), and epidermal growth factor (20 ng/ml; R and D Systems). Medium was changed every other day and growth factors added at intervals of 24 hours. Nestin expressing cells were selected and amplified. Subsequent karyotype analysis confirmed their GBM origin. CSC differentiation was carried out in the aforementioned medium modified with the addition of CNTF (20 ng/ml; Calbiochem) and the removal of growth factors. Glioma cell lines, U343 (UCSF Brain Tumor Tissue Bank, San Francisco, CA), U87 (ATCC, Rockville, MD), U251 (NCI, Bethesda, MD), and CCF52 (Cleveland Clinic Foundation, Cleveland, OH) were grown in DMEM (Invitrogen) treated with 50  $\mu$ M of all trans-retinoic acid (50  $\mu$ M; Sigma) and/or 10 nM of okadaic acid, a protein phosphatase-1 inhibitor (10 nM; EMD Biosciences) every three days.

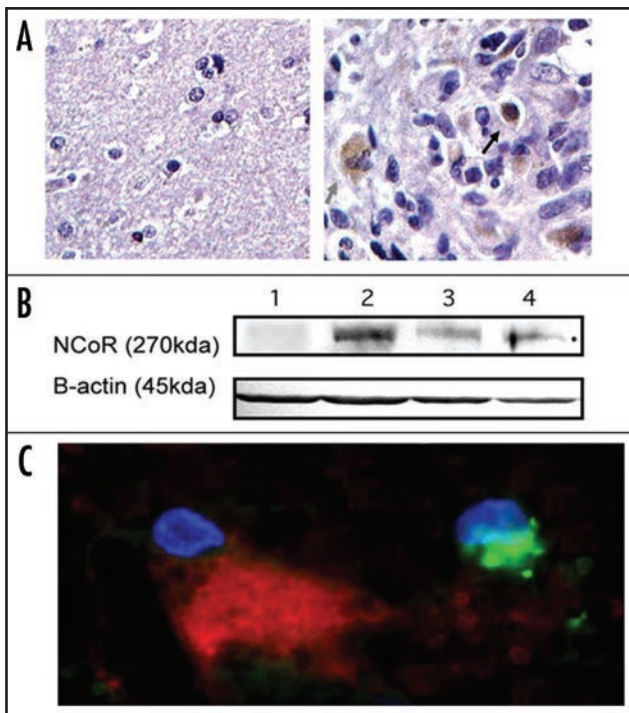


Figure 2. (A) Expression of N-CoR in GBM by immunohistochemistry. N-CoR protein is present in both nucleus and cytoplasm in GBM. Right panel: arrows point to N-CoR staining in the nucleus (black arrow) and cytoplasm (grey arrow). Left panel: no N-CoR is seen in normal tissue. Magnification = 400x. (B) Expression of N-CoR in GBM by Western blot analysis. N-CoR is present in GBMs on lanes 2–4 (molecular weight 270 kDa). N-CoR is absent in normal white matter (lane 1).  $\beta$ -actin was used as internal positive quantitative control. (C) Subcellular localization of N-CoR correlates with glial differentiation: N-CoR (green) and GFAP (red) immunolabeling in GBM. Cell on right demonstrating nuclear N-CoR localization shows no cytoplasmic GFAP. Cell on left with absent N-CoR labeling shows cytoplasmic expression of GFAP. Magnification = 630x.

## RESULTS AND DISCUSSION

N-CoR was found to be expressed only in GBM tissue in our comparative proteomic analysis of seven GBM and seven topographically matched normal brain white matter tissue specimens (Fig. 1). Expression of N-CoR in GBM was confirmed by immunohistochemistry and Western blotting (Figs. 2A and 2B). To demonstrate a role for N-CoR in CSC differentiation state, we investigated the relationship between subcellular N-CoR localization and astroglia-like differentiation. Expression of glial fibrillary acidic protein (GFAP), an established marker of astroglial differentiation and the subcellular localization of N-CoR was assessed by indirect immunofluorescence microscopy on GBM primary cell cultures, established cell lines (A172, CCF3, CCF4, CCF52, HS683, U87, U251, U343 MG-A), and frozen and paraffin-embedded tissue sections of GBM. Nuclear expression of N-CoR correlated with the absence of GFAP expression in the cells of primary cultures and tissue sections, whereas cytoplasmic expression of N-CoR correlated with positive expression of GFAP (Fig. 2C). Taken together, these observations indicate that nuclear N-CoR localization may function as a surrogate marker of undifferentiated GBM cells.

To examine the role of N-CoR in GBM development and differentiation, we treated cultured CSC isolated from GBM with ciliary neurotrophic factor (CNTF), an agent that has previously been shown to induce the astrocytic differentiation of NSC *in vitro*.<sup>13,14</sup>

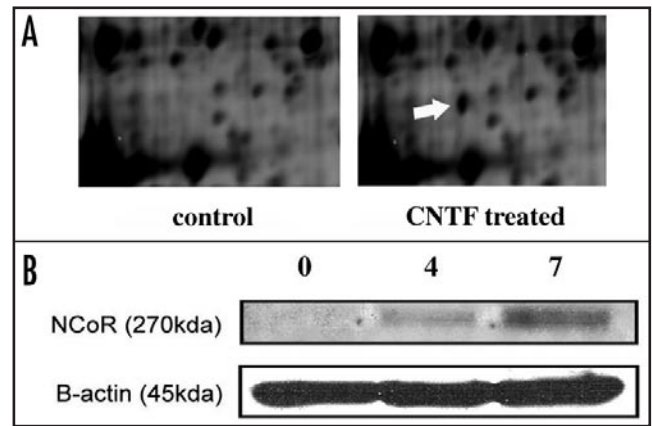


Figure 3. (A) GFAP expression by CNTF-treated CSC. GFAP expression was induced in glioma stem cells by treatment with CNTF and detected by two dimensional gel electrophoresis and liquid chromatography-mass spectroscopy. Arrow points to the GFAP spot. (B) Cytoplasmic N-CoR fraction increased by CNTF treatment of CSC. Cytoplasmic level of N-CoR expression in CSC shows gradual increase from day 0 to day 7 upon CNTF treatment.  $\beta$ -actin is shown as a quantitative internal control.

We then assayed GFAP expression along with subcellular N-CoR localization. As observed with NSC, CSC cultured with CNTF were induced to express GFAP (Fig. 3A). Western blot analysis of the cytoplasmic fraction proteins of CNTF-treated CSC indicated translocation of N-CoR to the cytoplasm (Fig. 3B). Both cytoplasmic N-CoR and GFAP expression peaked at day 7 following CNTF stimulation. These data demonstrate that GBM derived CSC are capable of differentiating along the astrocyte lineage following cytokine stimulation and suggest a treatment strategy based on inducing N-CoR mediated differentiation.

To determine the feasibility of pharmacologically targeting the N-CoR pathway, we investigated a variety of compounds with N-CoR inhibitory effects. Retinoids, metabolites of vitamin A, have been examined therapeutically in a variety of cancers including gliomas.<sup>15</sup> In acute promyelocytic leukemia, retinoids are used therapeutically to induce differentiation of the cancer cells.<sup>16</sup> N-CoR interacts with the retinoic acid receptor/retinoid-X receptor (RAR/RXR) heterodimer to exert its repressive activity and is released upon ligand (retinoic acid) binding to the receptor complex.<sup>17-19</sup> We hypothesized that one of the potential therapeutic effects of RA on malignant gliomas may be the induction of differentiation of the proliferating CSC compartment by the binding of retinoic acid to the RAR/RXR heterodimer, followed by dissociation of N-CoR from the RAR/RXR complex, and subsequent translocation of N-CoR to the cytoplasm. This would explain the previous observation of increased GFAP expression and moderate growth inhibition in a glioma cell line (U343 MG-A) treated with RA.<sup>20</sup> and represents a novel mechanism of action of RA in glioma therapy. In addition, since the cytoplasmic translocation of N-CoR is triggered by phosphatidylinositol-3-OH kinase/Akt1 kinase-dependent phosphorylation, we examined the effect of okadaic acid, a protein phosphatase-1 inhibitor. By preventing the action of protein phosphatase-1, okadaic acid increases the phosphorylation of N-CoR and promotes its cytoplasmic translocation. We also hypothesized that the combined effect on the N-CoR pathway of these two agents would, at a minimum, be additive.

To test this, we treated the U343 MG-A cell line with 50  $\mu$ M all-trans retinoic acid and/or 10 nM of okadaic acid. Three cultures for each of four treatments were grown for sixteen days with cell

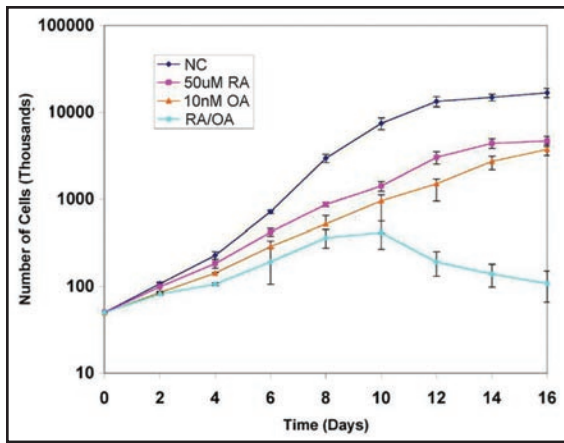


Figure 4. Logarithmic growth curve of glioma cell line, U343 MG-A, treated with retinoic acid (orange), okadaic acid (pink), combination of RA/OA (aqua) and control (dark blue) for 16 days. Individual treatments with RA and OA show a modest inhibition of growth. Combination of RA/OA shows synergistic reduction in cell growth. Error bars indicate 1 SD.

counts obtained at baseline and for each of the eight subsequent even numbered days. Curve fitting indicated exponential cell count growth for each treatment except for the OA+RA treatment group. Log transformations were applied to the cell counts, and a repeated measures analysis of variance (ANOVA) model was used to evaluate the treatment differences over time. Pair wise treatment comparisons used Sidak's statistic to account for multiple testing. ANOVA models were used to examine differences among the three cultures within each treatment group. Within each treatment group, the cultures were highly similar. Model based F-statistics indicated significant differences across time ( $p = 0.006$ ) and for time by treatment interactions ( $p = 0.001$ ). The differences in log cell counts among the four treatments were significant with an  $F_{3,8} = 163.2$  and the resulting  $p$ -value less than 0.0001. All pair wise treatment differences (Sidak's test) were significant with  $p$ -values less than 0.0001, except the RA vs OA difference ( $p = 0.079$ ). Cell counts monotonically increased for OA, RA, and controls through day 16. The number of cells with the combination treatment (OA + RA) increased until day 10 and then decreased for the remaining duration of the study (Fig. 4). Retinoic and low-dose okadaic acids each have a modest effect on cell growth. In combination however, there is a striking synergy in the inhibition of cell growth.

These observations demonstrate a role for N-CoR in GBM derived CSC differentiation and suggest a new treatment paradigm for gliomas. In addition, this study suggests a novel mechanism of action of retinoic acid in the treatment of GBM. To our knowledge, this is the first example of a therapeutic strategy directly targeting the undifferentiated compartment of GBM. Differentiation and growth inhibition of glioma cells is achieved by the synergistic combination of two compounds acting at different sites along the N-CoR pathway.

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