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The dopamine D_2 receptor contributes to the spheroid formation behavior of U87 glioblastoma cells

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Abstract

Background—Glioblastoma multiforme (GBM) is a common, and lethal, central nervous system cancer. This cancer is difficult to treat because most anti-cancer therapeutics do not readily penetrate into the brain due to the tight control at the cerebro-vascular barrier. Numerous studies have suggested that dopamine D_2 receptor antagonists, such as first generation antipsychotics, may have anticancer efficacy *in vivo* and *in vitro.* The role of the D_2R itself in the anticancer effects is unclear, but there is evidence suggesting that D_2R activation promotes stem-like and spheroid forming behaviors in GBM.

Objectives—We aimed to observe the role of the dopamine D_2 receptor (D_2R) and its modulators (at selective concentrations) in spheroid formation and stemness of glioblastoma multiforme (GBM) cell line, U87MG to clarify the validity of the D_2R as a therapeutic target for cancer therapy.

Methods—Spheroid formation assays and western blotting of the glioblastoma cell line, U87MG, were used to observe responses to treatment with the D_2R agonists sumanirole, ropinirole, and PHNO and the D_2R antagonists thioridazine, pimozide, haloperidol, and remoxipride. Extreme limiting dilution analysis was done to determine the impact of sumanirole and remoxipride treatment on sphere-forming cell frequency. Proliferation was also measured by crystal violet staining. Stable lentiviral transduction of *DRD2* or *shDRD2* were used to validate the role of the D_2R in assay behaviors.

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8.5 Author Contributions

JSW conceived, designed, carried out, and analyzed all experiments with the aid of JLR, EJK, and MVG. JSW wrote the manuscript. JDN, GLM and RJH contributed to experimental design and analysis and edited the manuscript.

8. Statements

8.2 Statement of Ethics

The authors have no ethical conflicts to disclose. No human or animal research was conducted a part of these studies

8.3 Disclosure Statement

The authors have no conflicts of interest to declare.

Results—D_2R antagonists thioridazine, pimozide, haloperidol, and remoxipride decrease spheroid formation behaviors at a selective 100 nM concentration, while D_2R agonists PHNO, sumanirole and ropinirole increase the formation of spheroids. Similarly, 100nM remoxipride decreased sphere-forming cell frequency. These results were recapitulated with genetic overexpression and knockdown of the D_2R, and combination experiments indicate that the D_2R is required for the effects of the pharmacological modulators. Furthermore, spheroid proliferation and invasive capacity increased under treatment with 100 nM sumanirole and decreased under treatment with 100 nM thioridazine. Expression levels of the stemness markers Nestin and Sox2, as well as those of differentiation marker GFAP, were not altered by 100 nM thioridazine or sumanirole for 72 h or continuous treatment with these compounds for 7 d during a spheroid formation assay.

Conclusions—Signaling activity of the dopamine D_2 receptor may be involved in the spheroid formation phenotype in the context of the U87MG cell line. However, this modulation may not be due to alterations in stemness marker expression, but due to other factors that may contribute to spheroid formation, such as cell-cell adhesion or EGFR signaling.

Keywords

dopamine; glioblastoma; spheroids; stem cell; neurotransmitters

2. Introduction

A common central nervous system cancer, glioblastoma multiforme (GBM) has a remarkably poor prognosis [1]. Less than ten percent of patients will survive for five years after diagnosis [2]. Even among patients who engage in clinical trials, only about a quarter of patients will survive for two years [2]. Additionally, while responding to treatment, patients often suffer from debilitating symptoms such as headaches, memory loss, seizures, and mood swings depending on tumor location [3]. There are many complications of GBM treatment, including limitations on resection (due to tumor location or diffuseness) and exclusion of potential therapeutics from the tumor by the cerebro-vascular or blood-brain barrier (BBB) [2, 3]. Thus, current surgical options, chemotherapeutics, and radiotherapy can only marginally improve patient survival and not cure GBM [1–3]. It is clear that new options are needed for GBM patients.

One potential treatment modality for GBM, as well as for other treatment-resistant cancers, is to target cancer stem cells (CSCs), a purported group of slow-cycling, undifferentiated cells capable of continual self-renewal [4]. CSCs are linked to increased tumor metastasis and chemoresistance [5, 6]. GBM cells grown in spheroid-inducing, serum-free media are enriched in stemness markers and are thought to provide a better model for *in vivo* behaviors of GBM as they mimic the highly proliferative, undifferentiated state of most GBMs [7]. Spheroid formation and spheroid culture in this type of system have become a common method of studying GBM due to enhanced behavioral similarities to *in vivo* tumors [7]. Treatments that are able to cross the BBB and are selective for CSCs over nonmalignant cells may offer a high-efficacy, low-adverse effect option for patients with GBM that could potentially extend survival or improve quality of life.

Interestingly, dopamine D_2-like receptor (D_2R) targeting compounds were identified as a potential selective CSC-targeting therapeutics in acute myeloid leukemia, lung cancer, and GBM through pharmacological screens [8–10], and dopamine itself contributes to GBM stem-like self-renewal characteristics [11, 12]. These receptors are $G_{\alpha i/o}$-coupled GPCRs that are found throughout the nervous system, though they are also present elsewhere in the body [13]. High expression of D_2R has been tied to poor prognosis in numerous cancers [14–18], and D_2R knockdown impairs the growth of the GBM cell line, U87MG [19]. Critically, D_2R antagonists such as those used in the above studies can cross the BBB and readily access CNS tumors. Many of these compounds are also FDA-approved treatments for conditions such as schizophrenia with well-characterized adverse effect profiles, pharmacokinetics, and pharmacodynamic profiles [13, 20]. If the D_2R is involved in stemness, treatment with already-available, relatively safe compounds could extend survival for GBM patients. Here, we use sphere formation assays to study the role of this receptor, and its activity, in GBM.

Although the D_2R and its antagonists have been identified in numerous screens, no study has yet clearly shown a direct relationship between D_2R antagonist cytotoxicity and D_2R receptor activity in order to elucidate the role of the receptor in cancer cell growth. Moreover, these studies were carried out with relatively nonselective concentrations (typically micromolar, when D_2R receptor affinities are typically in the low nanomolar range for these compounds) of D_2R antagonists, a concentration at which binding to other receptors and off target enzymes is likely. In this work, we further characterized the role of D_2R in GBM spheroid formation and stem-like molecular signatures in the context of the commonly used GBM cell line, U87MG. To that end, we employ a combination of genetic modulation and the application of varying chemotypes of D_2R modulators at concentrations selective for D_2R binding to reduce the likelihood of off target effects interfering in the study.

3. Materials and Methods

Reagents and cell culture

U87MG cells were obtained from ATCC (Manassas, GA), and all experiments were performed within 20 passages of receipt. U87MG-shDRD2 and U87MG-OE-DRD2 were generated according to manufacturer's protocols with lentiviruses, respectively DRD2 GIPZ-shRNA viral particles (Dharmacon, Lafayette, CO) or myc-DDK tagged DRD2 lentiORF particles (Origene Technologies, Rockville, MD). For monolayer cultures, cells were maintained in MEM (Gibco, Waltham, MA) + 10% FBS (Hyclone, Logan, UT). Spheroids were generated and maintained in spheroid media [DMEM/F12 (1:1) + 20 ng/mL epidermal growth factor (Sigma Aldrich, St. Louis, MO), 5 μg/mL insulin (Sigma Aldrich), 10 ng/mL basic fibroblast growth factor (Sigma Aldrich) and 0.4% bovine serum albumin (Research Products International, Mount Prospect, IL). Media was mixed fresh for each experiment, and 1x B-27 (Gibco) was added to spheroid media immediately before use. All cultures were incubated at 37°C and 5% CO_2. D2R modulators were the generous gift of Dr. Richard B. Mailman (Penn State College of Medicine, Hershey, PA) aside from sumanirole, which was obtained from Sigma Aldrich (St. Louis, MO).

Spheroid forming assays

Sphere-forming assays were carried out as previously described [21]. Briefly, cells were plated at 200 cells/well in given concentrations of compounds in spheroid media in low adhesion 96 well plates (Corning Costar, Corning, NY) and incubated for 7 d before quantification. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as rounded aggregates of cells with a smooth surface and poor cell to cell definition. Loose cellular aggregates of well-defined cells were not included in spheroid counts.

Immunoblotting

Western blotting was performed as previously described [22] using the ScanLater (Molecular Devices, San Jose, CA) visualization system. 4–12% bis/tris gels (Life Technologies, Carlsbad, CA) were loaded with even amounts of protein (8–12 μg/well) as determined by Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA) and run at 175 V for 60–90 min in MOPS buffer (Life Technologies). Transfer was carried out on ice at 20 V for 120 min. Primary antibodies (Cell Signaling Technology, Danvers, MA) included: Nestin, GFAP, Sox2, and vinculin (at dilutions of 1:1000, 1:500, 1:500, and 1:1000 respectively, all from Cell Signaling Technology, Danvers, MA). ScanLater Eu-conjugated secondary antibodies (Molecular Devices) were used for visualization with a SpectraMax i3x multimode plate reader (Molecular Devices). Images were quantified with ImageStudio Lite software (Licor, Lincoln, NE).

Invasion assays

Invasion assays were carried out with Corning BioCoat Matrigel Invasion Chamber 8.0 micron 24 well plates (Corning, NY) according to manufacturer's instructions. U87 spheroid cells were plated in transwell inserts at 15 ×10^4 cells/well in DMEM/F12 not supplemented with growth factors, and complete sphere media was used as a chemoattractant. Invasion assays were carried out for 48 h before insert removal and cell counting. Inserts were washed, and staining with DiffQuik (Siemens, Munich, Germany). Photos were taken of the inserts and quantified with ImageJ.

MTT assays

MTT assays were carried out as previously described [22], with the following exception. Due to the suspension nature of these cells, spheroid cells were plated at 35000 cells/well in 50 μL of complete spheroid media, then treated with 50 μL of 2x treatment media as noted before 48h incubation.

Crystal Violet

Crystal violet was used to observe cell proliferation due to concerns with the potential for limited BrdU delivery to the center of spheroids. For this assay, 20,000 spheroid cells/well in 1 mL media were plated in 24 well plates in complete spheroid media with the conditions noted. At 48, 96, and 168 h, cells were harvested by centrifugation and fixed with methanol for 15 min at room temperature before three PBS rinses. These cells were then stained with crystal violet solution (0.5% in 20%methanol, 80% water) for 30 min before three PBS

rinses and centrifugations (until the PBS had no traces of crystal violet). The crystal violet was then solubilized in methanol and the absorbance at 590 nm was observed for technical triplicates for each independent sample. These values were then normalized to the reading at time zero and reported as fold change.

Extreme Limiting Dilution Assays

Extreme limiting dilution assays were done based on protocols previously described [23, 24]. U87MG cells were plated at 100, 20, 10, 5, and 1 cell(s)/well in 200μl spheroid media in low adhesion 96 well plates (Corning Costar, Corning, NY) with 100nM sumanirole, remoxipride or vehicle. For this assay, plates were incubated for 9 d before quantification rather than 7 d as done for the sphere-forming assays described above to account for the lower plating densities used. At this time point there was maximum spheroid formation without significant cell death. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as above. Analysis was done using the online extreme limiting dilution analysis (ELDA) webtool [25].

Statistics

All data shown are representative of 2–3 independent experiments. Spheroid counts were carried out in quadruplicate or more for each experiment, whereas cell counts and invasion assays were carried out in triplicate. Time course data was analyzed with two way ANOVA followed by a Dunnett's posthoc, whereas spheroid count data and immunoblot signals were analyzed with one way ANOVA plus Dunnett's posthoc. For spheroid forming assays, at least eight replicate wells were counted; results are presented as box plots, with the whiskers representing minimum and maximum values and the box representing quartiles. For extreme limiting dilution assays, 240 replicate wells were counted for cells plated at 100 cells/well, 480 replicate wells were counted for cells plated at 20, 10, and 5 cells/well and a minimum of 70 replicate wells were counted that were verified to contain a single cell at plating. Analysis was done with the ELDA webtool that uses maximum likelihood estimations (MLE) in generalized linear models (GLM) to determine the sphere-forming cell frequency followed by chisquare tests for significance. ELDA results are presented as 95% confidence intervals. All other data points shown are presented as mean ± SEM.

4. Results

Selective concentrations of D_2R modulators alter sphere formation of U87MG.

First, we clarified the function of D_2R modulators in a common model of stemness by measuring spheroid-forming capability of the U87MG cell line. For this assay, we used a standard 100 nM concentration, which is closer to expected receptor affinity and thus should provide higher selectivity for D_2R than concentrations of these compounds that have historically been used [14, 26–31]. Over the course of 7 d, 100 nM of D_2 antagonists thioridazine, pimozide, haloperidol, and remoxipride reduced spheroid formation, whereas 100 nM of agonists sumanirole, ropinirole, and PHNO increased the number of spheroids formed (Figure 1A). At these concentrations, there is no significant effect on MTT measurements of overall cell metabolic activity in either monolayer or spheroid cultures (Figure 1B-C). Thus, at non-cytotoxic concentrations, multiple chemotypes of D_2

antagonists reduce the number of spheres formed while multiple chemotypes of agonists increase it in the context of U87MG. To determine if this effect is mirrored by alterations in the frequency of sphere-forming cells extreme limiting dilution analysis (ELDA) was done. For this assay cells were plated at decreasing cell densities and the quantity of sphere-positive wells for each density was used to calculate the frequency of sphere-forming cells in the U87MG population. Over the course of 9 d, 100nM of remoxipride significantly reduced sphere-forming cell frequency, whereas 100nM of sumanirole did not significantly increase the frequency of sphere-forming cells (Figure 1D). Thus, while the D_2 agonist sumanirole did increase the number of spheres formed per well it did not alter how many cells in the U87MG population could form spheres. In contrast the D_2 antagonist remoxipride reduced both the number of spheres and the frequency of cells that could form spheres in this population.

Genetic modulation of D_2R alters sphere formation of U87MG.

While such pharmacological evidence is strongly supportive of a D_2R-mediated reduction in spheroidforming capacity, we utilized genetic manipulation of the *DRD2* gene to observe the role of the D_2R in spheroid-forming phenotypes. Stable, lentivirally-transduced cell lines were used due to the 7 d time period of these assays. Stable transduction of sh-RNA against *DRD2* were able to reduce protein expression by about half, whereas stable overexpression of *DRD2* increased expression nearly fourfold over that of the parental line (Figure 2A, $p<0.01$ by one way ANOVA with Dunnett's posthoc). Overexpression of *DRD2* slightly increased cell proliferation, while knockdown slightly decreased it over the course of 96 h as measured by cell counts (Figure 2B, $p<0.01$ by two way ANOVA with Dunnett's posthoc). However, the differences in number of spheres formed is more pronounced. Knockdown reduced spheroid formation by nearly half, and overexpression doubled it (Figure 2C, $p<0.001$ by one way ANOVA with Dunnett's posthoc). While it is possible that this effect is due to the proliferative changes in the monolayer culture, the differences in magnitude make it more likely that the presence of D_2R more greatly facilitates survival in the more restrictive environment of spheroid media.

Effects on the sphere-forming phenotype are responsive to D_2R expression levels.

To further control for the possibility of off-target pharmacological effects, we assayed the role of the receptor further with a combination of pharmacological competition and genetic manipulation. First, we ascertained the effects of cotreatment with D_2R antagonists and agonists. In these studies, we found that cotreatment with 100 nM PHNO, sumanirole, or ropinirole was able to at least partially block the reduction in spheroid formation caused by treatment with 100 nM thioridazine, pimozide, and haloperidol (Figure 3A). We then compared the behaviors of wild type (Figure 1A), *DRD2* overexpressed (Figure 3B) and *DRD2* knockdown (Figure 3C) U87MG cell lines under treatment with D_2R modulators. When *DRD2* was overexpressed (Figure 3B), the ability of antagonists to reduce sphere formation was impaired ($p<0.05$ for all four antagonists by one way ANOVA with Dunnett's posthoc). Of the agonists, only 100 nM sumanirole significantly increased spheroid formation ($p<0.05$). It is possible that overexpression led to the near-saturation of signal through the D_2R at a basal level, thus ablating any further effects from agonist treatment. When *DRD2* was knocked down, none of the compounds, either agonist or

antagonist, were able to alter the spheroid-formation phenotype of the cells (Figure 3C, not significantly different from control by one way ANOVA and Dunnett's posthoc). This suggests that the observed effect requires the D_2R, and is thus not off-target.

Invasive and proliferative behaviors are affected by D_2R modulators.

The combination of results from pharmacological and genetic modulation in this assay suggested that there was a clear D_2R effect on the spheroid-forming phenotype, but we wished to ascertain if this phenotype was due to increased stemness. It is possible that increased sphere formation is related to increases in adhesion proteins or overall proliferation rate more than to stemness markers. We chose to study the glial differentiation factor, glial fibrillary acidic protein (GFAP), and two common stemness markers, Nestin and Sox2 (sex determining region Y-box 2) in both preformed spheres treated for 72h (Figure 4A,C) and spheres formed over 7 d in media containing the D_2R modulators (Figure 4B,C). As elsewhere, we used the more selective 100 nM concentration for all treatments. There was a trend in reduced expression of Nestin and Sox2 for thioridazine treated cells in the preformed sphere condition, but only the reduction in Sox2 expression was significant ($p=0.002$). In the sphere-forming condition, no significant changes were observed, as expression levels of these proteins were highly variable between replicates, suggesting the potential for significant drift over the seven day period, perhaps due to variable compensatory mechanisms in the surviving cells.. However, significant changes were observed in crystal violet staining, which was used as a surrogate marker of spheroid proliferation (Figure 4D). Sumanirole was able to increase total cell membrane staining at 96 h and 168 h, but thioridazine significantly decreased it ($p<0.01$ for both compounds at 96 and 168 h). Spheroids were also more able to invade in a 48 h transwell assay when treated with sumanirole than thioridazine or remoxipride (Figure 4E, $p<0.001$ by two way ANOVA with Dunnett's posthoc). Taken together, these data suggest that there is an effect on cellular proliferation and invasive behavior with D_2R modulation, but this effect is independent of the regulation of stemness markers Nestin and Sox2.

5. Discussion/Conclusions

In this work, we have observed a clear spheroid-formation phenotype tied to D_2R activity in U87MG cells. Pharmacological and genetic manipulation are able to modulate spheroid formation in this cell line in a manner which can be ablated with the addition of antagonistic factors. It is worth noting that overexpression and knockdown of this receptor was sufficient to ablate both agonist and antagonist effects. In the case of overexpression, it is possible that the effects of agonism were overwhelmed by increased low-level constitutive signaling from the D_2R. It appears that these receptors are engaging in appreciable signaling in the absence of supplied D_2R ligand, though it is possible that the cells are producing dopamine on their own or a factor present in the growth media is providing some level of agonism. Indeed, recent reports suggest that GBM cells can secrete dopamine themselves [32]. Similarly, heightened receptor availability negated the effects of pharmacological antagonism.

These findings modify our understanding of D_2R in GBM spheroids, as we have seen clear spheroid-formation effects at selective concentrations of D_2R modulators, but we were

unable to observe alterations in expression of Nestin,or the differentiation marker, GFAP, but Sox2 expression was significantly reduced in spheroids which were treated with thioridazine for 72h. This suggests that while there are changes in sphere formation, they may not be fully attributable to increases in stemness markers. In the absence of increased stemness characteristics in the sumanirole treated condition, it appears that cell viability and spheroid growth in the context of serum-free, suspension growth must be largely due to another factor. These could include, but are not limited to, increased cell-cell adhesion, reduced susceptibility to death signals, or an enhanced reliance on other growth signaling systems which are favored in this media, such as EGFR signaling.

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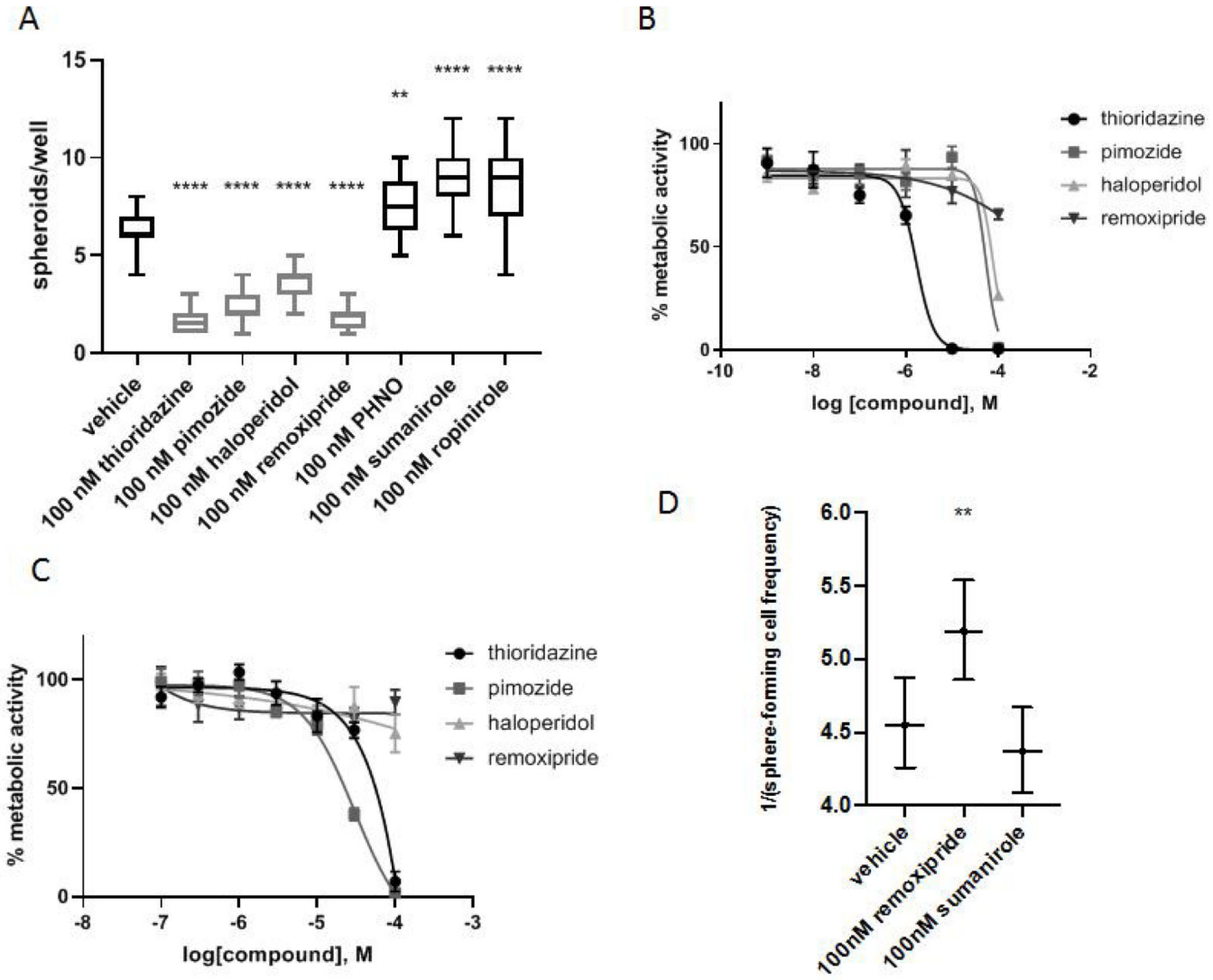

Figure 1.

Spheroid formation is altered by selective concentrations of D2 modulators. A, 100 nM of D2R antagonists thioridazine, pimozide, haloperidol, and remoxipride significantly reduce spheroid formation of U87MG cells in a 7d assay. 100 nM of D2R agonists PHNO, sumanirole, and ropinirole significantly increased the number of spheroids formed. B, 100 nM concentrations of D2 antagonists thioridazine, haloperidol, pimozide, and remoxipride are nontoxic to monolayer cultures of U87MG. C, 100 nM concentrations of D2 antagonists are nontoxic to U87MG cells cultured as spheroids. Spheroid formation assays were analyzed by one-way ANOVA with Dunnett's posthoc. D, 100nM remoxipride significantly reduced the sphere-forming cell frequency of U87MG cells in a 9 d assay. 100nM sumanirole did not significantly reduce the sphere-forming cell frequency. Upper and lower bars represent the 95% confidence interval. Analysis was done with the ELDA webtool. **, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$.

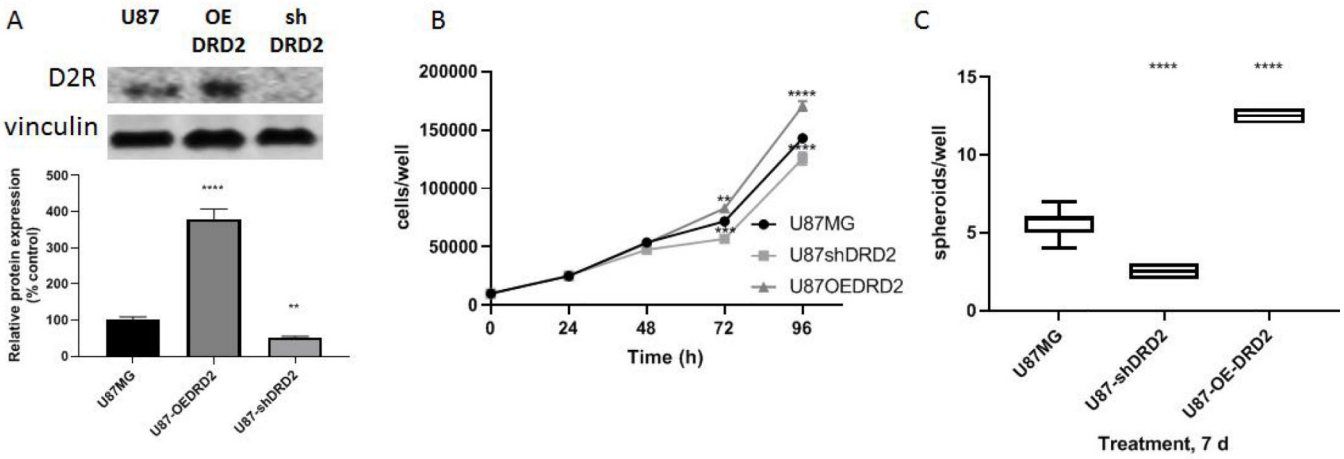

Figure 2.

Genetic modulation of D_2R alters spheroid formation capacity. A, Stable transduction with lentiviral vectors produced significant alterations in D_2R protein expression level. Transduction with *shDRD2* reduced protein expression by half (knockdown, KD on blot), whereas stable overexpression of *DRD2* increased protein expression by ~3.5 fold (overexpression, OE on blot). B, Overexpression of *DRD2* slightly increases cell proliferation rates as measured by cell counts, while knockdown slightly decreased it over the course of four days. C, Expression levels of D_2R correlate with spheroid formation capacity. Knockdown reduced spheroid formation by half, while overexpression more than doubled it. Spheroid formation assays and western blot data were analyzed by one-way ANOVA with Dunnett's posthoc, while cell counts were analyzed with two-way ANOVA with Dunnett's posthoc. **, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$.

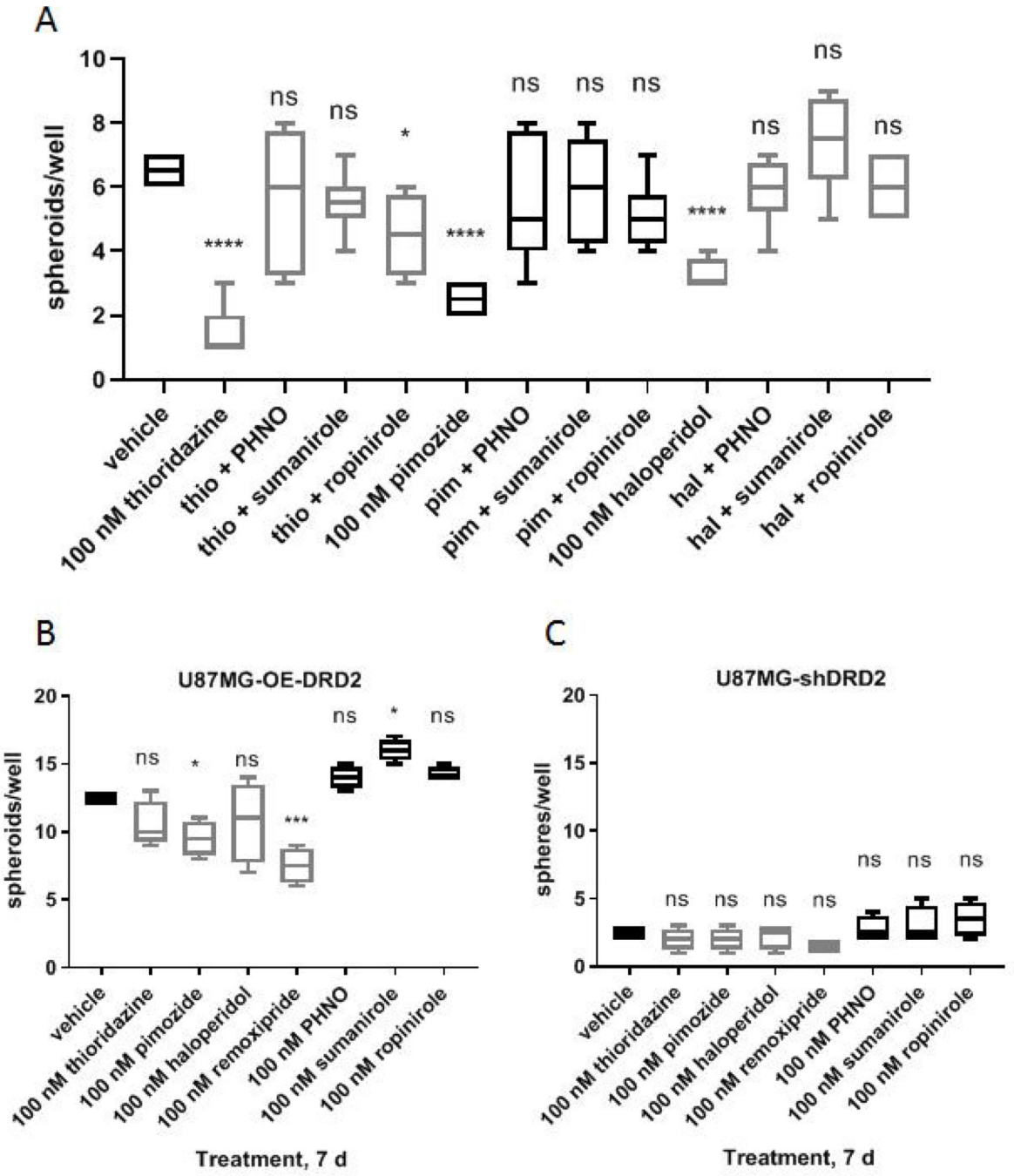

Figure 3.

The D_2R is involved in pharmacologically induced spheroid formation phenotypes. A, cotreatment with 100 nM D_2R agonists PHNO, sumanirole, and ropinirole partially ablated the reduction in spheroid number induced by 100 nM of D_2 antagonists thioridazine, pimozide, and haloperidol over the course of 7 d. B-C, modulation of D_2R expression impacted compound response in a 7d spheroid formation assay. Wild type (Figure 1A) responses to antagonists were reduced by overexpression of D_2R (B), while knockdown

blocked all compound responses (C). Spheroid formation assays were analyzed by one-way ANOVA with Dunnett's posthoc. *, $p<0.05$;**, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$.

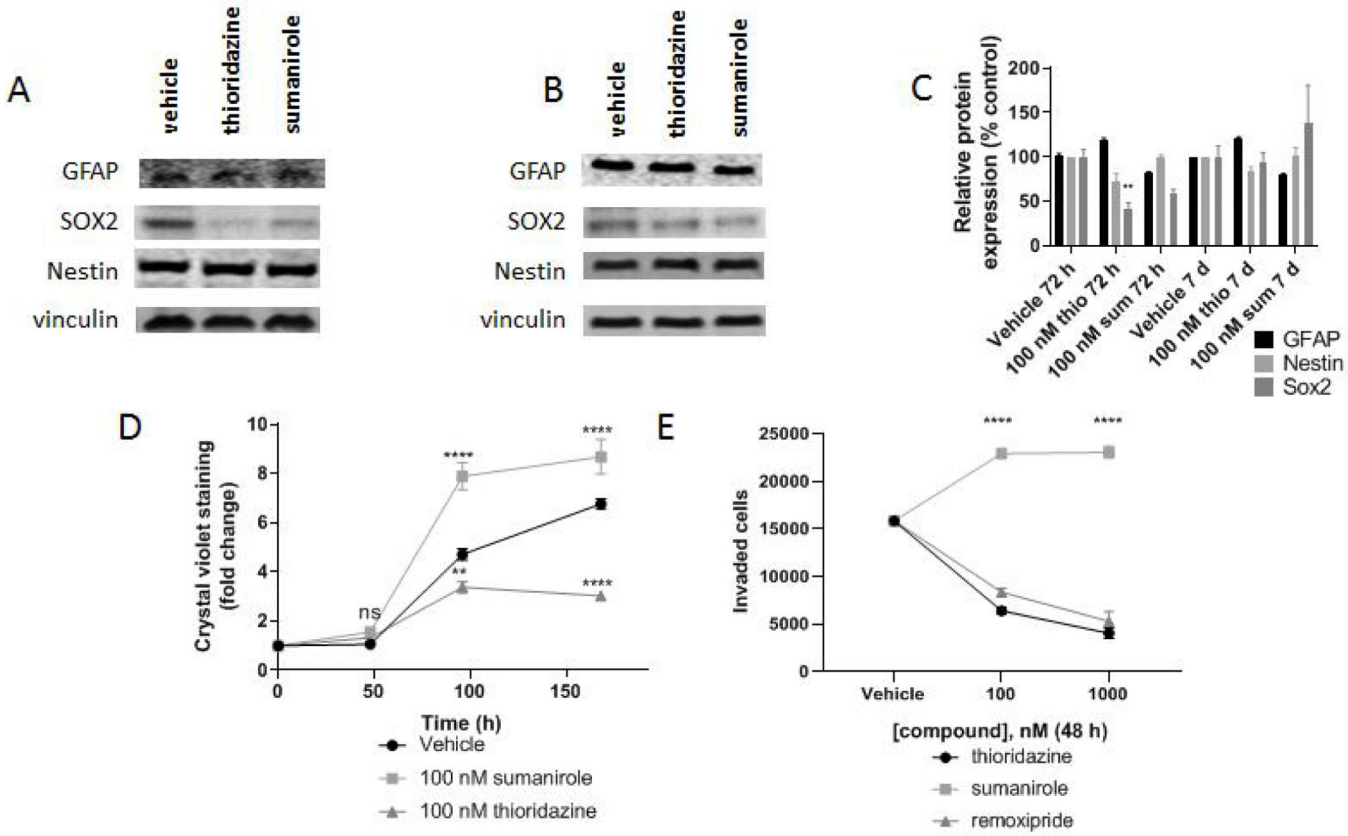

Figure 4.

D_2R modulators alter spheroid behaviors but not stemness markers. Pharmacological modulation of D_2R activity alters malignant behaviors of spheroids, but do not change stemness marker expression levels. A, U87MG spheroids were treated with vehicle, 100 nM sumanirole, or 100 nM thioridazine for 72 h. Expression of stemness markers Nestin and glial differentiation marker GFAP were not significantly altered, while expression of Sox2 was reduced in thioridazine-treated cells. B, U87 MG spheroids were formed over the course of 7d in the presence of vehicle, 100 nM sumanirole, or 100 nM thioridazine. Expression of markers was unchanged by treatment. C, Quantification of A and B. Densitometry values were normalized to loading control, vinculin. D, Spheroid proliferation was measured by crystal violet staining over 7d under treatment with vehicle, 100 nM sumanirole, or 100 nM thioridazine. D_2R agonist sumanirole increased spheroid proliferation at 96 and 168 h, whereas 100 nM thioridazine significantly reduced it. E, Invasion of cells from U87MG spheroids was measured via transwell assay after 48 h under treatment with 0, 100, and 1000 nM concentrations of sumanirole, thioridazine, and remoxipride. Sumanirole significantly increased invasion at 100 and 1000 nM, while thioridazine and remoxipride inhibited it in a concentration dependent manner. Western blots were analyzed via one way ANOVA, while two-way ANOVA was used for cell proliferation and invasion assays. Dunnett's posthoc was used for each. *, $p<0.05$;**, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$.