Original Article

DRD1 and DRD4 are differentially expressed in breast tumors and breast cancer stem cells: pharmacological implications

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Background: Abnormal expression of dopamine receptors (DRs) has been described in multiple tumors, but their roles in breast cancer are inconclusive or contradictory since evidence of pro- and anti-tumoral effects have been reported. Herein, we analyzed the expression of DRs in breast cancer, especially in the subpopulation of cancer stem cells (CSCs), and evaluated the functional role of the receptors by pharmacological targeting.

Methods: Expression of *DRD1*, *DRD2*, *DRD3*, *DRD4* and *DRD5* was investigated in human breast tumors and cancer cell lines using public databases. Correlation between gene expression and clinical outcome was studied by Kaplan-Mayer analyses. By flow cytometry, we assessed DRD1, DRD2, and DRD4 expression in cultures of MCF-7 (luminal) and MDA-MB-231 (triple-negative) cells. Using the previously reported SORE6 reporter system we examined the differential expression of DRD1, DRD2, and DRD4 in CSCs and tumor-bulk cells. The effect of pharmacological modulation of DRs on stemness and cell migration was studied by quantification of the reporter-positive fraction and wound healing assays, respectively.

Results: *DRD1*, *DRD2* and *DRD4* transcripts were expressed in breast tumors. *DRD4* was overexpressed compared to normal tissue and showed prognostic value. *DRD1*, *DRD2* and *DRD4* transcripts were also found in MCF-7 and MDA-MB-231 cells, but only DRD1 and DRD4 proteins were detected. DRD4 was underexpressed in CSCs compared to tumor-bulk cells, whereas DRD1 was found only in the CSCs fraction, suggesting that those receptors may have relevance in stemness control. Subtoxic concentrations of DRD1-targeting compounds did not induced significant changes in the CSCs pool. On the other hand, DRD4 inhibition by Haloperidol slightly increased the CSCs content but also reduced cell migration.

Conclusions: Pharmacological modulation of DRD1 in MCF-7 or MDA-MB-231 cells seems to be irrelevant for stemness maintenance. DRD4 reduced expression in breast CSCs or its inhibition by Haloperidol favors CSCs-pool expansion. DRD4 inhibition can also reduce cell migration, indicating that DRD4 plays different roles in stem and non-stem breast cancer cells.

Keywords: Dopamine receptor D1 (DRD1); dopamine receptor D4 (DRD4); breast cancer stem cells; dopamine receptor agonist; dopamine receptor antagonist

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Introduction

The family of dopamine receptors (DRs) includes five G protein-coupled receptors (GPCRs)—DRD1, DRD2, DRD3, DRD4 and DRD5—with different anatomical distribution, expression levels, dopamine affinity, signal transduction, and effector targets (1,2). Changes in the expression and/or function of DRs have been reported in multiple neurological pathologies (3,4), but their role in cancer progression is still unclear (2).

In breast cancer, the most frequent cancer type in women worldwide (5), the role of DRs is controversial. The idea that DRs expression favors more aggressive phenotypes is supported by the fact that expression of DRD1 and DRD2 are increased in malignant tumors compared to benign ones and normal mammary tissue (6). Furthermore, patients with DRD1 overexpression have reduced overall and recurrence-free survival compared to patients with no expression (7). Accordingly, exposure of triple-negative breast cancer cells to a DRD1 selective antagonist inhibits proliferation and motility, and triggers cell death (8). Similarly, it has been reported that *DRD2* is overexpressed in human breast cancer samples and cell lines, and its downregulation suppresses proliferation and induces apoptosis *in vitro* (6). However, the pharmacological activation of DRD2 lacks of effect in mouse models of triple-negative breast tumors (7).

On the other hand, there is also evidence supporting an anti-tumoral role of DRs. For example, exogenous administration of dopamine reduces tumor growth and angiogenesis in animal models (9-11). DRD1 agonists reduce viability and promote apoptosis in triple-negative breast cancer cells, reducing xenotransplant growth (7), and reduce migration, invasion and lung metastasis (12). In agreement, DRD1 antagonists promote xenotransplant growth (10).

Given that previous studies have focused on DRD1 and DRD2, herein we analyzed the expression of all five DRs in breast tumors and breast cancer cell lines using public datasets. Our results showed that *DRD3* and *DRD5* transcripts are undetectable in most of the samples, but *DRD1*, *DRD2* and *DRD4* transcripts were expressed with high variability. Experimental quantification of DRD1, DRD2 and DRD4 proteins in MCF-7 (luminal) and MDA-MB-231 (triple-negative) cells showed that DRD4 is consistently expressed in the cell membrane, but DRD1 was found only intracellularly. To analyze the possible differential expression of DRs in subsets of cancer cells, we employed the previously reported SORE6 reporter system (13) and evaluated the membrane expression of DRs in cancer stem cells (CSCs) and tumor-bulk cells. The expression of DRD1 was increased but that of DRD4 was reduced in CSCs from of both cell lines, suggesting that DRs may have relevance in stemness control. To address such possibility, we treated breast cancer cells with agonists or antagonists of DRs. Subtoxic concentrations of DRD1-targeting drugs did not induced significant changes in the CSCs fraction, but DRD4 inhibition might increase such fraction. Finally, we identified that DRD4 pharmacological inhibition reduced the cell migration of MDA-MB-231 cells, indicating that the role of DRD4 in each breast cancer cell subpopulation requires further analysis. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-783/rc).

Methods

Analysis of gene expression and clinical outcome

Comparison of expression of DRs in breast tumors versus normal tissue and analyses of the relationship between DRs expression with clinical outcome were analyzed in The Cancer Genome Atlas (TCGA)/Genotype-Tissue Expression (GTEX) cohort using the UCSC Xena browser (14). Analysis of coexpression correlation in the same cohort was performed using cBioPortal (15,16). Expression of genes of interest in breast cancer cell lines was assessed using expression data from the Cancer Cell Line Encyclopedia (CCLE) (17) accessed through Cancer Dependency Map (DepMap) portal (18).

Cell lines

We employed MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) breast cancer cells; both obtained from American Type Culture Collection (ATCC). Only cells below passage 20 were employed for our experiments. MDA-MB-231 cells were routinely cultured in Leibovitz's L-15 with 10% fetal bovine serum (FBS) at 37 ℃, whereas MCF-7 cells were maintained in Eagle's Minimum Essential Medium (EMEM) with 10% FBS and 0.01 mg/mL human recombinant insulin, at 37 ℃ in an atmosphere with 5% CO_2. Lentivirus generation was performed in HEK293 cells (CRL-1573, ATCC) cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, at 37 ℃ in an atmosphere with 5% CO_2.

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Compounds and treatments

We employed the compounds SKF-38393 (sc-264306, Santa Cruz Biotechnology), Quinpirole (Q102, Sigma-Aldrich), SCH-23390 (D054, Sigma-Aldrich), and Haloperidol (sc-203596, Santa Cruz Biotechnology). Stock solutions of SKF-38393, Quinpirole, and SCH-23390 were prepared in Mili Q water, whereas Haloperidol was dissolved in DMSO (D4540, Sigma-Aldrich). Solutions were stored at −70 ℃ with light protection until usage.

Identification of CSCs

Quantification of the CSC-subpopulation was performed in sublines generated by lentiviral transduction of a reporter system containing six concatenated repeats of SOX2/OCT4 response elements driving the expression of destabilized green fluorescent protein (SORE6-GFP) (13). A construction with a minimal cytomegalovirus promoter (mCMV)-GFP was employed to generate control cell lines. Both reporter constructions were kindly donated by Dr. L.M. Wakefield (National Cancer Institute, Bethesda, MD, USA). Briefly, MDA-MB-231 or MCF-7 cells were exposed to lentiviral supernatants diluted 1:1 in fresh medium for 72 h. Transduced cells were positively selected through a sequential treatment with puromycin 0.5 µg/mL (P4512, Sigma-Aldrich) for additional 72 h and GFP-based cell sorting (FACS Aria II Cell Sorter). The percentage of GFP+ cells after two-dimensional (2D) culture was quantified with the Attune NxT cytometer.

Immunostaining and flow cytometry

Expression of DRs was analyzed using anti-human DRD1 Alexa Fluor 405 (FAB8276V, R&D Systems), anti-human DRD2 Alexa Fluor 647 (sc-5303, Santa Cruz Biotechnology), or anti-human DRD4 PE (sc-136169, Santa Cruz Biotechnology). We employed as isotype controls Isotype $IgG_{2a,\,k}$ Alexa Fluor 405 (IC003V, R&D Systems), Isotype $IgG_{2a,\,k}$ Alexa Fluor 647 (557857, BD Pharmingen) and Isotype $IgG_{2a,\,k}$ PE (555574, BD Pharmingen), respectively. Cells were collected with phosphate-buffered saline (PBS) with 0.02 % ethylenediaminetetraacetic acid (EDTA), washed with PBS and stained with monoclonal antibodies (mAbs) or isotype diluted in PBS with 5% FBS for 30 min at 4 ℃ in the dark. After washing, cells were acquired in a Attune NxT cytometer and data were analyzed with FlowJo software V.10.0. Detection of intracellular DRs was performed in cells that were fixed/ permeabilized using Cytofix/Cytoperm solution (554714, BD Bioscience) and washed with Perm/Wash buffer (554723, BD Bioscience) before incubation with the same antibodies listed above.

Wound healing assay

Migration was evaluated as previously described (19). Briefly, MDA-MB-231 cells were seeded in 6-well plates at a density of 9×10^5 cells/well. The next day, the monolayer was wounded with a 200 µL-pipette tip, the wells were washed, and the culture exposed to the corresponding treatments in Leibovitz's L-15 with 2% FBS and 10 µM Cytosine β-D-arabinofuranoside (C1768-100MG, Sigma-Aldrich). The cultures were photographed at time zero and 24 h later in four positions per experimental condition. The percentage of wound closure was calculated by analyzing the micrographs with ImageJ (20) and normalizing the cell-free area in each position against the corresponding area at time zero. The experiments were repeated three independent times.

Statistical analysis

Gene expression (RNAseq) data were compared by Welch's *t*-test. Gene coexpression correlation was assessed by Spearman test and Pearson test. Survival curves were analyzed by log-rank test. Mean fluorescence intensity (MFI) of SORE6-GFP+ and SORE6-GFP− cell subpopulations were compared using Student's *t*-test. For assays comparing the effects of multiple concentrations of DR-targeting compounds *vs.* control (protein expression or migration) we employed ANOVA followed by Dunnett's test.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Institutional Committee of Ethics and Research, Facultad de Medicina UNAM FMED/CI/RGG/377/2017. Individual consent for this retrospective analysis was waived.

Results

Expression of DRs in breast cancer

To identify changes in DRDs gene expression in breast

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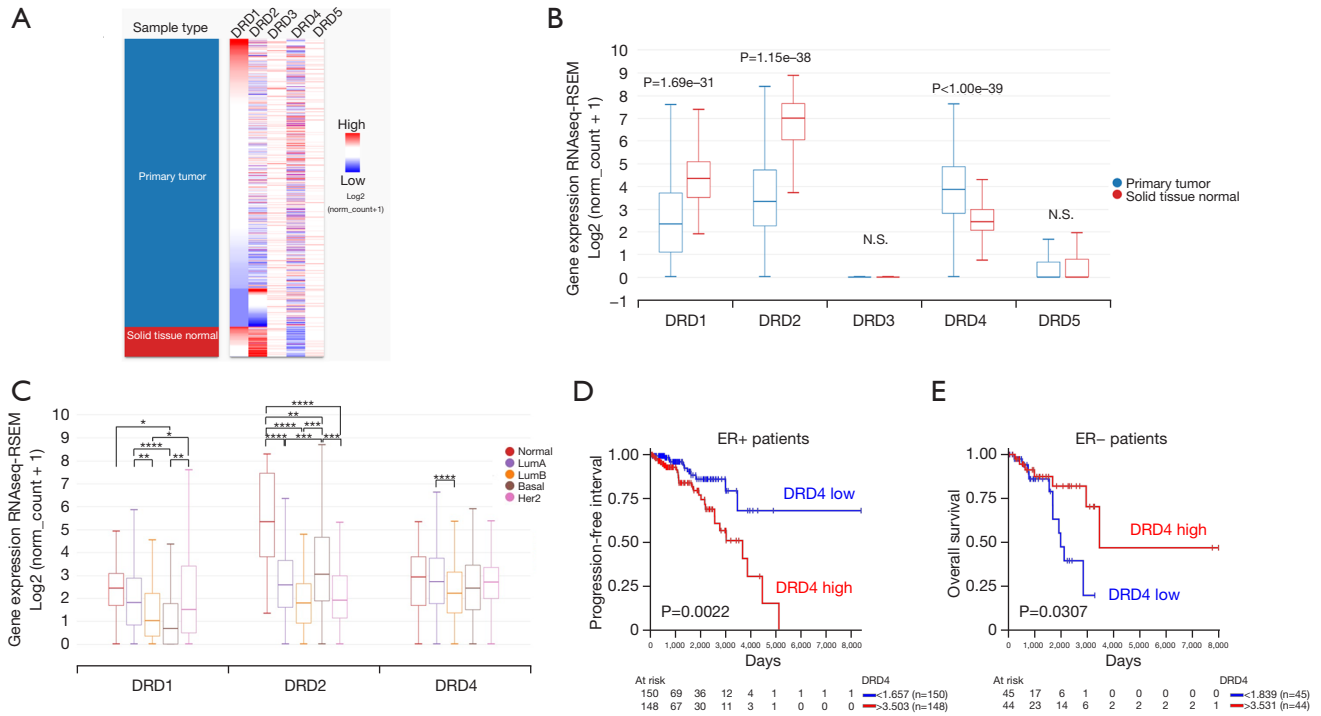

Figure 1 Analysis of DRs expression in breast tumors and clinical correlation. (A) Heatmaps of the mRNA levels of DRDs in breast tumors and their corresponding normal tissues. (B) Comparison of the expression of DRs in breast tumors *vs.* normal tissue (cohort: TCGA TARGET/GTEx; n=1,205). Statistical analysis was performed using the Welch's *t*-test. (C) Analysis of DRDs by breast cancer molecular subtype. (D) Progression-free interval in ER-positive breast cancer patients from TCGA cohort. (E) Overall survival in ER-negative breast cancer patients from TCGA cohort. All plots were generated using the UCSC Xena browser. Kaplan-Meyer analyses shown in (D) and (E) compare Q1 *vs.* Q4 and reported P was calculated with log-rank test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001 (Welch's *t*-test). ER, estrogen receptor; DR, dopamine receptor; RSEM, RNA-Seq by Expectation Maximization; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; N.S., not significant.

tumors, we compared the mRNA expression of all five DRDs in tumors *vs.* normal tissue using data from TCGA/GTEx database. *DRD3* and *DRD5* were expressed at very low levels in primary breast tumors and normal mammary tissue. *DRD1* and *DRD2* mRNA levels were reduced in breast tumors compared to normal tissue. On the other hand, *DRD4* expression was significantly higher in tumor samples (*Figure 1A,1B*).

In the TCGA cohort, we found differences in DRDs expression by molecular subtype (*Figure 1C*). *DRD1* was underexpressed in basal tumors compared with all other subtypes but luminal B. *DRD2* mRNA was overexpressed in normal-like and basal tumors. For *DRD4*, we found significant differences only between luminal subtypes. In the same cohort, *DRD1* or *DRD2* expression did not correlated with changes in clinical outcome (data not shown). On the other hand, increased *DRD4* expression correlated with decreased progression-free interval in patients with estrogen receptor (ER)-positive tumors (*Figure 1D*) and augmented survival time in patients with ER-negative tumors (*Figure 1E*). These results suggest that DRD4 expression plays a dual role depending on the ER status. However, we found the *DRD4* expression showed a weak, but statistically significant, negative correlation with *ESR1* (ER) expression and negligible correlation with *ERBB2* (HER2) or progesterone receptor (*PGR*) (Figure S1).

DRs expression in breast cancer cell lines

Then, we analyzed the expression of DRDs genes in breast cancer cell lines using public data from the CCLE (17). In agreement with the TCGA data, *DRD3* and *DRD5* transcripts were undetectable in most of the cell lines studied. Particularly, in MCF-7 and MDA-MB-231, the

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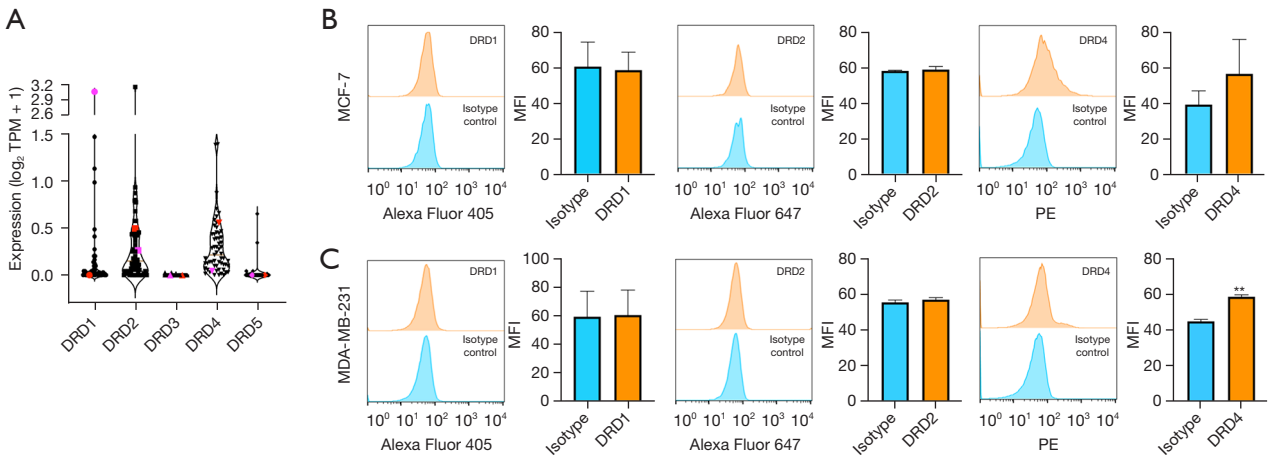

Figure 2 Expression of DRs breast cancer cell lines. (A) mRNA expression of DRDs in 62 breast cancer cell lines form the Cancer Cell Line Encyclopedia. MCF-7 and MDA-MB-231 cell lines are shown in magenta and red, respectively. (B,C) Protein quantification of DRD1, DRD2, and DRD4 in MCF-7 (B) and MDA-MB-231 (C) by flow cytometry. Graphs in (B) and (C) show MFI (average ± SEM) from 2–3 independent experiments. **, P<0.05 (Student's *t*-test). DR, dopamine receptor; MFI, mean fluorescence intensity; SEM, standard error of the mean; TPM, transcript per million.

two cell lines employed in this work, both genes were not expressed (*Figure 2A*) and, therefore, were not considered for further studies. *DRD2* was expressed in most the cell lines analyzed, whereas *DRD4* was detected in all the cell lines and had a higher average expression. *DRD1* expression showed large variability, with very high transcript number in MCF-7 and no expression at all in MDA-MB-231.

The expression of the proteins DRD1, DRD2, and DRD4 in the membrane of MCF-7 and MDA-MB-231 cells was assessed by flow cytometry. DRD1 and DRD2 were not detected in the two cell lines studied (*Figure 2B,2C*) but they were found in the positive controls U87-MG and HEPG2 cells (Figure S2). On the other hand, membrane DRD4 was detected in both breast cancer cell lines (*Figure 2B,2C*), as well as in the positive control HEPG2 (*Figure S2*).

Given that *DRD1* transcript has been detected by real-time PCR (RT-PCR) (21) and Western blot in MCF-7 cells (7), we analyzed the expression of DRD1, DRD2 and DRD4 in permeabilized MCF-7 cells. We found that DRD1 and DRD4, but not DRD2, were indeed located intracellularly (Figure S3).

DRD1 and DRD4 are differentially expressed in breast CSCs

To analyze the differential expression of DRDs in breast CSCs, we employed sublines stably expressing the reporter system SORE6-GFP. In those cells, GFP is expressed in cells with transcriptionally active SOX2/OCT4, corresponding to the CSCs subpopulation (13). We found that approximately 4% and 9% of the cells were GFP+ in MCF-7 and MDA-MB-231, respectively (*Figure 3A*). When comparing the expression of DRs in CSCs with the rest of the population, we found a discreate but significant increase in the expression of DRD1 in the SORE6-GFP+ fraction for both cell lines. We also found that DRD4 expression is reduced in SORE6-GFP+ cells, which was statistically significant for MCF-7 (*Figure 3B,3C*). These results suggest that DRD1 and DRD4 are differentially expressed in stem *vs.* non-stem cells.

Effect of DRD-targeting drugs in the CSCs pool

The relevance of the DRs in the CSCs biology was assessed by pharmacological activation or inhibition of the receptors. MCF-7 (*Figure 4A*) and MDA-MB-231 (*Figure 4B*) cells were treated either with: (I) the DRD1/DRD5 agonist SKF-38393; (II) the DRD1/DRD5 antagonist SCH-23390; (III) the DRD2/DRD3/DRD4 agonist Quinpirole; or (IV) the DRD2/DRD3/DRD4 antagonist Haloperidol. Although any of the treatments induced significant changes in the SORE6-GFP+ fraction, we identified a tendency to increase in MDA-MB-231 cell treated with Haloperidol (*Figure 4B*). The four drugs lacked cytotoxicity at the concentrations

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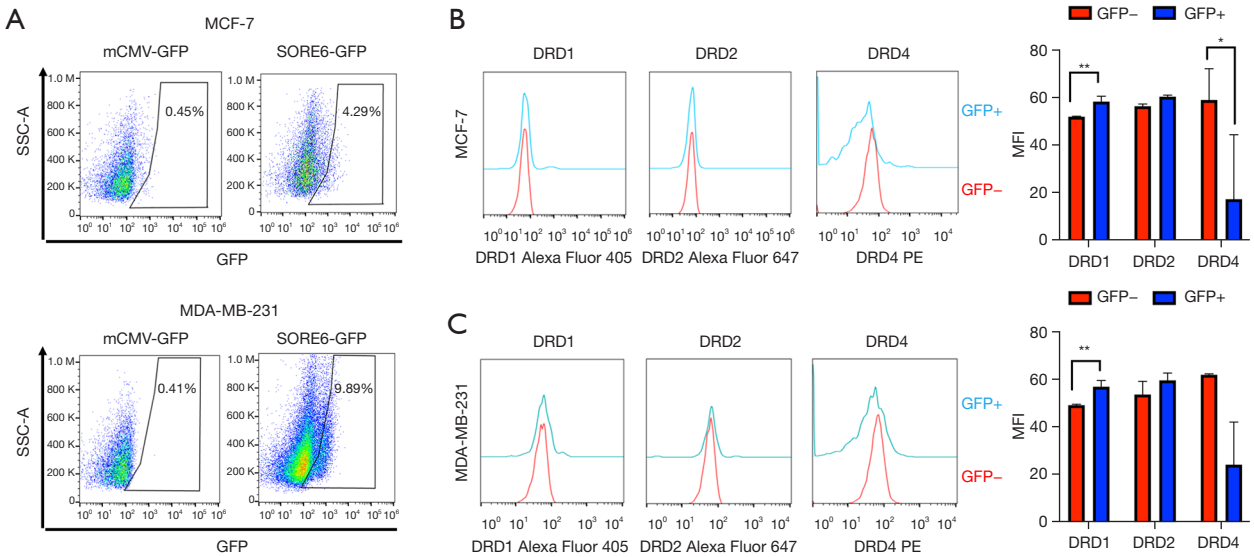

Figure 3 Quantification of DRs in breast cancer cell subpopulations. (A) MCF-7 and MDA-MB-231 transduced with SORE6-GFP reporter were employed to identify the breast cancer stem cell fraction (SORE6-GFP+). Cells transduced with mCMV-GFP reporter were employed as negative controls for staining. (B,C) Expression of DRDs in breast cancer stem (SORE6-GFP+) and tumor bulk (SORE6-GFP–) cells in MCF-7 (B) and MDA-MB-231 (C). *, P<0.05; **, P<0.01 (Student's *t*-test). DR, dopamine receptor; MFI, mean fluorescence intensity; SORE, SOX2/OCT4 response element; mCMV, minimal cytomegalovirus promoter; GFP, green fluorescent protein; PE, phycoerythrin; SSC-A, side scatter-area.

evaluated, as demonstrated by the quantification of the 7-aminoactinomycin D-positive (7AAD+) fraction (*Figure 4C,4D*).

DRD4 inhibition reduces migration in MDA-MB-231 cells

Previous reports show that DR-modulation modifies the migration of breast cancer cells (8,12). Given that DRD4, but not DRD1 or DRD2, was expressed in the highly migratory cell line MDA-MB-231, we analyzed the effect of the DRD4-targeting drugs Quinpirole and Haloperidol in cell migration (*Figure 5*). DRD4 inhibition with 10 µM Haloperidol reduced the cell migration (*Figure 5C,5D*). No changes were observed after Quinpirole treatment (*Figure 5A,5B*).

Discussion

Expression of DRs has been found altered in multiple types of cancers (2) and several authors have proposed that DRs may become therapeutic targets for improving clinical responses in cancer patients (22-25). Herein, we identified that DRD1 is downregulated in human breast tumors. These data agree with a previous work reporting that only one third of human breast tumors have clear immunoreactivity to anti-DRD1, and that such patients have reduced overall survival (7). Our analysis of TCGA data indicate that DRD1 is underexpressed in basal tumors, which contrast with the reported importance of DRD1-mediated signaling in triple-negative breast cancer cells (7,8,26). In agreement, the triple-negative model selected for this study, the cell line MDA-MB-231, did not express *DRD1* mRNA nor DRD1 protein, suggesting that this cell line is not a good model for studying the role of DRD1 in breast cancer biology. Surprisingly, in the luminal cell line MCF-7 DRD1 protein was detected only intracellularly. As other membrane GPCRs, DRD1 requires membranal localization to be activated by extracellular agonists (27). Thus, further studies must investigate if the inadequate translocation of DRD1 occurs in other luminal models and if intracellular DRD1 overexpression plays a role of in breast cancer cell biology.

The expression of DRD1 was increased in the SORE6-GFP+ cells, which corresponds to CSCs (13). CSCs are key in the development of drug resistance and metastasis

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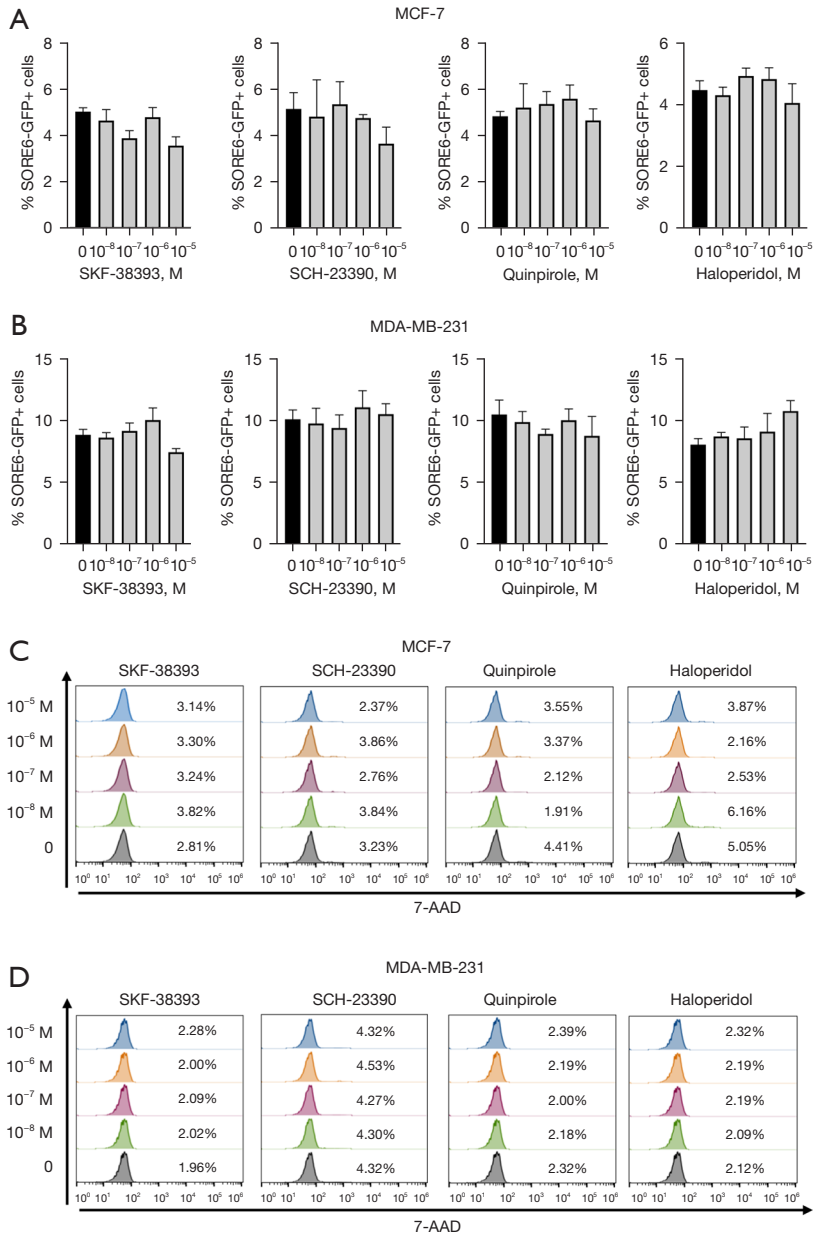

Figure 4 Effect of DR agonists or antagonists on the cancer stem cell pool. (A,B) Quantification of the SORE6-GFP+ fraction after exposure of MCF-7 (A) or MDA-MB-231 (B) to different concentrations of the annotated drugs for 72 h. Graphs show average ± SEM from 3–4 independent experiments. (C,D) Quantification of cell death in response to the drugs in MCF-7 (C) or MDA-MB-231 (D). A representative experiment for each drug/cell line is shown from 3 independent replicates. DR, dopamine receptor; SORE, SOX2/OCT4 response element; 7-AAD, 7-aminoactinomycin D; SEM, standard error of the mean; GFP, green fluorescent protein.

(28,29) and characterizing the signals that influence their phenotype is a major goal in breast cancer research (30). Previous studies reported that DRD1 activation by agonists reduce the CSC-pool in Adriamycin-resistant MCF-7 (10) and MDA-MB-231 (12) cells. Those reports differ from our findings, since the use of the DRD1/DRD5 agonist SKF-38393 in this work did not change the SORE6-GFP+ fraction, even a micromolar concentrations. The disparities could be caused by the usage of different methods and experimental endpoints. For example, Yang *et al.*

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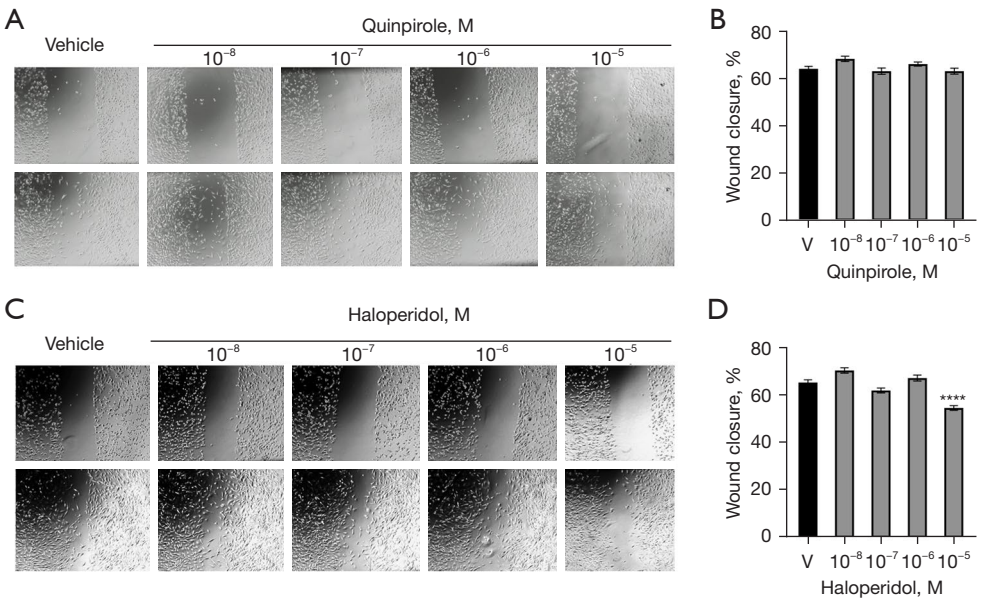

Figure 5 Effect of DRD4 modulation on the breast cancer cell migration. (A,B) Representative pictures (×4) of the effect of the DRD2/DRD3/DRD4 agonist Quinpirole in the migration of MDA-MB-231 cells (24 h) (A) and the corresponding quantification (average ± SEM) from 3 independent replicates (B). (C,D) Representative pictures of the effect of the DRD2/DRD3/DRD4 antagonist Haloperidol (C) and quantification (average ± SEM) from 3 independent experiments (D). Water or DMSO concentration in vehicle controls was 0.05%. ****, P<0.0001 (Dunnett's test). DR, dopamine receptor; SEM, standard error of the mean; DMSO, dimethyl sulfoxide; V, vehicle.

quantified the CSCs fraction using the $CD44^+/CD24^-$ immunophenotype and aldehyde dehydrogenase (ALDH) activity (12). Nevertheless, our results do not support the idea that pharmacological modulation of DRD1 could be beneficial for breast cancer patients.

We also found that DRD4 is overexpressed in breast tumors and can be detected in the membrane of both cellular models employed. However, SORE6-GFP+ cells had reduced DRD4 expression compared with the rest of the cancer cells. These results correlate with previous works reporting that female schizophrenic patients treated with Haloperidol or other DRD2/DRD4 antagonists have a higher risk of developing breast cancer (31,32). On the other hand, the drug thioridazine, another DRD2 antagonist with low selectivity, is active against breast, leukemia, and colorectal CSCs (33,34), but it is unclear if those effects are caused by DR inhibition or by modulation of other receptors. In our experiments, Haloperidol produced a non-significant increase in the SORE6-GFP+ fraction of MDA-MB-231 cells, supporting the hypothesis that DRD4 inhibition promotes the acquisition of a more malignant phenotype and worst clinical outcome. Surprisingly, DRD4 underexpression correlated with poor clinical only in ER-negative tumors, but had an opposite trend in ER-positive tumors. This dual role of DRD4 and the relationship with ER status requires further studies.

Given that DRD4 is expressed not only in the CSC-pool but also in tumor-bulk cells, we evaluated the effect of pharmacological modulation of the receptor in the migration of MDA-MB-231 cells. DRD4 inhibition with Haloperidol decreased migration in wound healing assays. In agreement, the drug SYA013, an Haloperidol analog, suppresses cell migration and invasion of MDA-MB-231 cells (35). However, SYA013 also induces apoptosis, whereas we did not detect increased cell death in cultures exposed to Haloperidol.

Conclusions

Pharmacological modulation of DRD1 in MCF-7 or MDA-MB-231 cells seems to be irrelevant in the maintenance of stemness, even when CSCs but not tumor-bulk cells have detectable levels of the receptor in the cell membrane. On the contrary, DRD4 reduced expression in breast CSCs or its inhibition by Haloperidol favors CSC-pool expansion. DRD4 inhibition can also reduce cell migration, indicating

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that DRD4 plays different roles in stem and non-stem breast cancer cells.

Acknowledgments

The results shown in *Figure 1* and Figure S1 are in whole or part based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga.
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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-783/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-783/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-783/coif). MAVV reports that research funding and APC was/will be provided by Universidad Nacional Autónoma de México, employer of MAVV. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Institutional Committee of Ethics and Research, Facultad de Medicina UNAM FMED/CI/RGG/377/2017. Individual consent for this retrospective analysis was waived.

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