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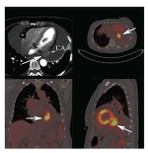

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MiR-422a weakened breast cancer stem cells properties by targeting *PLP2*

Running title: MiR-422a acted on BCSCs by targeting *PLP2*

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Abstract

Objective: This study investigated miR-422a and *PLP2* expressions in breast cancer cells and breast cancer stem cells (BCSCs). Besides, their influences on polymorphism changes were observed.

Methods: Flow cytometry and fluorescence-activated cell sorting was performed and $CD24^-/CD44^+$ cells were sorted from breast cancer cells and recognized as BCSCs. Microarray was applied to search for the differentially expressed miRNAs and mRNAs between MCF7 and BCSCs. The aberrant expression of miR-422a and PLP2 was further confirmed by RT-qPCR and the direct targeted relationship was verified by dual-luciferase reporter assay. After *in vitro* transfection, the expression of miR-422a and *PLP2* were manipulated and biological functions of BMSCs were compared with CCK-8, colony formation and sphere formation assay. The tumorigenesis ability of transfected BMSCs was also investigated in NOD/SCID tumor mice models.

Results: BMSCs were successfully established from MCF7 cells and miR-422a expression was downregulated while *PLP2* level decreased in BMSCs. MiR-422a directly targets the 3'UTR of *PLP2* and suppressed its expression. Besides, the up-regulation of miR-422a contributed to weakened ability of proliferation and microsphere formation of BMSCs, while *PLP2* overexpression facilitated those biological abilities. Tumorigenesis of BMSCs in mice models was impaired by either overexpression of miR-442a or silencing of *PLP2*.

Conclusion: Up-regulation of miR-422a attenuated microsphere formation, proliferation and tumor formation of breast cancer stem cells via suppressing the *PLP2* expression.

Keywords: breast cancer stem cells, miR-422a, *PLP2*

Introduction

Breast cancer was the primary cause of cancer-related deaths worldwide [1]. Sustained proliferation, activated invasion, migration and resistance to cell death contributed to its rapid recurrence and low survival rate. Cancer stem cells are potential origins of breast cancer and may dictate tumor phenotype [2]. They share properties including self-renewal, tumor initiation, indefinite replicative potential, and the ability to generate differentiated progeny. Breast cancer stem cells (BCSCs) could be recognized by activities of CD44+/CD24− and enzyme aldehyde dehydrogenase (ALDH+) [3]. Besides, BCSCs possessing both the CSC markers (CD44+CD24− and ALDH+) have shown the greatest tumor-initiating capacity in previous studies [4]. SOX6 has previously reported to be important for the maintenance of stem cells and OCT4 as well a weighted regulator of stemness in

various cancers, both as representative tumor markers [5, 6]. Therefore, stemness properties downregulation is of great significance in breast cancer studies.

Recently, miRNAs (small non-coding RNAs) have been reported to function as oncogenes or tumor suppressors. They are capable of regulating breast cancer at the post-transcriptional level [7]. MiR-422a has been proved to suppress cell proliferation in non-small cell lung cancer by targeting at *KLK4* [8]. MiR-422a downregulation could aggravate cell proliferation, migration and invasion of osteosarcoma [9]. The aberrant expression of miRNAs may also regulate the stemness of cancer stem cells and contribute to the tumorigenicity [10, 11]. Via the control of DNMT3b protein, miR-221 facilitates the self-renewal and differentiation in breast cancer stem cells and promotes the pathogenesis of breast cancer [12]. However, the potential role of miR-422a in cell stemness has not been explored, which would possibly span miRNA studies in stem properties of breast cancer.

Proteolipid protein 2 (*PLP2*), also known as A4 protein, has been shown to be upregulated in several cancers, including melanoma cancer, alzheimer's disease, renal cell carcinoma and so on [13, 14]. Its overexpression enhances cell proliferation, adhesion, and invasion in melanoma cells [13]. It is also involved in CCR1 signaling pathway and stimulates the migration of human osteosarcoma cells [15]. However, except from the study of Longo A *et.al*, no correlation between *PLP2* and breast cancer cells have been reported before [16]. Our microarray results found the aberrant expression of PLP2 in breast cancer stem cells compared with the origin MCF-7 cells, the influence of PLP2 on stemness was then explored.

Herein, we investigated influences of miR-422a and *PLP2* on breast cancer stem cells. By changing miR-422a and *PLP2* expressions, polymorphism changes *in vitro* and tumor growth *in vivo* were recorded, which suggested BCSCs properties fluctuations. These discoveries may provide a novel insight into breast cancer studies from an aspect of reducing stemness properties.

Methods

Cell culture and transfection

MCF-10A and MCF-7 cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 ml penicillin, 100 ml streptomycin and 2 mM L-glutamine. Isolated BCSCs were cultured in serum-free RPMI1640 medium containing bFGF、EGF、B27 (Shanghai Novoprotein technology co. LTD, China). When cells in culture had grown to 80% confluence, the tissue culture medium was replaced with medium containing 1.6 mM (24 wells) or 3 mM (6 wells) of the plasmid per well. MiR-422a mimics, negative mimics, *PLP2*-pcDNA3.1, pcDNA3.1 plasmid vector and *PLP2* shRNA were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into cells using Lipofectamine 2000 (Life Technologies, USA) according to manufacturer's instructions. Groups were set as follows: NC group included cells transfected with negative mimics; miR-422a mimics group included cells transfected with miR-422a mimics; *PLP2* group included cells transfected with *PLP2*-pcDNA3.1; miR-422a mimics+*PLP2* group included cells transfected with miR-422a mimics and *PLP2*-pcDNA3.1.

Bioinformatics analysis

GSE68271 and GSE99394 were used to screen out differentially expressed miRNAs and mRNAs. R language was applied in the formation of heat map and volcano plot. MiRanda (http://www.microrna.org/microrna/getMirnaForm.do) was used to predict the target site of miRNA. A higher mirSVR score indicated a more stable complementarity and a higher PhastCons score indicated a greater conservation.

BCSCs isolation

These cells were sorted into two sub-populations based on surface markers CD44+ and CD24-: cells with CD24-/CD44+ (BCSCs) and non CD24-/CD44+ (non BCSCs). Breast cancer cells were trypsinized, suspended into single-cell mixtures and washed with PBS. After incubation on ice for 30 min with monoclonal antibodies specific for human cell surface markers including CD44 and CD24 (PE conjugated) (eBioscience, San Diego, CA, USA). After washing, cells were analyzed

using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) using Cell Quest Pro software (BD Biosciences).

RT-qRCR

Total RNA was extracted with Trizol reagent (Life Technologies, USA) and reverse-transcribed with PrimeScript II 1st Strand cDNA Synthesis Kit. RT-qPCR was then performed using SYBR Premix Ex Taq according to the manufacturer's protocol to detect the expression level of miR-422a and *PLP2*. The expression of RNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were provided by Sangon (shanghai, China). All primers were detailed in Table 1.

Western blot

Total protein was separated on by SDS-PAGE polyacrylamide gel electrophoresis polyacrylamide and then electro-transferred onto PVDF membranes at constant voltage 80 V. After 1 h blocking with BSA, the membrane were incubated with antibodies. Primary antibodies included mouse anti-PLP2 (1:5000; BD Biosciences, San Jose, CA, USA), mouse anti-OCT4 (1:1000, Abcam, Cambridge, MA, USA) and mouse anti-SOX2 (1:2000, Abcam). Secondary antibodies included rabbit anti-mouse IgG-HRP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse anti-GAPDH (Kangchen, Shanghai, China) was used as an internal parameter. All antibodies were diluted with 5% milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and incubated for either 1 h at room temperature or overnight at 4 ^{o}C. Protein bands were visualized with ECL Western blotting substrate (Pierce, Rockford, IL, USA).

Dual-Luciferase Reporter Assay

For luciferase reporter experiments, the 3'UTR sequence of *PLP2* predicted to interact with miR-422a or a mutated sequence within the predicted target sites was synthesized and inserted into the Mlu I and Hind Ⅲ sites of a pGL3 vector (Promega, Madison, WI, USA) (wt forward

5’-GAATTCGCGAACTTCCCTCA-3’, wt reverse 5’- GGATCCTTTGATGAAAGGATTACT -3’, mut forward: 5'-AGTTAGATTTCAGAGTCCAGGCCCTAGGTTGG-3', mut reverse: 5'-ACCTAGGGCCTGGACTCTGAAATCTAACTCC-3'). These constructs were known as pGL3-PLP2-3'UTR-wt or pGL3-PLP2-3'UTR-mut, respectively. For the reporter assay, the HEK 293T cells (Cell library of the Chinese academy of sciences，Shanghai, China)\ cells were plated onto 12-well plates, and then co-transfected with the above-mentioned constructs and 5 ng of pRL-TK (Promega), with or without miR-214 or miR-con using Lipofectamine 2000 reagent (Invitrogen). Approximately 48 h later, the cells were harvested. The luciferase and Renilla signals were determined using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions.

Spheroid formation assay

Breast cancer stem cells were transfected with miRNAs or plasmids for 24 h in six-well plates. Single‐cell suspensions were prepared and cells were plated on six-well ultralow attachment plates (Corning-Costar Inc., Corning, NY, USA) at a density of 1,000 cells/ml. Cells were grown in DMEM supplemented with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen). After 7 days of culturing, the mammospheres with no less than 50. 0 um diameter were counted.

Colony Formation Assay

MCF-7 cells were trypsinized, counted, and seeded for colony formation assay in 6-well plates at 300 cells per well. During colony growth, the culture medium was replaced every 3 days. At 7 days after seeding, the colonies were stained with 0.02% crystal for 1 h and then were counted in 5 random chosen fields under an inverted phase-contrast microscope (Olympus IX73; Olympus, Tokyo, Japan). The colony was counted only if it contains more than 50 cells. Each treatment was carried out in triplicate.

CCK-8 Assay

Cells were plated in 96-well plates at a density of 3×10^4 per well. At 24, 48, 72 and 96 h post-plating, 10 μl Cell Counting Assay Kit-8 solution (Sigma, St. Louis, MO) was added to each

well and incubated for 2 h, and the absorbance at 490 nm was measured using a microplate reader. Results represented the average of samples from three independent studies per well.

NOD/SCID Xenograft Model

Cells were cultured in antibiotic-free normal growth medium supplemented with FBS till 80% confluency. *PLP2* shRNA and pre-miR-422a were sub-cloned into pCMV-Red-Firefly-Luc plasmid and the lentivirus was packed in 293T cells. PCMV-Red-Firefly-Luc vector was utilized as negative control. BCSCs were infected with the harvested lentivirus (MOI=10). After the selection with Doxycycline (2μg/ml) for two weeks, stably expressing miR-442a or *PPL2*-knockdown BCSCs were injected into female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (6 weeks old) fat pad at a concentration of 2×10^6 /ml and 5×10^6 / ml. Tumor growth *in vivo* was measured by Bioluminescent IVIS imaging system 200 (Xenogen CA, USA). The animals were sacrificed after 80 days. Animal maintenance and experiments were performed in accordance with the animal care guidelines of the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All animal experiments were approved by the Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Statistical analyses

Each experiment was done in triplicate. Data were expressed as the mean ± standard deviation (x±s). When two groups were compared, the differences between groups were analyzed using Student's t-test and when more than two groups were compared, a one-way analysis of variance (ANOVA) was used. Statistical analyses were performed in GraphPad Prism 6.0 (GraphPad Softwares Inc, San Diego, CA, USA). A probability level of 0.05 indicated statistical significance.

Results

MiR-422a was lowly expressed while PLP2 was highly expressed in BCSCs

Flow cytometry were applied to selected breast stem cells in breast cancer cells with stem properties marker CD24-CD44+. Besides, OCT4 and SOX6 protein expression were tested to validate the stemness of isolated BCSCs (Figure S1A-B). MiRNA expressions in breast cancer cell line MCF-7

and BCSCs were evaluated by microarray analysis (Table S1). Among 33 down-regulated miRNAs, miR-422a barely been investigated before and was selected for further study (Figure 1A). GSE99394 also indicated twenty most significantly overexpressed genes, including PLP2 (Figure 1B). As suggested in Figure S1C, miR-422a was significantly correlated with TLR2 signaling pathway. MRNA expressions in breast cancer cell line MCF-7 and BCSCs were evaluated by microarray analysis at dataset (Table S2). Besides, differentially expressed mRNAs shown in Table S2 indicated that they were significantly related to RNA binding and B cell homeostasis (Figure S1C). Then, qRT-PCR validated that miR-422a expression was significantly higher in CD24-CD44+ marking BCSCs than in normal epithelial cells (P=0.0038) and breast cancer cells (P=0.0027). Besides, no obvious difference was observed between non-BCSCs group and breast cancer group (Figure 1C). QRT-PCR and western blot also confirmed the prediction: PLP2 had a higher mRNA and protein expression in CD24-CD44+ marking BCSCs compared with breast cancer cells (P=0.001, P=0.0048) and normal epithelial cells (P=0.004, P=0.003). Therefore, PLP2 was high expressed in BCSCs compared with normal breast cancer cells (all P<0.05, Figure 1D-1F).

MiR-422a down-regulated *PLP2* expression in BCSCs

MiRanda predicted SOX6 as well as *PLP2* as miR-422a targets. However, SOX6 expression didn't show significant differences in BCSCs and PLP2 was therefore the only suitable target (Figure S2C-2D). MiR-422a might bind to PLP2 wt at position 282 (Figure 2A). A mirSVR score of -0.2454 which was lower than -0.1 indicated that the binding was stable and a PhastCons score of 0.5865 suggested a relatively high conservation. To confirm the direct target relationship between miR-422a and *PLP2*, dual luciferase reporter assay was performed. It turned out that miR-422a mimics significantly decreased the luciferase activity in pGL3-*PLP2* wt group but caused minor changes in pGL3-*PLP2* mut group (P<0.05, Figure 2B), indicating that miR-422a directly targeted at *PLP2* and inhibited its expression. Their regulation relationship was further confirmed in Figure 2C-2F. MiR-422a mimics increased its expression in miR-422a mimics group and *PLP2*-pcDNA3.1 up-regulated PLP2 expression. Meanwhile, miR-422a mimics significantly down-regulated PLP2 expression while PLP2 overexpression exerted little influence on miR-422a expression. However, miR-422a mimics+ PLP2-pcDNA3.1 group showed no much changes of PLP2. Therefore, miR-422a could directly target at *PLP2* and inhibit the expression of *PLP2* in BCSCs.

MiR-422a suppressed BCSCs properties by *PLP2* down-regulation

As shown in Figure 3, the transfection of miR-422a mimics and PLP2 overexpression vector significantly changed stem cell population. MiR-422a overexpression greatly reduced CD44+CD24- cell population in isolated BCSCs, indicating a decrease on BCSCs stemness. On the contrary, PLP2 overexpression increased stem cell population in isolated BCSCs which meant an enhancement on BCSCs stemness (Figure 3A). Altered protein expressions of OCT4 and SOX2 were also recorded. MiR-422a could elevate OCT4 and SOX2 expressions and overexpression of *PLP2* would suppress expressions of stemness markers (Figure 3B). However, the combination of miR-422a and PLP2 failed to either change stem cells population or influence expressions of those proteins.

MiR-422a inhibited while PLP2 promoted BCSCs proliferation

To investigate the role of miR-422 and *PLP2* in BCSCs, the cell proliferation rate of co-transfected compounds was determined using tumorsphere formation assay, CCK-8 and colony formation assay. Influences of miR-422a and PLP2 on cell proliferation in normal breast cancer cells were also proved to show similar trend in BCSCs. MiR-422a mimics significantly inhibited colony formation and cell viability and PLP2 greatly improved colony formation and cell viability (Figure S2A-2B). Since this study focused on BCSCs, we only study changes in stem cells. In tumorsphere formation assay, the number of glomus cells with no less than 50 μm diameter was counted. Knockdown efficiency of shRNAs was firstly validated in Figure S2E-2F which indicated shRNA1 as the most efficient inhibitor of PLP2 expression and therefore was chosen in following PLP2 knockdown experiments (Figure S2E-2F). As shown in Figure 4A, the number decreased in miR-422a mimics group, increased in *PLP2* group, but showed no marked changes in the miR-422a mimics+*PLP2* group. Therefore, miR-422a mimics suppressed the sphere formation efficiency in BCSCs, while *PLP2* exerted the opposite effect. Similarly, fewer colonies were observed in miR-422a mimics group while more colonies were detected in *PLP2* group, which indicated that miR-422a inhibited cell proliferation, PLP2 promoted cell proliferation, but miR-422a and PLP2 together exert no obvious effect on cell proliferation ($P>0.05$, Figure 4B). CCK-8 at the same time proved the effect of miR-422a and PLP2: OD value in miR-422a mimics group was significantly lower and that in *PLP2* group was remarkably higher than NC and mix group, suggesting

effectiveness on stemness suppression of miR-422a overexpression and *PLP2* suppression (P>0.05, Figure 4C).

Upregulation of miR-422a or knockdown of *PLP2* inhibited tumorigenesis of BMSCs *in vivo*

For investigating the impact of miR-442a and *PLP2* expression on the tumor formation ability of BMSCs, we first established the stably miR-422a overexpressing and *PLP2*-knockout BCSCs. The successful manipulating of miR-422a and *PLP2* expression in BCSCs was confirmed in Figure 5A. IVIS image of mice at day 35 were shown in Figure 5B. NOD/SCID mice in NC group inoculated with 2×10^6 /ml cells into fat pad formed 1.5 cm diameter tumors in 35 days, while those in lv-miR-422a group and lv-shPLP2 group failed to form palpable tumors (both P<0.001). However, NOD/SCID mice in lv-NC group inoculated with 5×10^6 /ml cells formed tumors of 1.5cm diameter in only 28 days, but tumors in lv-shPLP2 grew more slowly (both P<0.01). Total photon counts in lv-shPLP2 group reached 1000 after 49 days while those in miR-422a overexpressing group were barely zero in total 70 days. Therefore, increased level of miR-422a or downregulation of *PLP2* could attenuate the tumor formation of BCSCs.

Discussion

In the present study, we have discovered that miR-422a suppressed BCSCs properties by suppressing *PLP2*. After overexpressing miR-422, microsphere formation, cell proliferation abilities *in vitro* and tumor initiation rate *in vivo* were significantly down-regulated in BCSCs. Therefore, miR-422a might function as a suppressor in tumorigenesis of breast cancer. This discovery would not only contribute to miRNA investigation in breast cancer but also facilitate the development of treatment strategies for breast cancer.

BCSCs could cause treatment relapse as they have higher migratory potential than differentiated, non-tumorigenic, breast cancer cells. They are of great capacity for self-renewal and differentiation, which lead to initiation, progression, metastasis and recurrence of tumor [17]. BCSCs could enable the re-establishment of tumors, therefore targets silencing stemness properties are needed. Instead of stemness correlated proteins or EMT involved proteins, this study focused on PLP2, an integral membrane protein that localizes to the endoplasmic reticulum in colonic epithelial cells.

Downregulated miR-422a expression has been found in several types of cancers, including osteosarcoma (OS), hepatocellular carcinoma and colorectal cancer [18]. MiR-422a was revealed as a valuable prognostic marker for osteosarcoma patients since it was correlated with large tumor size, advanced TNM stage, distant metastasis and grade of tumor [18]. Consistently, Downregulated miR-422a expression in BCSCs might be of great value in breast cancer treatment.

Then we further investigated how miR-422a functioned in BCSCs. MiRanda predicted various mRNAs that could be regulated by miR-422a, including *PLP2*, a differentiation correlated gene. Besides, their combination was relatively stable and highly conserved, which was why the relationship between miR-422a and *PLP2* was further investigated in BCSCs and mice models. *PLP2* had been shown to involve in various cancers, including breast cancer, hepatocellular carcinoma, osteosarcoma, and melanoma [13]. Zhu *et al.* reported that miR-664 directly targeted the 3'UTR region of *PLP2* in T-cell acute lymphoblastic leukemia [19]. Ding *et al.* suggested that *PLP2* was a bona fide target of miR-664 in cutaneous malignant melanoma [13]. This study proved that *PLP2*, which was downregulated by miR-422a, exhibit tumor initiating potential in BCSCs.

In vitro experiments proved that miR-422a mimics weakened whereas *PLP2* enhanced the tumorsphere formation ability and cell proliferation. *In vivo* experiments confirmed functions of miR-422a and *PLP2* in tumor outgrowth in mice models, which suggested that miR-422a overexpression could inhibit the tumorigenesis of BCSCs via downregulating the expression of *PLP2*.

Limitations have to be taken into consideration in this study. BCSCs sorted out in this study might not be replicable in other researches. Besides, some other targets except PLP2 could be regulated by miR-422a and other pathways affected by miR-442a and PLP2 was not explored in this study.

Moreover, the underlying mechanism responsible for the aberrant miR-442a was attractive but not attended in this manuscript due to the limited financial support. However, those could be involved in our further study.

Conclusion

In conclusion, we have proved that miR-422a directly targeted at *PLP2* in BCSCs and suppressed stemness properties. MiR-422a mimics weakened ability of tumorsphere formation, cell proliferation *in vitro* and tumor growth *in vivo* in BCSCs by downregulating *PLP2*. Therefore, miR-422a may be a potential therapeutic target for breast cancer treatment.

Conflict of Interest Statements for Authors

We declare that we have no conflict of interest.

Author contribution

Yanmei Zou, Yuandong Chen and Shuo Yao contributed to research conception and design as well as manuscript drafting; Guangrui Deng, Dian Liu, Yihua Wang and Xun Yuan analysed and interpreted data; Shunfang Liu, Jie Rao and Huihua Xiong made statistical analysis; Xianglin Yuan, Shiying Yu, Feng Zhu and Hua Xiongrevised the manuscript and took the role of funding collectors. In addition, all authors approved final manuscript.

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Figure legends

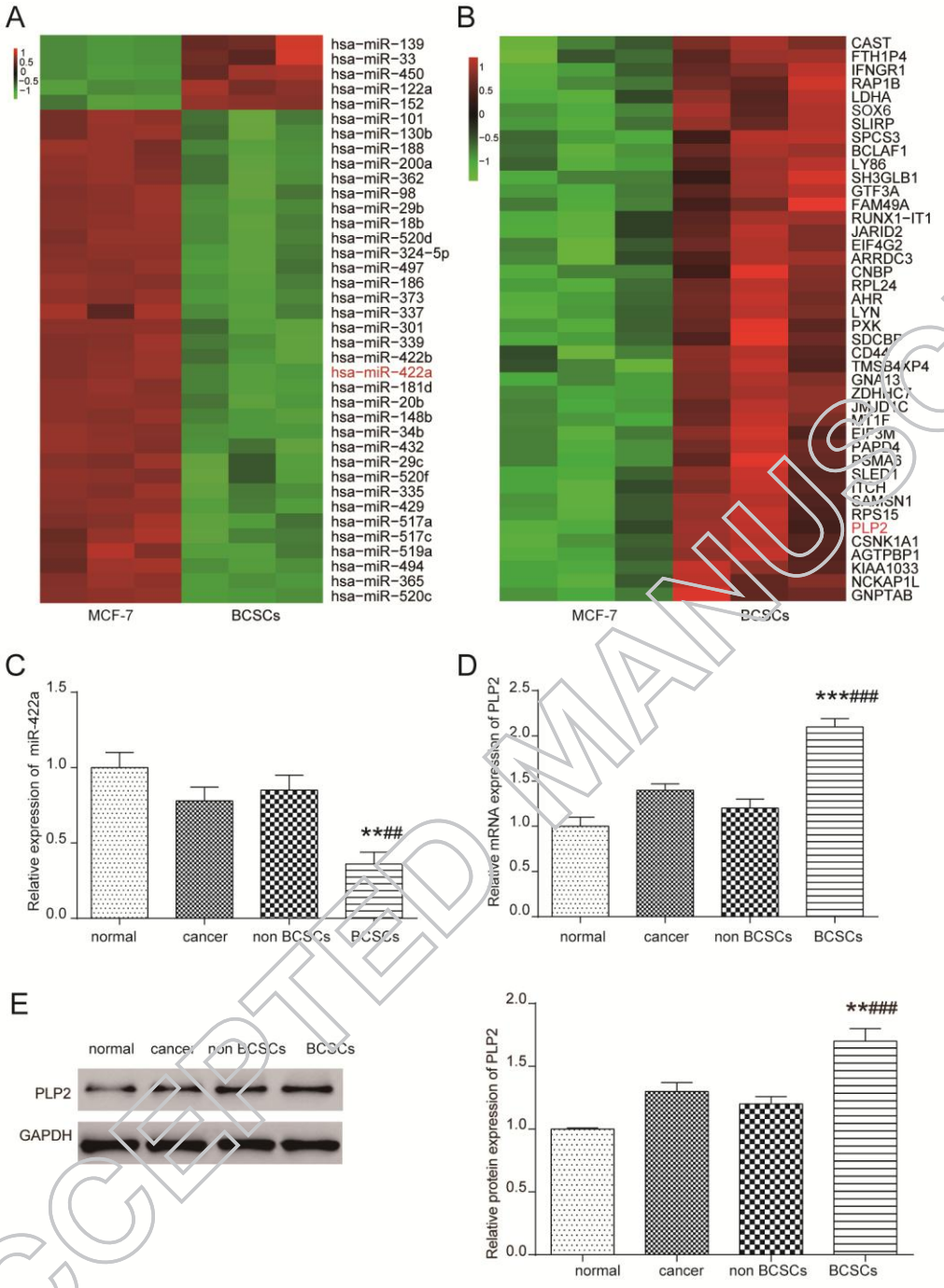

Figure 1. MiR-422a was lowly expressed while *PLP2* was highly expressed in BCSCs. (A) Microarray analysis indicated that miR-422a was lower expressed in BCSCs than in breast cancer cell line MCF-7. (B) Microarray analysis indicated that *PLP2* was higher expressed in BCSCs than in breast cancer cells. (C) qRT-PCR of miR-422a expression verified that miR-422a was lower

expressed in BCSCs than in breast cancer cells. (D-E) qPCR and western blot analysis verified that *PLP2* was higher expressed in BCSCs than in breast cancer cells. $^{**}P< 0.01$, $^{***}P<0.001$ indicated significant difference compared with normal epithelial cells. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ indicated significant difference compared with breast cancer cells.

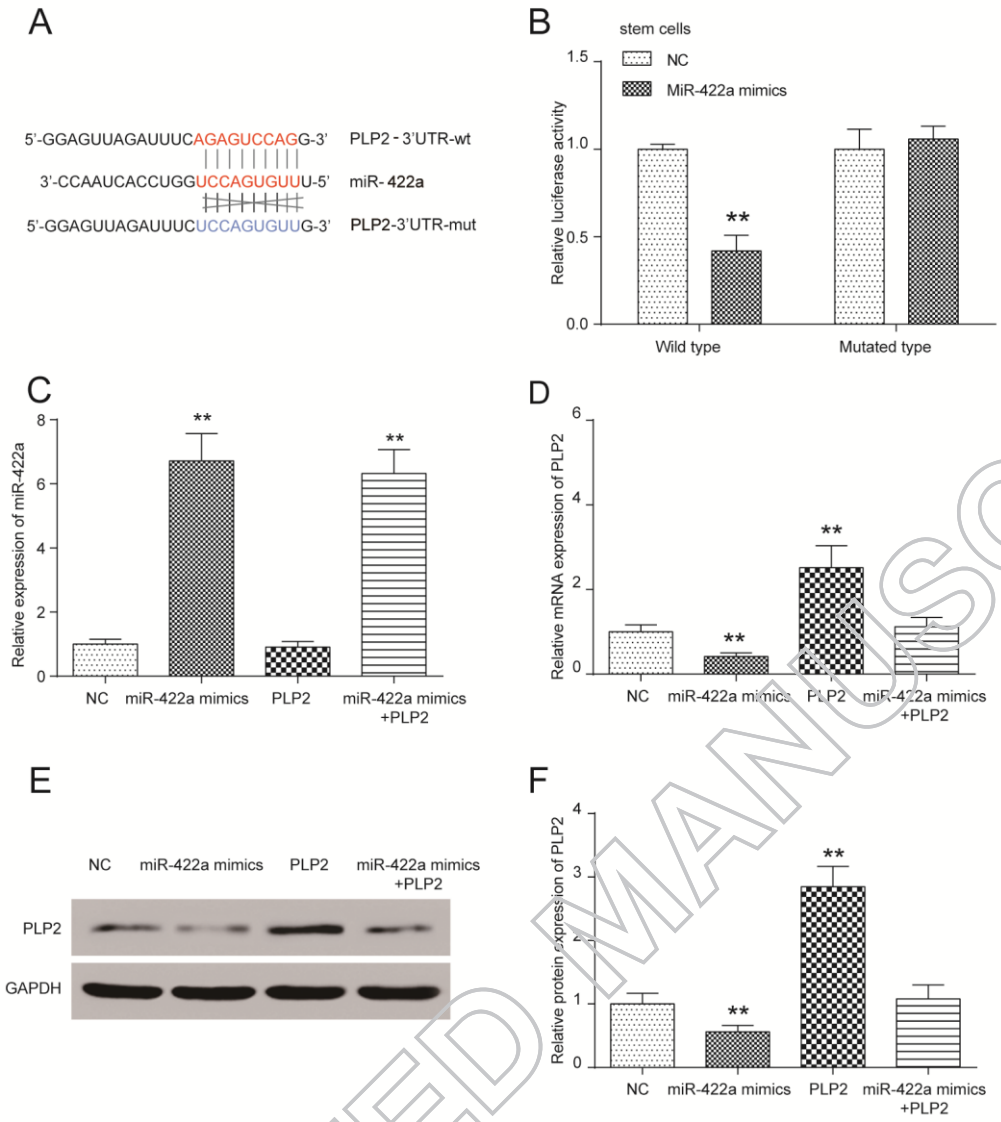

Figure 2. MiR-422a downregulated *PLP2* expression in BCSCs. (A) MiRanda predicted site 285 as binding sites. (B) Dual-Luciferase reporter assay verified that miR-422a directly targeted at *PLP2* wild type and downregulated luciferase activity ($P<0.05$). (C-F) MiR-422a expression was increased in miR-422a mimics group and miR-422a mimics+*PLP2* group. *PLP2* expression was decreased in miR-422a mimics group and increased in *PLP2* group, but was not influenced significantly in miR-422a mimics+*PLP2* group. $^{**}P< 0.01$ indicated significant difference compared with NC group.

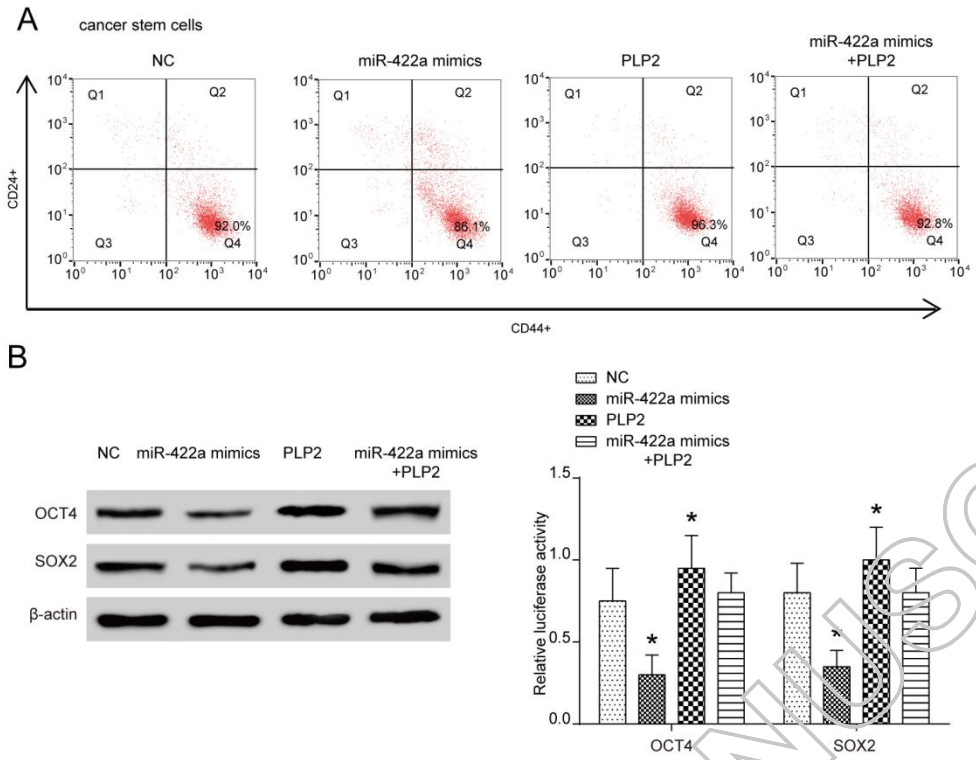

Figure 3. MiR-422a suppressed BCSCs properties by *PLP2* downregulation. (A) BCSCs population decreased in miR-422a mimics group and increased in PLP2 overexpression group. (B) Stemness markers OCT4 and SOX2 expression were significantly suppressed in miR-422a mimics group and elevated in PLP2 overexpression group.

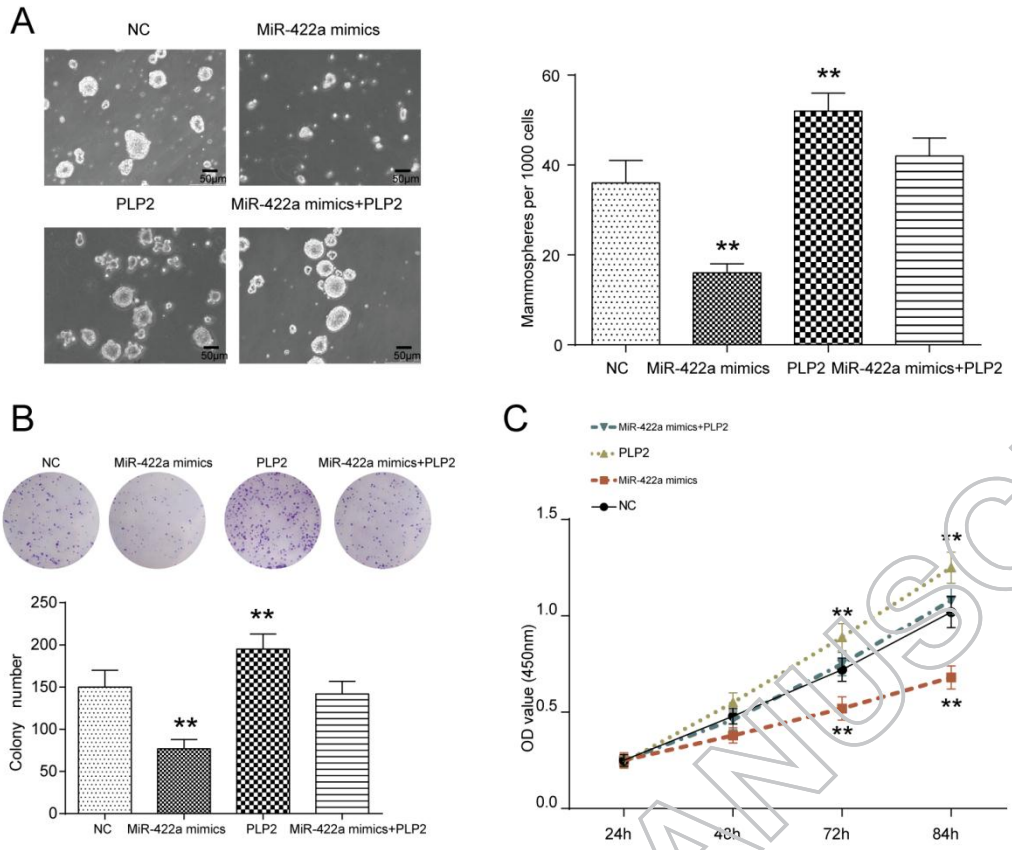

Figure 4. MiR-422a inhibited while *PLP2* promoted BCSCs proliferation. (A) Bright field images of sphere formation assays displayed that miR-422a mimics led to a decrease while *PLP2* overexpression led to an increase in tumorsphere formation. (B) MiR-422a inhibited cell proliferation and *PLP2* accelerated the proliferation while joint expression of miR-422a and *PLP2* revealed no significant changes in cell formation assay. (C) Cell proliferation was significantly repressed in miR-422a mimics group while upregulated in *PLP2* group in CCK8 assay. Similar proliferation rate was detected in the mix group compared with NC group. $^{**}P< 0.01$ indicated significant difference compared with NC group. Scale bar: 50 μm.

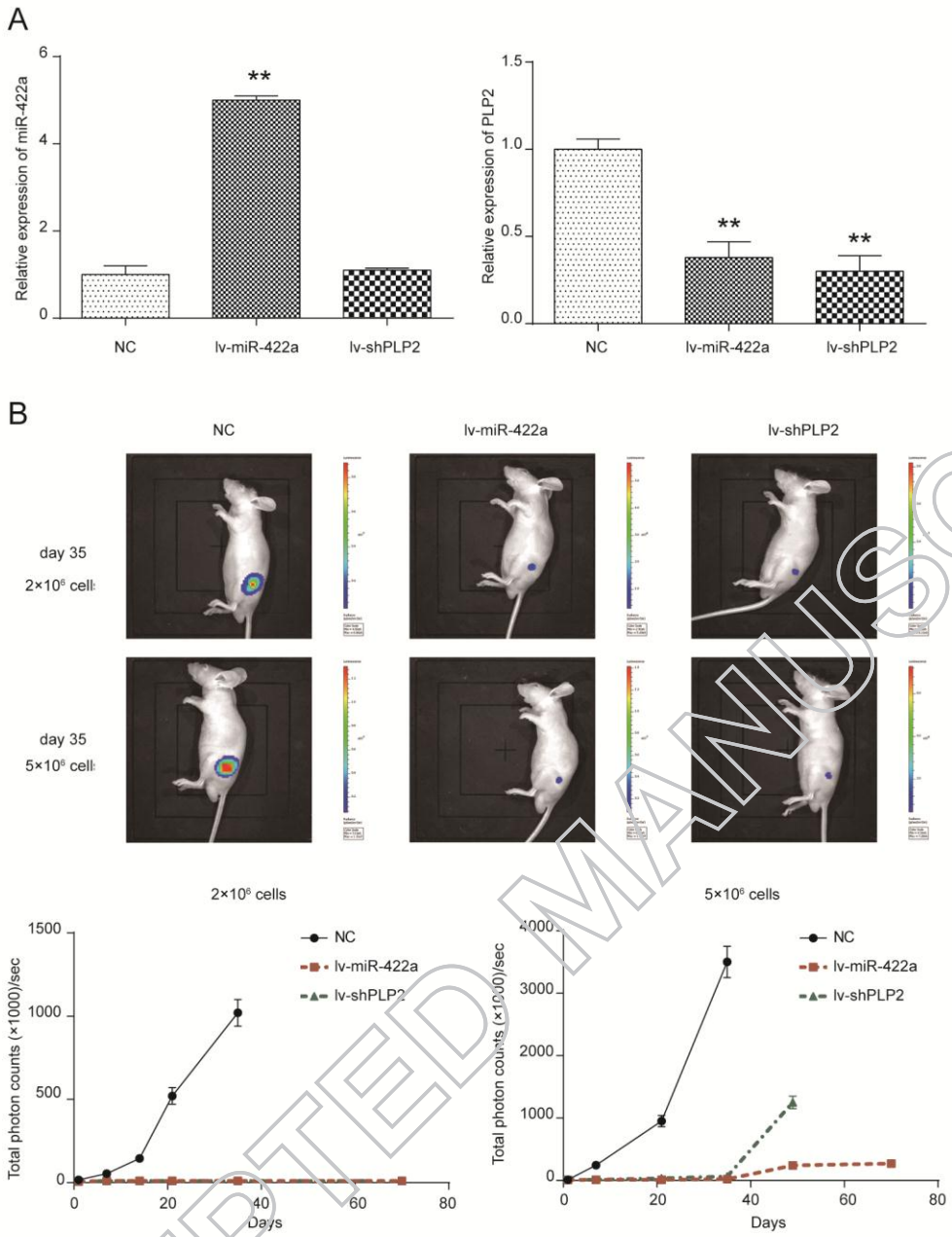

Figure 5. MiR-422a mimics and PLP2 shRNA attenuated BCSCs tumorigenesis in vivo. (A) MiR-422a expression was increased in miR-422a overexpressing group and PLP2 expression was decreased in lv-miR-422a group and lv-shPLP2 group. (B) Mice in lv-NC group inoculated with 2 × 10^6 /ml cells into fat pad formed 1.5 μm tumors in 35 days, while those in lv-miR-422a group and lv-shPLP2 group failed to form palpable tumors. Mice in lv-NC group inoculated with 5 × 10^6 /ml cells into fat pad formed 1.5 μm tumors in 28 days but lv-shPLP2 group as well as lv-shPLP2 group displayed no significant changes. $^{**}P<0.01$ indicated significant difference compared with NC group.

Table 1. Primers for qRT-PCR.

	Primers
MiR-422a	F: 5’-ACUGGACUUAGGGUCAGAAGGC-3’
MiR-422a	R: 5’-GCCUUCUGACCCUAAGUCCAGU-3’
U6	F: 5’-CTTCGGCAGCACATATAC-3’
U6	R: 5’-GAACGCTTCACGAATTTGC-3’
PLP2	F: 5’-CTCATAGCGGCAATCCTCTAC- 3’
PLP2	R: 5’-AAGGTGACATAGGCATCATAGC-3’
GAPDH	F: 5’-TGGTCACCAGGGCTGCTT-3’
GAPDH	R: 5’-AGCTTCCCGTTCTCAGCC-3’

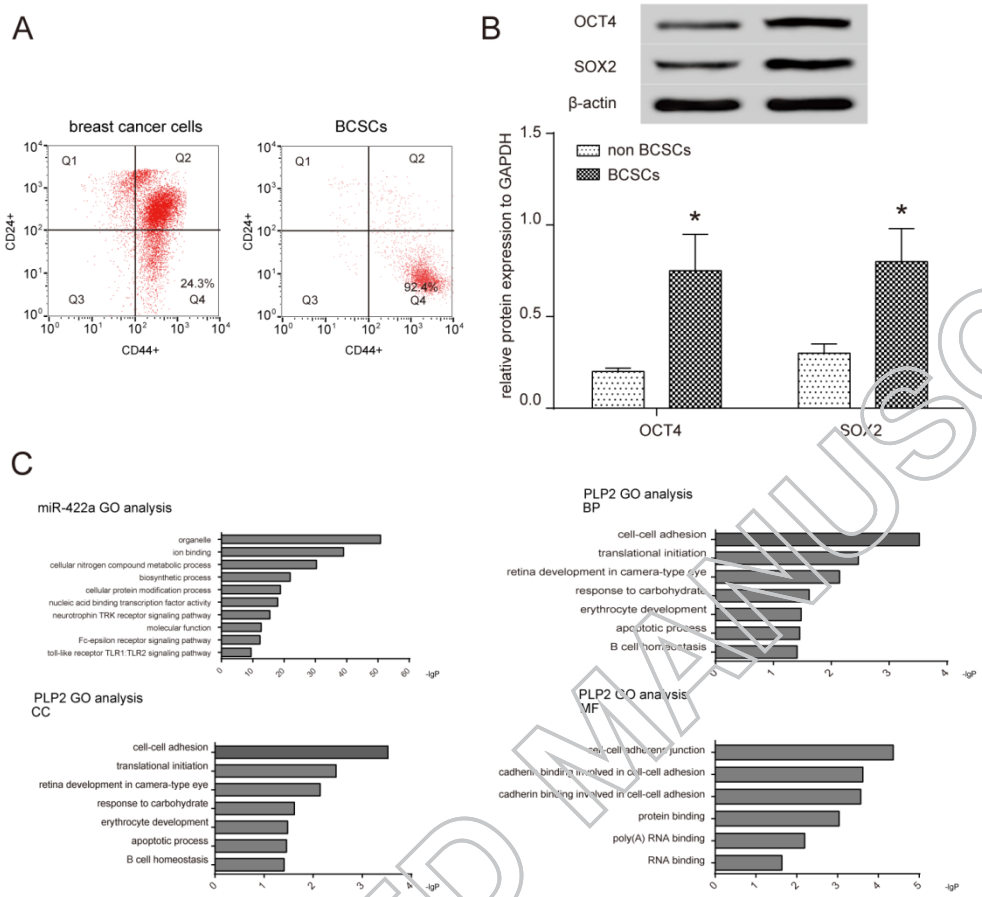

Figure S1. BMSCs isolation and gene Oncology analysis. (A) Breast cancer cells with CD24-CD44+ markers were isolated and named as BCSCs. (B) Stemness markers expression were validated by SOX6 and OCT4 expressions. (C) GO functional annotation of miR-422a and analysis of differentially expressed mRNAs were obtained from David website. MiR-422a was significantly correlated with TLR2 signaling pathway. Those differentially expressed mRNAs showed that they were significantly correlated with RNA binding and B cell homeostasis.

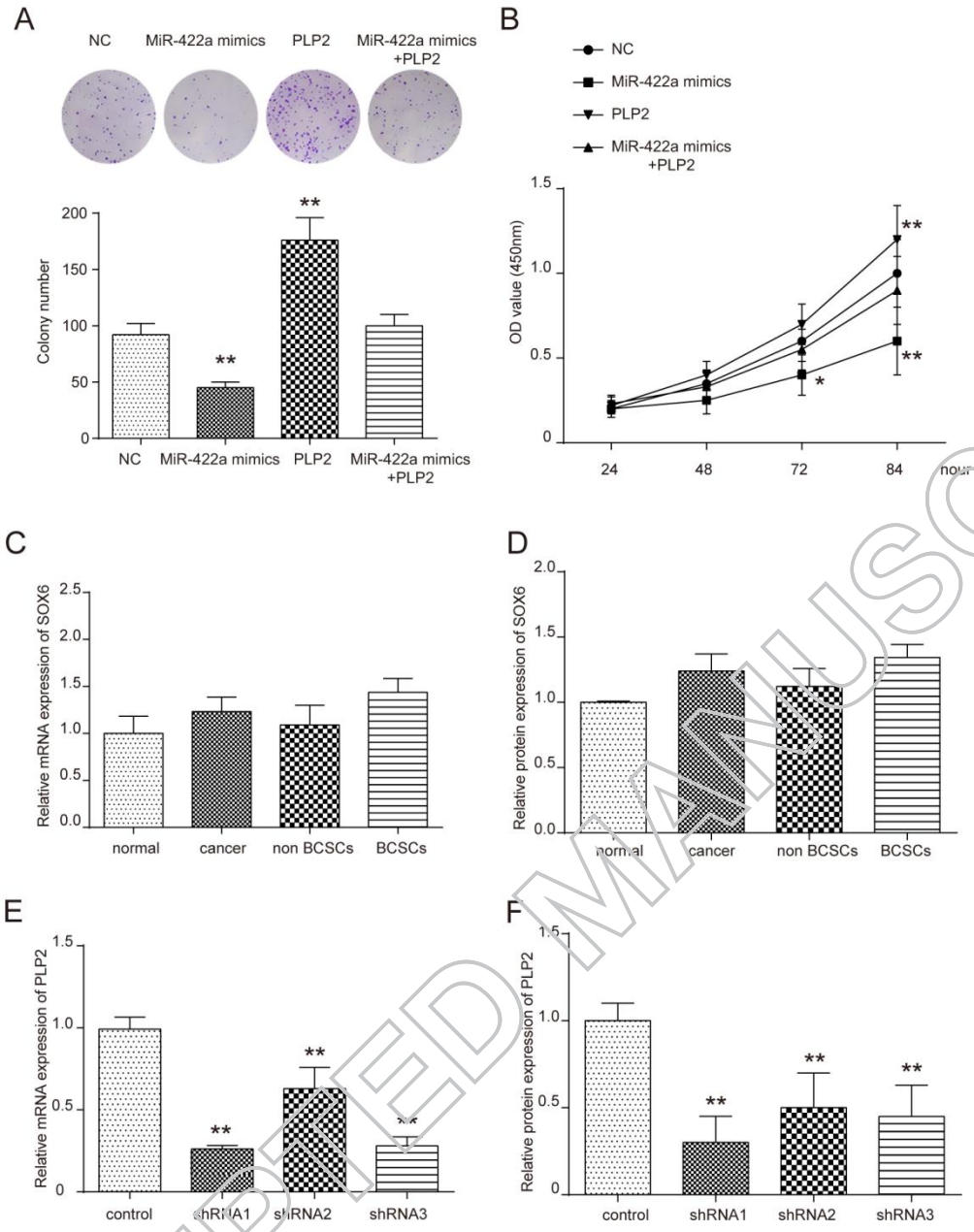

Figure S2. MiR-422a's influence on breast cancer cells, BCSCs stemness validation and shRNA efficiency detection. (A-B) MiR-422a mimics inhibited cell proliferation and cell vitality but PLP2 overexpression enhanced cell proliferation and cell vitality. (C-D) SOX2 mRNA and protein expressions were validated in isolated BCSCs and turned out to show no statistical significance. (E-F) shRNA1 turned out to show the best PLP2 knockdown efficiency.

Table S1 Fold change and P value for MiRNA selection.

genesymbol	logFC	P.Value	adj.P.Val
hsa-miR-122a	5.374164	4.74E-07	8.25E-05
hsa-miR-122a	4.892376	4.28E-06	0.000141
hsa-miR-122a	4.861325	1.05E-05	0.000214
mmu-miR-201	4.406877	7.44E-07	8.25E-05
mmu-miR-463	3.489278	9.35E-05	0.000931
mir128B-3	3.315814	7.53E-06	0.000189
hsa-miR-33	3.100923	9.27E-05	0.000931
mmu-miR-322	2.989234	8.32E-05	0.000873
hsa-miR-450	2.856665	1.23E-05	0.000231
hsa-miR-152	2.645847	8.31E-06	0.000193
hsa-miR-139	2.376154	8.94E-05	0.000913
hsa-miR-373	2.26873	9.11E-05	0.000925
hsa-miR-362	-2.01435	5.94E-05	0.000697
hsa-miR-497	-2.06461	3.68E-05	0.000494
hsa-miR-339	-2.10507	3.56E-05	0.000483
mir9-5	-2.12758	7.47E-05	0.00081
hsa-miR-18b	-2.13957	2.43E-05	0.000356
hsa-miR-130b	-2.18033	8.60E-05	0.000887
hsa-miR-494	-2.21356	2.79E-05	0.000398
hsa-miR-29c	-2.26227	7.42E-05	0.00081
hsa-miR-422a	-2.355	2.32E-05	0.000347
hsa-miR-186	-2.38734	1.30E-05	0.000236
hsa-miR-101	-2.39359	7.22E-05	0.0008

hsa-miR-98	-2.51916	2.08E-05	0.000324
hsa-miR-422b	-2.57973	2.21E-05	0.000333
hsa-miR-186	-2.60611	7.86E-06	0.000189
hsa-miR-324-5p	-2.62769	9.44E-06	0.000203
hsa-miR-432	-2.63316	1.81E-05	0.000292
hsa-miR-29c	-2.64127	6.41E-05	0.000731
hsa-miR-337	-2.79766	3.40E-05	0.000469
hsa-miR-186	-2.80658	1.09E-05	0.000214
hsa-miR-429	-2.81729	4.10E-06	0.000138
hsa-miR-422a	-2.82585	3.91E-06	0.000136
hsa-miR-34b	-2.8532	4.94E-06	0.000154
hsa-miR-422a	-2.89079	1.70E-05	0.000283
hsa-miR-148b	-2.91432	5.72E-06	0.00016
hsa-miR-34b	-2.9589	5.03E-05	0.000627
hsa-miR-520d	-3.05071	1.38E-05	0.000243
hsa-miR-520f	-3.06325	3.87E-05	0.000516
hsa-miR-148b	-3.06374	2.47E-06	0.000115
hsa-miR-34b	-3.0649	1.36E-06	8.95E-05
mir9-3	-3.08028	2.94E-05	0.000412
hsa-miR-520c	-3.09395	3.17E-06	0.000125
hsa-miR-335	-3.11518	7.05E-06	0.000186
hsa-miR-148b	-3.12657	7.81E-06	0.000189
hsa-miR-517a	-3.13294	9.36E-06	0.000203
hsa-miR-373	-3.2179	1.53E-06	9.31E-05
hsa-miR-520d	-3.25862	3.06E-06	0.000124
hsa-miR-517c	-3.26123	2.09E-05	0.000324

hsa-miR-20b	-3.2985	5.41E-05	0.000655
hsa-miR-520c	-3.40129	3.91E-06	0.000136
hsa-miR-98	-3.41061	6.55E-05	0.000741
hsa-miR-301	-3.50705	7.05E-06	0.000186
hsa-miR-520c	-3.52317	5.87E-07	8.25E-05
hsa-miR-335	-3.52933	1.51E-05	0.000263
hsa-miR-519a	-3.5642	8.64E-06	0.000198
hsa-miR-200a	-3.59041	7.18E-06	0.000186
hsa-miR-200a	-3.61014	5.77E-06	0.00016
hsa-miR-181d	-3.62824	1.10E-05	0.000214
hsa-miR-188	-3.75625	3.24E-06	0.000125
hsa-miR-365	-3.79001	8.38E-07	8.25E-05
hsa-miR-20b	-3.80431	2.18E-06	0.000114
hsa-miR-365	-3.914	1.26E-05	0.000235
hsa-miR-29b	-3.9404	8.38E-07	8.25E-05
hsa-miR-188	-4.11855	1.34E-06	8.95E-05
hsa-miR-188	-4.177	1.16E-05	0.000222
hsa-miR-365	-4.18536	2.50E-07	8.25E-05
hsa-miR-181d	-4.24222	7.81E-07	8.25E-05
hsa-miR-181d	-4.50955	3.78E-07	8.25E-05

Table S2 Gene selection.

genesymbol	logFC	P.Value	adj.P.Val
CAST	2.06578	0.0003	0.131433
BCLAF1	2.076871	0.000113	0.113065
CNBP	2.081516	0.000782	0.151136
ITCH	2.105401	0.000695	0.148555
LDHA	2.13313	0.000636	0.144501
GTF3A	2.136708	9.99E-05	0.112053
MT1F	2.183497	4.16E-05	0.111596
GNPTAB	2.189172	0.000255	0.128764
PAPD4	2.192218	7.03E-05	0.111596
GNA13	2.289873	8.38E-06	0.082637
SPCS3	2.390901	0.00062	0.144501
PXK	2.391712	0.000545	0.144501
JARID2	2.428098	0.000661	0.144621
CD44	2.474284	0.000911	0.158055
LYN	2.565293	0.000115	0.113065
FAM49A	2.598693	0.00087	0.158055
CSNK1A1	2.599158	0.000217	0.128764
JMJD1C	2.653646	2.90E-05	0.102256
TMSB4XP4	2.722732	0.000869	0.158055
ARRDC3	2.850496	0.00097	0.161839
SLED1	2.861462	0.000256	0.128764
ZDHHC7	2.904895	2.29E-06	0.082637
AGTPBP1	2.934979	0.000385	0.13664

SH3GLB1	2.972169	0.000902	0.158055
RPS15	3.001094	0.000525	0.144501
KIAA1033	3.029816	0.000141	0.121085
RUNX1-IT1	3.141228	0.000639	0.144501
NCKAP1L	3.263682	0.000235	0.128764
EIF4G2	3.61522	0.000283	0.128938
EIF3M	3.737349	0.000282	0.128938
LY86	3.804418	0.000236	0.128764
FTH1P4	3.828316	0.000964	0.161839
RAP1B	3.830355	0.000195	0.128558
SDCBP	3.916592	0.000589	0.144501
PLP2	3.979422	0.000986	0.161839
SAMSN1	4.095245	0.000382	0.13664
AHR	4.246521	9.39E-05	0.111596
SLIRP	4.327446	4.36E-05	0.111596
PSMA6	4.831971	0.000239	0.128764
RPL24	4.918023	4.93E-05	0.111596
IFNGR1	5.588224	0.000159	0.121085
SOX6	5.830208	3.65E-05	0.111596