Received: 6 February 2018 | Accepted: 4 May 2018

DOI: 10.1002/jcb.27117

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Epigallocatechin-3-gallate inhibited cancer stem cell–like properties by targeting hsa-mir-485-5p/RXRα in lung cancer

Pan Jiang | Chuyue Xu | Lijun Chen | Aochang Chen | Xiaoyue Wu | Ming Zhou | Ijaz Ul Haq | Zahula Mariyam | Qing Feng

Department of Nutrition and Food Hygiene, Key Laboratory of Toxicology, School of Public Health, Nanjing Medical University, Nanjing, China

Correspondence
Qing Feng, Department of Nutrition and Food Hygiene, Key Laboratory of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211100, Jiangsu, China.
Email: qingfeng@njmu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81472977; Priority Academic Program Development of Jiangsu Higher Education Institutions

Abstract

Non–small-cell lung cancer (NSCLC) appears to be a significant threat to public health worldwide. MicroRNAs have been identified as significant regulators for the development of NSCLC. Previous reports have suggested that hsa-mir-485-5p is dysregulated in various cancers. RXRα, as a kind of nuclear receptor, is an effective target of cancer treatment. Cancer stem cells (CSCs) are recognized as the main cause for tumor metastasis, recurrence, and chemotherapy resistance. However, the mechanism by which hsa-mir-485-5p and RXRα modulate CSCs in NSCLC remains unknown. Here, we found that hsa-mir-485-5p was decreased in serum samples from patients with NSCLC and NSCLC cells. Meanwhile, epigallocatechin-3-gallate (EGCG), an effective anticancer compound extracted from green tea, can enhance hsa-mir-485-5p expression. Hsa-mir-485-5p mimics markedly inhibited NSCLC cell growth and induced cell apoptosis. However, inhibition of hsa-mir-485-5p significantly enriched CSC-like traits. Moreover, bioinformatics analysis predicted the binding correlation between hsa-mir-485-5p and RXRα, which was confirmed by a dual-luciferase reporter assay. We observed that RXRα was increased in NSCLC and EGCG could inhibit RXRα levels dose dependently. In addition, RXRα upregulation or activation expanded the CSC-like properties of NSCLC cells, whereas RXRα inhibition or inactivation could exert a reverse phenomenon. Consistently, in vivo experiments also validated that EGCG could repress the CSC-like characteristics by modulating the hsa-mir-485-5p/RXRα axis. Our findings may reveal a novel molecular mechanism for the treatment of NSCLC.

KEYWORDS

cancer stem cells, epigallocatechin-3-gallate, hsa-mir-485-5p, non–small-cell lung cancer, RXRα

1 | INTRODUCTION

Lung cancer is well known as a leading cause of cancer-related death worldwide, with a high mortality rate.[1,2] Approximately 85% of patients with cancer are diagnosed with non–small-cell lung cancer (NSCLC), one of the most predominant pathological types of lung cancers.[3] Although considerable progress has been made in the treatment of NSCLC, the 5-year overall survival rate of patients with NSCLC still remains poor, mainly due to

the lack of early diagnosis and effective treatments. Therefore, novel and effective molecular biomarkers are urgently needed for the diagnosis and treatment of NSCLC.

MicroRNAs (miRNAs) belong to a class of small noncoding RNAs, which can modulate various posttranscriptional processes.[4,5] MiRNAs can play crucial roles in many cellular biological processes, such as cell proliferation, differentiation, and apoptosis.[6] Many studies have established that hsa-mir-485-5p plays tumor-suppressive roles in multiple cancers. Hsa-mir-485-5p can suppress glioma tumorigenesis and serve as a novel candidate for glioma therapeutic treatment.[7] Hsa-mir-485-5p expression was reduced in patients with gastric cancer and it predicts a poor prognosis.[8] In hepatocellular carcinoma, hsa-mir-485-5p can suppress cell proliferation and invasion by inhibiting stanniocalcin 2.[9] However, the biological roles of hsa-mir-485-5p in NSCLC remain uninvestigated.

RXRs act as members of the steroid/thyroid hormone superfamily, with 3 RXR subtypes: α, β, and γ. These are nuclear transcription factors that are essential in embryonic development, differentiated phenotypes, metabolism, and cell death.[10,11] Among the 3 RXR subtypes, RXRα can play unique roles in various physiological processes, including tumorigenesis. According to many previous studies, RXRα is elevated in multiple human cancers, such as human prostate cancer,[12] breast cancer,[13] and thyroid cancer.[14] In lung cancer, it has been reported that dual targeting of RXRα and HDAC can lead to pleiotropic antitumor activities.[15] In our previous study, we reported that RXRα was increased in head and neck squamous cell carcinoma (HNSCC).[16] Nevertheless, the potential roles of RXRα in NSCLC are not clear.

Cancer stem cells (CSCs) are a subpopulation of stem-like cells, which have the ability of self-renewal and differentiation in solid tumors.[17,18] Targeting CSCs can provide a novel strategy for cancer therapy. For instance, miR-196a-5p can modulate gastric CSC characteristics via inhibiting Smad4.[19] MiR-335 can inhibit osteosarcoma stem cell–like properties by targeting POU5F1.[20] However, the correlation between hsa-mir-485-5p and CSCs in NSCLC remains unclear. Epigallocatechin-3-gallate (EGCG) is a type of catechin and it can exhibit many activities, including anti-inflammatory, antidiabetes, antiobesity, and antitumor.[21] EGCG can inhibit growth and induce apoptosis in esophageal cancer cells through demethylating and reactivating the p16 gene.[22] EGCG can attenuate the cell viability of NSCLC A549 cells through inhibiting Bcl-xL.[23] In addition, EGCG can specifically target CSCs in several types of cancers.[24] In colorectal cancer, EGCG can inhibit cancer stem–like cells and promote 5-fluorouracil chemosensitivity.[25] EGCG can repress nasopharyngeal CSC self-renewal and migration, and reverse the epithelial to mesenchymal transition through inactivating nuclear factor-κB (NF-κB) p65.[26]

We aimed to investigate the mechanism of EGCG in modulating lung CSCs. In our previous study, we found that RXRα was increased in HNSCC tissues and it could induce HNSCC stemness.[16] Here, we found that hsa-mir-485-5p was decreased and RXRα was increased in NSCLC. EGCG can upregulate hsa-mir-485-5p and downregulate RXRα, respectively. Hence, we hypothesized that EGCG can inhibit stemness by targeting the hsa-mir-485-5p/RXRα axis in NSCLC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

A549, H460, H1299, and HEK-293T cells were purchased from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank (Shanghai, China). H460 and HEK-293T cells were maintained in Dulbecco modified Eagle medium (Gibco, Carlsbad, CA). A549 and H1299 cells were cultured using RPMI 1640 medium supplemented with 10% of heat-inactivated fetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Cells were incubated in a 5% CO_2 incubator at 37°C.

2.2 | Tumor specimens

NSCLC serum samples and health controls were obtained from patients in the First Affiliated Hospital of Nanjing Medical University. Institutional approval was obtained from the review boards of Nanjing Medical University. Before serum acquisition, we obtained written informed consent from each patient. Institutional approval was obtained from the Ethical Review Board of Nanjing Medical University (Nanjing, China). The experiment was performed according to the approved guidelines.

2.3 | Western blot assay

Equal proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen, Life Technologies Corp, Carlsbad, CA) and transferred onto nitrocellulose filter membranes (GE Healthcare UK Ltd, Bucks, UK). The membranes were incubated with primary antibodies. The next day, the membranes were treated with horseradish peroxidase–conjugated secondary antibodies for 1 hour. The primary antibodies included anti-RXRα, anti-CD133, anti-CD44, anti–SRY (sex-determining region Y)-box 2 (anti-Sox2),

anti-Nanog, anti–octamer-binding transcription factor 4 (anti-Oct4), anti–matrix metallopeptidase 9 (anti-MMP-9), anti–E-cadherin, anti–β-actin, and anti–glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (1:1000; Abcam, Cambridge, UK).

2.4 | Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted using RNAiso Plus (Takara Biotechnology, Dalian, China). RNA was reverse-transcribed using the PrimeScript RT Master Mix (Takara Biotechnology) and a quantitative polymerase chain reaction (qPCR) was performed using SYBR Premix Ex Taq II (Takara Biotechnology). The qPCR primers are shown in Table 1. GAPDH was used as an internal messenger RNA (mRNA) control. U6 was used as a reference miRNA control. qPCR was carried out using the Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA).

2.5 | Luciferase activity assay

The wild-type (WT) or mutant (MUT) RXRα-binding hsa-mir-485-5p was synthesized and subcloned into the pGL3 basic vector (Promega, Madison, WI). Mimics or inhibitors of hsa-mir-485-5p (RiboBio, Guangzhou, China) were cotransfected with 10 μg pLUC-WT-RXRα or pLUC-MUT-RXRα using Lipofectamine 2000 reagent (Invitrogen).

2.6 | Flow cytometry analysis

Cells were collected, resuspended, and incubated in a freezer for 30 minutes with fluorescence-conjugated monoclonal antibodies purchased from BD Biosciences (San Jose, CA) against human CD44 allophycocyanin (APC) and its isotype immunoglobulin G1. For cell apoptosis analysis, cells were harvested and stained using fluorescein isothiocyanate annexin V and propidium iodide. Cell apoptosis was observed using the FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (FlowJo LLC, Ashland, OR).

2.7 | Transfection

Hsa-mir-485-5p mimics, inhibitors, or their negative controls (Invitrogen) were transfected. Human RXRα or control (RiboBio) small interfering RNAs (siRNAs) were transfected into the cells. The human RXRα plasmid pECE-RXRα or the control plasmid pECE (Invitrogen) was transfected.

2.8 | Nude mouse xenograft studies

Twelve mice were obtained from the Shanghai Animal Laboratory Center and housed in the Experimental Animal Center. Mice were injected subcutaneously into the front dorsum with parental A549 cells (5×10^6 each). Two groups were created: the control group (6 mice, normal saline, 0.1 mL per 10 g) and the EGCG group (6 mice, EGCG, 20 mg/kg). Two weeks later, the mice were administered the indicated doses of EGCG by an intraperitoneal injection every 3 days. After 6 weeks, mice were killed by cervical dislocation. This study was carried out in a strict accordance with the requirements in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.9 | Immunohistochemistry staining

Immunohistochemistry staining was performed by the Department of Pathology, The First Affiliated Hospital of Nanjing Medical University. Image-Pro Plus software (version 6.0; Media Cybernetics, Bethesda, MD) was used to analyze the staining results.

2.10 | Statistical analysis

Data were presented as the mean ± standard deviation of at least 3 independent experiments. Comparisons between quantitative variables were performed using the Student *t* test or 1-way analysis of variance. Data were considered statistically significant when the $P < .05$. GraphPad Prism v 6.0 software (GraphPad Software, San Diego, CA) was used for the statistical analysis.

TABLE 1 Primers used for the real-time polymerase chain reaction

Genes	Forward (5′-3′)	Reverse (5′-3′)
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG
RXRα	TTCGCTAAGCTCTTGCTC	ATAAGGAAGGTGTCAATGGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MiR-485	CCAAGCTTCACCCATTCCTAACAGGAC	CGGGATCCGTAGGTCAGTTACATGCATC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3 | RESULTS

3.1 | EGCG enhanced hsa-mir-485-5p expression in NSCLC cells

Hsa-mir-485-5p has been reported to play tumor-suppressive roles in multiple cancers. To determine hsa-mir-485-5p expression in patients with NSCLC, serum samples were collected and subjected to a quantitative reverse-transcription PCR (qRT-PCR) assay. In Figure 1A, it is shown that hsa-mir-485-5p was significantly decreased in patients with NSCLC. Meanwhile, compared with human normal lung epithelial BES-2B cells, hsa-mir-485-5p was downregulated in NSCLC cells (Figure 1B). Then, the cells were treated with different doses of EGCG for 24 hours and we observed that hsa-mir-485-5p was upregulated in A549 (Figure 1C), H460 (Figure 1D), and H1299 (Figure 1E) cells in a dose-dependent manner. These data suggested that hsa-mir-485-5p can serve as a tumor suppressor and EGCG can induce its expression in vitro.

3.2 | EGCG rescued CSC-like properties triggered by the silencing of hsa-mir-485-5p

To detect whether hsa-mir-485-5p was involved in NSCLC growth, hsa-mir-485-5p mimics were transfected into A549 cells. The MTT assay was used to measure the cell survival of A549 cells and it was shown that hsa-mir-485-5p mimics could slow down the cell growth (Figure 2A). In addition, overexpression of hsa-mir-485-5p can markedly improve the apoptosis capacity of A549 cells (Figure 2B). As reported in other studies, various CSC-like markers, such as CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9, are responsible for lung CSCs. Hence, to evaluate whether hsa-mir-485-5p played a role in the stemness of NSCLC cells, hsa-mir-485-5p inhibitors were tranfected into A549 cells for 24 hours. We observed that hsa-mir-485-5p inhibition can markedly increase $CD44^+$ cell ratios in A549 cells (Figure 2C). Then, A549 cells were treated with 20 μM EGCG for 24 hours after hsa-mir-485-5p inhibitors were transfected (Figure 2C). Intriguingly, it was demonstrated that 20 μM EGCG could reverse the stemness that was induced by hsa-mir-485-5p inhibitors (Figure 2C). Additionally, hsa-mir-485-5p mimics can repress CD133, CD44, and Oct4 protein levels in A549 cells (Figure 2D). A tumor-sphere–formation assay can isolate and enrich CSCs. Here, A549 cells were placed in a serum-free suspension culture containing the appropriate growth factors. After 1 week, stable tumor spheres were observed in A549 cells and then cells were transfected with hsa-mir-485-5p mimics. As shown in Figure 2E, the volume of tumor spheres was markedly repressed

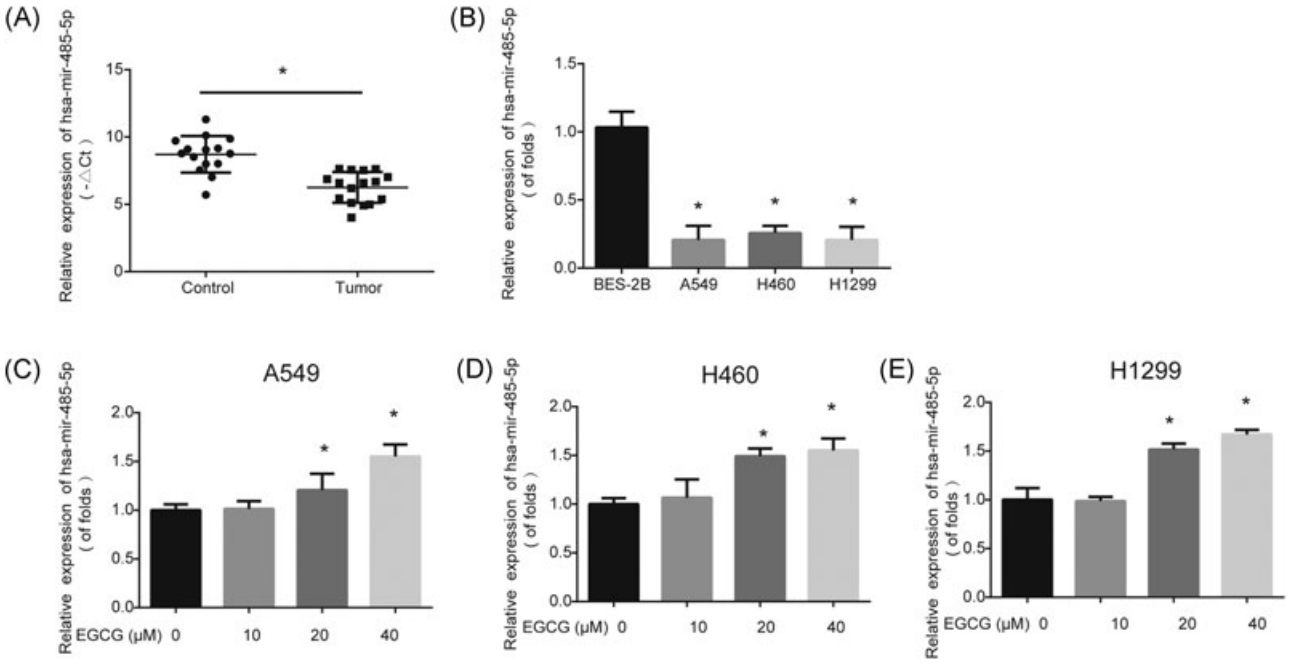

FIGURE 1 EGCG increased hsa-mir-485-5p expression, which was downregulated in NSCLC serum samples and NSCLC cells. (A) Hsa-mir-485-5p levels in NSCLC serum samples (n = 16) and healthy volunteer controls (n = 15). Quantitative polymerase chain reaction analyses were performed to test hsa-mir-485-5p expression with U6 as a loading control. (B) Hsa-mir-485-5p expression in NSCLC cells, including A549, H1299, H460, and human normal bronchial epithelial cells BES-2B. Hsa-mir-485-5p expression in A549 (C), H460 (D), and H1299 (E) cells. Cells were treated with 0, 10, 20, and 40 μM EGCG for 24 hours, respectively. Error bars represent the mean ± SD of at least 3 experiments. *$P < .05$. EGCG, epigallocatechin-3-gallate; NSCLC, non–small-cell lung cancer

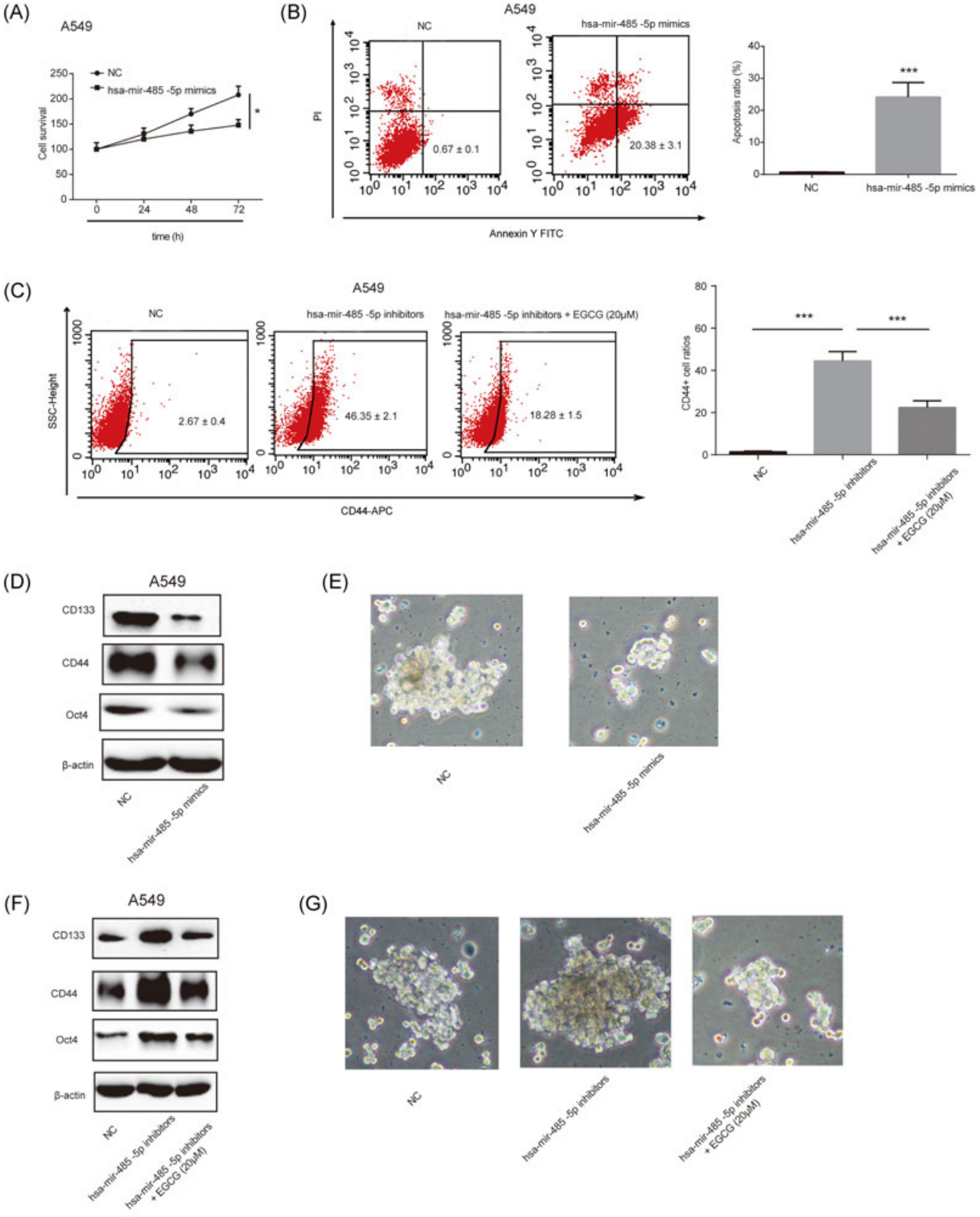

FIGURE 2 Continued

by overexpression of hsa-mir-485-5p mimics. Meanwhile, hsa-mir-485-5p inhibitors increased the protein expression of CD133, CD44, Oct4, and tumor spheroid volume, whereas EGCG could rescue this phenomenon (Figure 2F,G). Therefore, from these results, it was implied that EGCG could inhibit CSC-like properties through increasing hsa-mir-485-5p.

3.3 | Hsa-mir-485-5p directly targeted RXRα

To identify the potential mRNA target of hsa-mir-485-5p, bioinformatics analysis, including TargetScan, Starbase, miRanda, and the miRDB database, was carried out. First, in Figure 3A, the conserved binding region between hsa-mir-485-5p and RXRα was observed. To validate the association between them, a dual-luciferase reporter test was used. Luciferase reporter plasmids of WT-RXRα and MUT-RXRα–binding sites were constructed and are shown in Figure 3B. Next, we observed that cotransfection of the luciferase reporter plasmid containing WT-RXRα with hsa-mir-485-5p mimics in HEK-293T cells decreased the reporter activity (Figure 3C). Conversely, cotransfection of the luciferase reporter plasmid containing WT-RXRα with hsa-mir-485-5p inhibitors increased the reporter activity (Figure 3D). In addition, it was found that RXRα levels were markedly inhibited by hsa-mir-485-5p upregulation but significantly promoted by hsa-mir-485-5p inhibition in NSCLC cells (Figure 3E–H). These findings indicated that RXRα can serve as a direct target of hsa-mir-485-5p.

3.4 | EGCG inhibited RXRα expression in vitro

RXRα is involved in various cancers and then to investigate RXRα mRNA expression in NSCLC, a qRT-PCR analysis was carried out. In Figure 4A,B, we found that RXRα was markedly increased in patients with NSCLC and in A549, H460, and H1299 cells. Next, we treated the cells with 0, 10, 20, and 40 μM EGCG, respectively. qRT-PCR results demonstrated that RXRα mRNA expression was inhibited by EGCG in NSCLC cells (Figure 4C–E). In addition, RXRα protein levels were also repressed by EGCG in vitro in a dose-dependent manner (Figure 4F–H). These findings implied that RXRα can act as an oncogene in NSCLC in vitro, which can be suppressed by EGCG.

3.5 | Knockdown of RXRα reduced the CSC-like traits of NSCLC cells

To explore whether RXRα was correlated with the stemness of NSCLC, RXRα siRNA or siRNA control was transfected into NSCLC cells. First, after RXRα was knocked down for 24 hours, mRNA expression of RXRα was obviously repressed in A549, H460, and H1299 cells (Figure 5A–C). Subsequently, we observed that the protein expression of RXRα, CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9 was also markedly decreased by inhibition of RXRα (Figure 5D–F). Then, to investigate whether RXRα inactivation was involved in the stemness of lung cancer, UVI3003, which acts as an antagonist of RXRα, was used in a dose-dependent manner. As shown in Figure 5G, it was found that the indicated doses of UVI3003 could alleviate CSC-like traits by increasing the protein levels of E-cadherin and inhibiting CD133, CD44, Nanog, and Oct4 in NSCLC cells. Basically, from these results, we suggested that knockdown or inactivation of RXRα can ameliorate the CSC-like properties of NSCLC cells.

3.6 | Overexpression of RXRα enriched the CSC-like characteristics of NSCLC cells

To investigate whether overexpression of RXRα can induce stemness in NSCLC, RXRα plasmids were transfected into NSCLC cells for 48 hours. In Figure 6A–C, we observed that RXRα expression was markedly increased by the RXRα plasmid in A549, H460, and H1299 cells. Then, it was found that protein the expression of RXRα, CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9 was markedly increased by the upregulation of RXRα (Figure 6D–F). Additionally, a flow cytometry assay was performed to determine the $CD44^+$ cell ratios and the tumor-sphere–formation test was used to detect sphere-formation capacity after the transfection of the RXRα plasmid. Interestingly, the RXRα plasmid could enrich $CD44^+$ cell populations of A549 cells and increase the spheroid

FIGURE 2 EGCG rescued the stemness triggered by the inhibition of hsa-mir-485-5p in vitro. (A) Effects of hsa-mir-485-5p mimics on the growth of A549 cells. Cells were treated with hsa-mir-485-5p mimics. The MTT assay was carried out to test the cell survival. (B) Effects of hsa-mir-485-5p mimics on the apoptosis of A549 cells. A flow cytometry assay was used to perform an analysis of apoptosis. (C) Flow cytometry analyses of the ratios of $CD44^+$ cells (mean ± SD, n = 3). Cells were transfected with hsa-mir-485-5pinhibitors for 24 hours and then treated with 20 μM EGCG. (D) CD133, CD44, and Oct4 protein levels in A549 cells. Cells were transfected with hsa-mir-485-5p mimics or their corresponding negative controls. (E) Microscopic observation of the sphere of A549 cells. All the images are ×100 magnified. (F) CD133, CD44, and Oct4 protein levels in A549 cells. (G) Microscopic observation of the sphere of A549 cells. Cells were treated with hsa-mir-485-5p inhibitors for 24 hours and then streated with 20 μM EGCG. Error bars represent the mean ± SD of at least triplicate experiments. $^*P < .05$, $^{***}P < .001$. EGCG, epigallocatechin-3-gallate; FITC, fluorescein isothiocyanate; Oct4, octamer-binding transcription factor 4; PI, propidium iodide

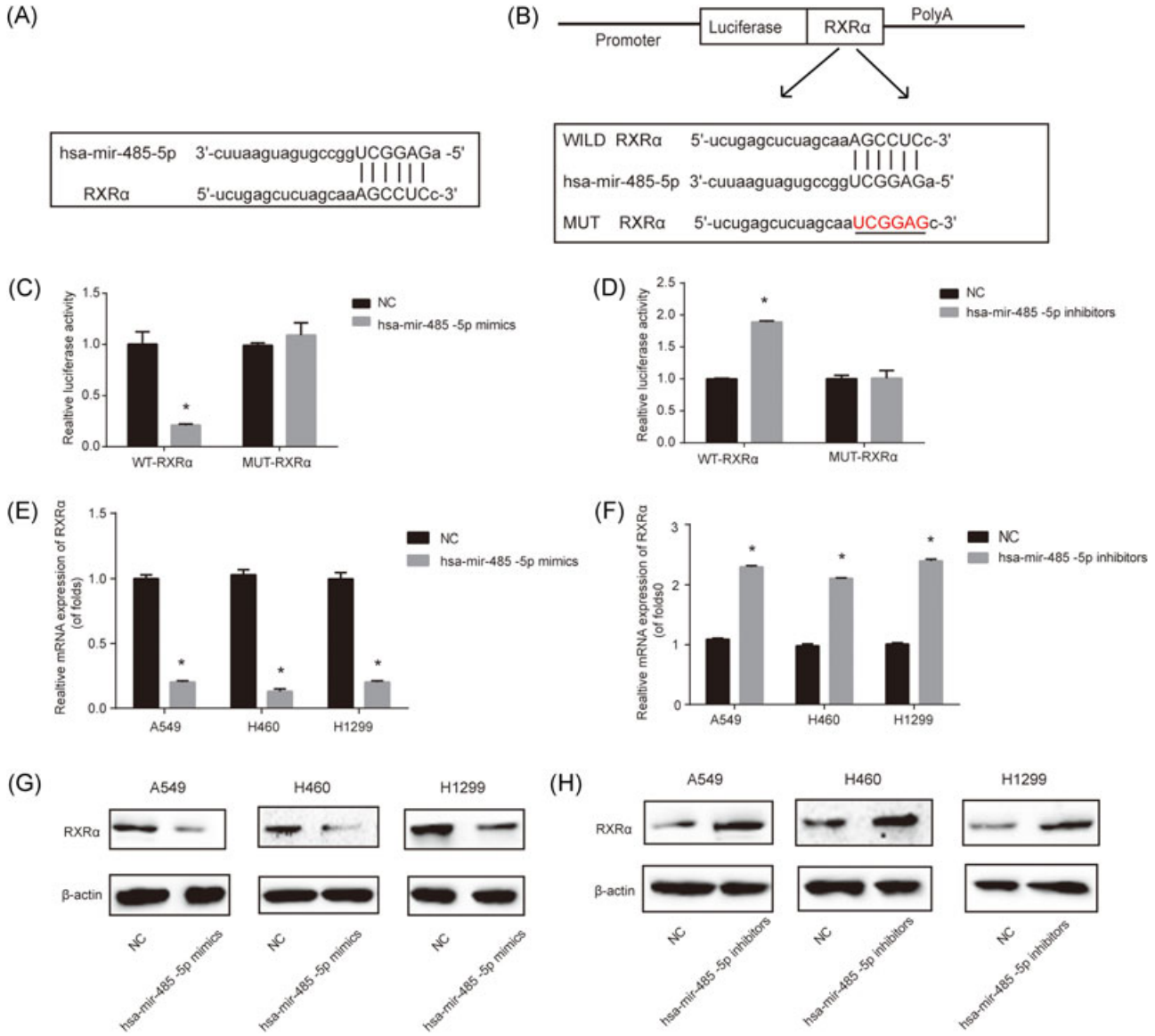

FIGURE 3 RXRα served as a direct target of hsa-mir-485-5p. (A) Binding region between hsa-mir-485-5p and RXRα. (B) The luciferase reporter constructs containing the WT-RXRα or the MUT-RXRα sequence. (C) WT-RXRα or MUT-RXRα was cotransfected into HEK-293T cells with hsa-mir-485-5p mimics or their corresponding negative controls. (D) WT-RXRα or MUT-RXRα was cotransfected into HEK-293T cells with hsa-mir-485-5p inhibitors or their corresponding negative controls. (E) mRNA expression of RXRα in NSCLC cells. Cells were transfected with hsa-mir-485-5p mimics or their corresponding negative controls. (F) mRNA expression of RXRα in NSCLC cells. Cells were transfected with hsa-mir-485-5p inhibitors or their corresponding negative controls. (G) Protein expression of RXRα in NSCLC cells. Cells were transfected with hsa-mir-485-5p mimics or their corresponding negative controls. (H) Protein expression of RXRα in NSCLC cells. Cells were transfected with hsa-mir-485-5p inhibitors or their corresponding negative controls. Error bars represent the mean ± SD of at least triplicate experiments. *$P < .05$. mRNA, messenger RNA; MUT, mutant; NC, negative control; NSCLC, non–small-cell lung cancer; WT, wild type

volume, respectively (Figure 6G,H). Next, to test whether the activation of RXRα could expedite the CSC-like properties of NSCLC cells, A549 cells were treated with different doses of 9-*cis*-retinoic acid (9-*cis*-RA), an agonist of RXRα, for 24 hours. Western blot analysis results demonstrated that 9-*cis*-RA could facilitate the stemness of NSCLC dose dependently (Figure 6I). Therefore, these findings indicated that upregulation or activation of RXRα could accelerate the stemness of NSCLC cells.

3.7 | EGCG inhibited CSC-like properties by targeting hsa-mir-485-5p/RXRα in vivo

An A549 cell nude mouse xenograft model was established to explore whether EGCG can restrain CSC-like phenotypes

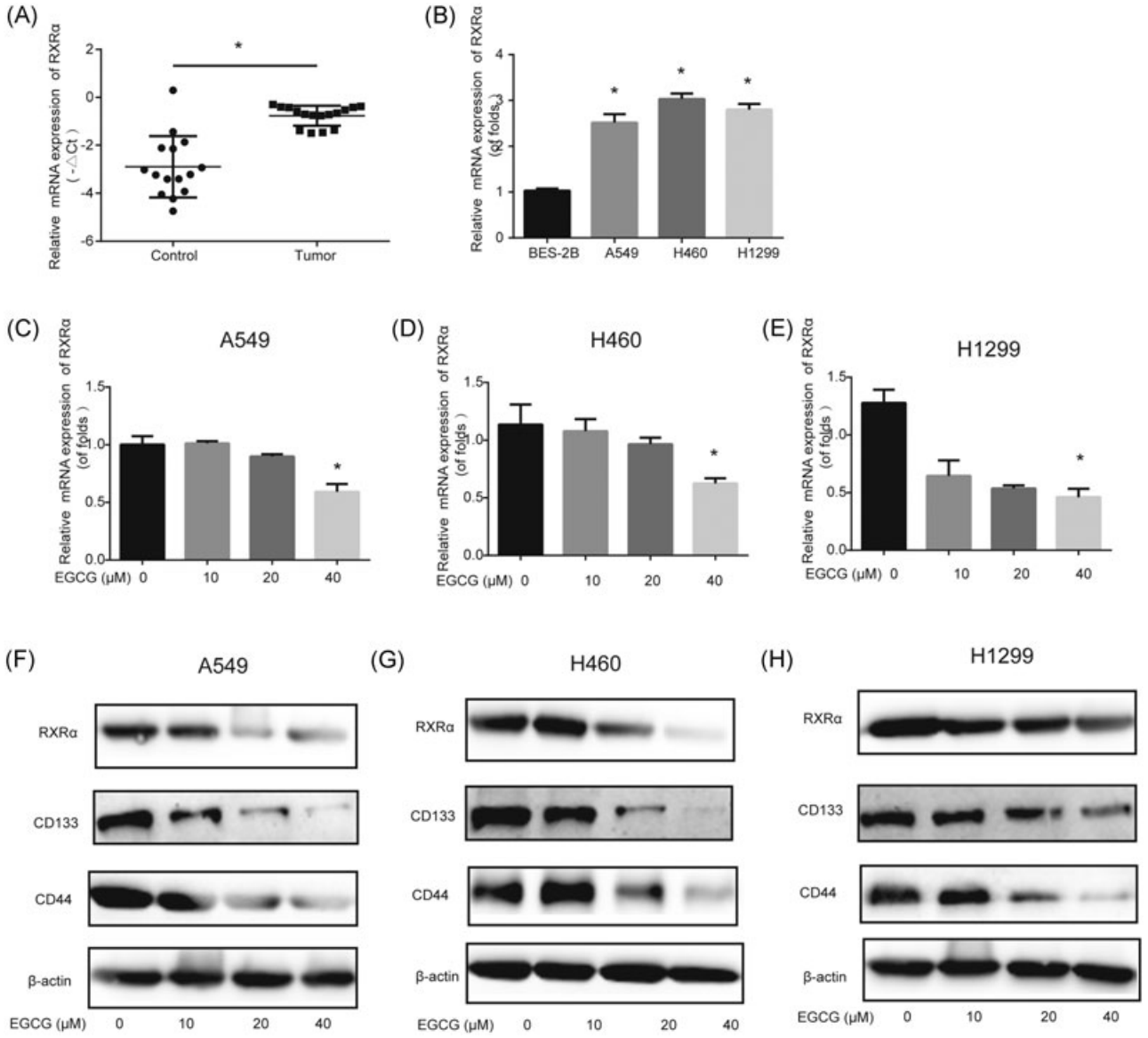

FIGURE 4 EGCG decreased RXRα expression, which was upregulated in NSCLC serum samples and NSCLC cells. (A) RXRα mRNA levels in the serum samples of patients with NSCLC (n = 16) and healthy volunteer controls (n = 15). qPCR analyses were carried out to detect RXRα mRNA expression. GAPDH acted as a loading control. (B) RXRα mRNA expression in NSCLC cells, including A549, H1299, H460, and BES-2B. RXRα expression in A549 (C), H460 (D), and H1299 (E) cells. Cells were treated with 0, 10, 20, and 40 μM EGCG for 24 hours, respectively. RXRα, CD133, and CD44 protein expression in A549 cells (F), H460 cells (G), and H1299 cells (H). Western blot analysis was used to determine RXRα, CD133, and CD44 protein expression. β-Actin was used as a reference control. Error bars represent the mean ± SD of at least triplicate experiments. *$P < .05$. EGCG, epigallocatechin-3-gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NSCLC, non–small-cell lung cancer

via targeting hsa-mir-485-5p/RXRα. Mice were divided into 2 groups (control group and EGCG group) and tumors were peeled from nude mice subcutis (Figure 7A). Immunohistochemistry assay results showed that Ki-67 was strongly repressed in the EGCG group (Figure 7B). Meanwhile, EGCG decreased RXRα mRNA levels and increased hsa-mir-485-5p expression, which was consistent with the in vitro data (Figure 7C). In addition, RXRα, CD133, and CD44 protein expression was markedly inhibited in the EGCG group compared with the control group. Hence, these results suggested that EGCG could inhibit stemness via modulating hsa-mir-485-5p/RXRα in vivo (Figure 7D).

4 | DISCUSSION

Here, we have shown that hsa-mir-485-5p was downregulated, whereas RXRα was upregulated in the serum samples and NSCLC cells of patients with NSCLC. RXRα knockdown can inhibit CSC-like properties,

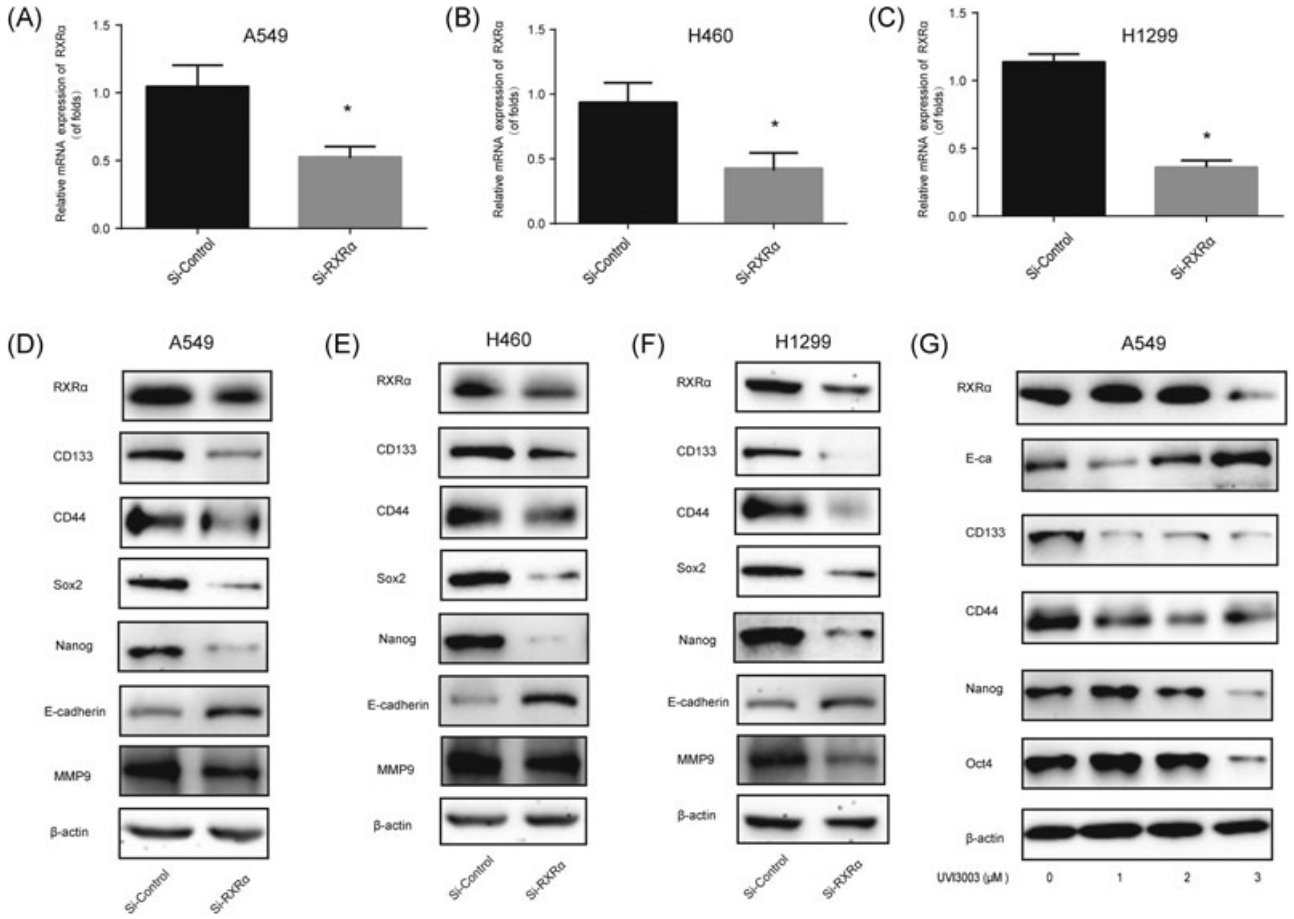

FIGURE 5 Knockdown and inactivation of RXRα attenuated the stemness of NSCLC cells. RXRα mRNA in A549 (A), H460 (B), and H1299 (C) cells. RXRα siRNA or its parental negative control was transfected into NSCLC cells. RXRα, CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9 protein expression in A549 (D), H460 (E), and H1299 (F) cells. (G) RXRα, E-cadherin, CD133, CD44, Nanog, and Oct4 protein expression in A549 cells. A549 cells were treated with 0, 1, 2, and 3 μM UVI3003 for 24 hours, respectively. Error bars represent the mean ± SD of at least triplicate experiments. $*P < .05$. MMP-9, matrix metallopeptidase 9; mRNA, messenger RNA; NSCLC, non–small-cell lung cancer; Oct, octamer-binding transcription factor 4; siRNA, small interfering RNA; Sox2, SRY (sex-determining region Y)-box 2

whereas RXRα overexpression can expand the stemness of NSCLC cells. In addition, EGCG could induce hsa-mir-485-5p expression and inhibit RXRα levels. EGCG can reverse the CSC-like characteristics that were triggered by inhibition of hsa-mir-485-5p. To our knowledge, this is a novel report of a potential mechanism for EGCG-inhibited CSC-like properties in NSCLC.

Subpopulations of cancer cells with increased renewal capacity are referred to as CSCs.[27] CSCs are emerging as crucial therapeutic targets for anticancer therapies. Recent data have demonstrated that NSCLC contains self-renewing subpopulations, which exhibits a stem-like phenotype and a high risk of tumor recurrence. Lung CSCs have been isolated from human cell lines and patient samples.[28-30] Therefore, CSC-related molecular changes can enable the development of specific agents for the eradication of tumor-maintaining CSCs. Natural products, such as EGCG, curcumin, resveratrol, sulforaphane, and withaferin-A, can diminish the clinically devastating properties of CSCs.[31] For example, curcumin and EGCG can inhibit breast CSCs through down-regulating STAT3–NF-κB signaling.[32] EGCG can repress the growth and tumorigenicity of nasopharyngeal tumor–initiating cells via attenuating STAT3 activation.[33] EGCG represses the properties of glioma stem–like cells and synergizes with temozolomide via downregulating P-glycoprotein.[34] In addition, EGCG can inhibit lung CSCs by mediating the Wnt/β-catenin pathway.[35] Previously, we had proven that curcumin can inhibit CSC-like properties by inactivating Wnt signaling.[16] In addition, we have reported that EGCG can enhance cisplatin sensitivity in NSCLC cells by modulating noncoding RNAs hsa-mir-98-5p and nuclear-enriched abundant transcript 1 (NEAT1).[36] Here, in our current study, we focused on whether noncoding RNAs were

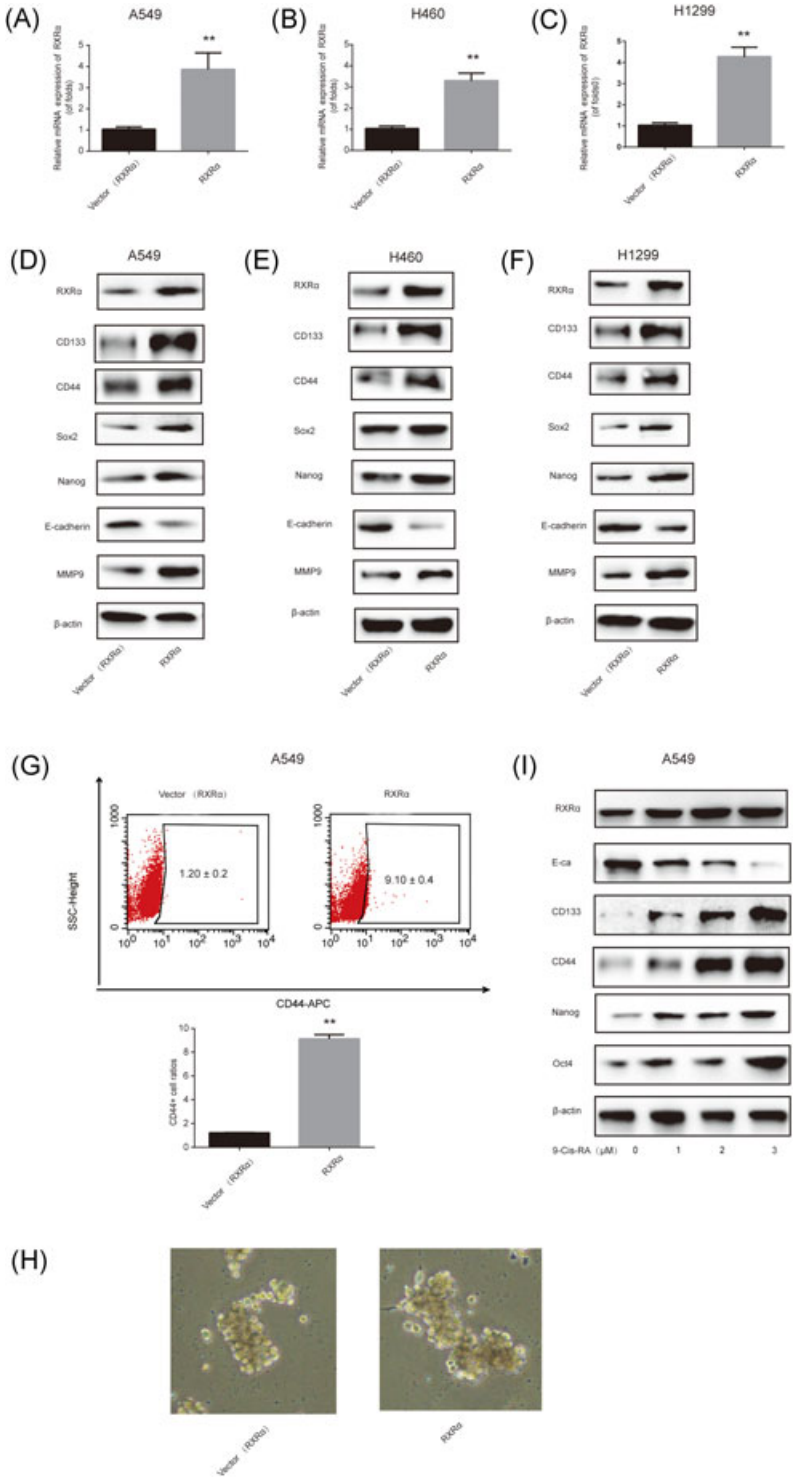

FIGURE 6 Overexpression and activation of RXRα exacerbated the CSC-like characteristics of NSCLC cells. RXRα mRNA in A549 (A), H460 (B), and H1299 (C) cells. NSCLC cells were transfected with vectors or RXRα plasmid. Twenty-four hours later, the cell samples were harvested and treated with qPCR. RXRα, CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9 protein expression in A549 (D), H460 (E), and H1299 (F) cells. (G) Flow cytometry analyses of the ratios of $CD44^+$ cells (mean ± SD, n = 3). (H) Microscopic observation of sphere of A549 cells. I, RXRα, E-cadherin, CD133, CD44, Nanog, and Oct4 protein expression in A549 cells. A549 cells were treated with 0, 1, 2, and 3 μM 9-*cis*-RA for 24 hours, respectively. Error bars represent the mean ± SD of at least triplicate experiments. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. APC, allophycocyanin; 9-cis-RA, 9-*cis*-retinoic acid; CSC, cancer stem cell; MMP-9, matrix metallopeptidase 9; mRNA, messenger RNA; NSCLC, non–small-cell lung cancer; Oct, octamer-binding transcription factor 4; qPCR, quantitative polymerase chain reaction; Sox2, SRY (sex-determining region Y)-box 2; SSC, side scatter

involved in the mechanism of EGCG in regulating lung CSCs.

Hsa-mir-485-5p has been identified as a significant tumor suppressor in various cancers, including breast cancer, gastric carcinoma, and ovarian cancer.[37-39] For instance, hsa-mir-485-5p can reverse epithelial to mesenchymal transition and promote cisplatin-induced cell death via targeting P21 (RAC1) activated kinase 1 (PAK1) in oral tongue squamous cell carcinoma.[40] Hsa-mir-485-5p–binding site single-nucleotide polymorphism rs8752 in the 15-hydroxyprostaglandin dehydrogenase (HPGD) gene is correlated with the risk of breast cancer.[41] However, its cellular function in NSCLC is not clear. Consistent with the roles of hsa-mir-485-5p in other cancers, we found that hsa-mir-485-5p was decreased in serum samples from NSCLC patients and NSCLC cells. Interestingly, we found that EGCG could induce its expression in

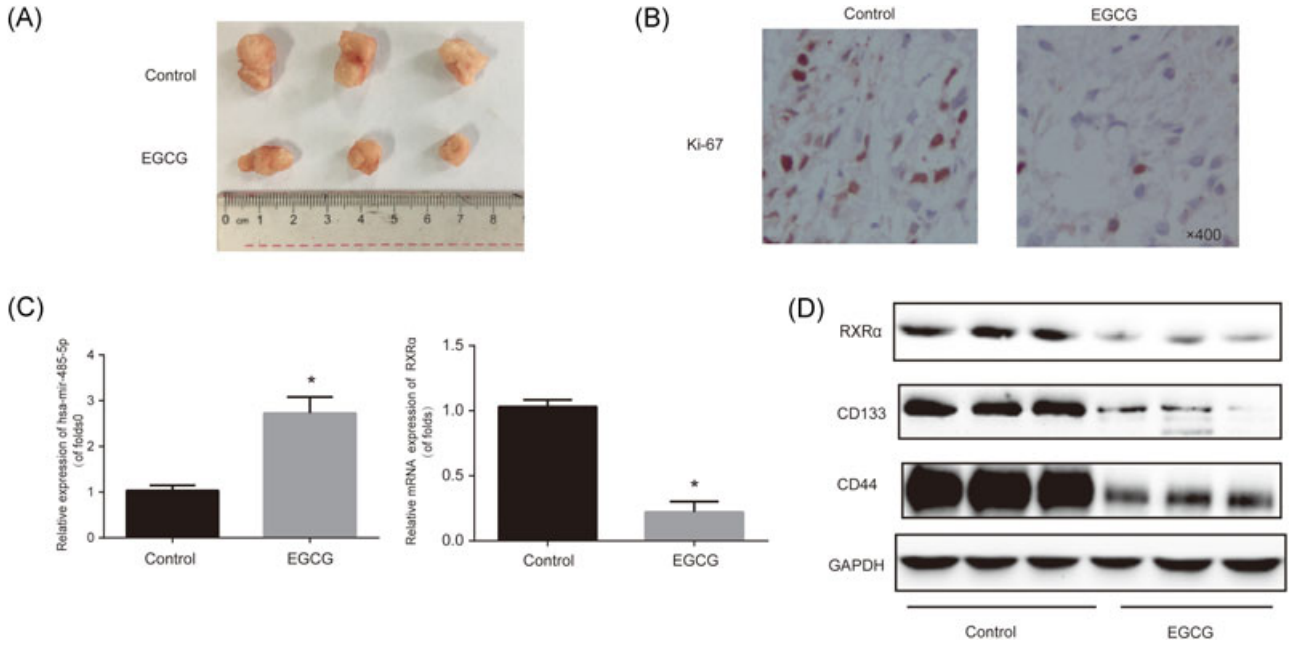

FIGURE 7 EGCG played an inhibitory role in the CSC-like properties by targeting hsa-mir-485-5p/RXRα in vivo. Twelve 5-week-old female BALB/c nude mice were injected with 5×10^6 A549 cells each. Two treatment groups were included: control (6 mice) and EGCG (6 mice). (A) Solid tumors were peeled from mouse subcutaneous tissue. (B) Immunohistochemistry staining of Ki-67 in tumor tissues. (C) Hsa-mir-485-5p and RXRα mRNA expression in tumor tissues. (D) RXRα, CD133, and CD44 protein levels in tumor tissues. GAPDH was used as a loading control. Error bars represent the mean ± SD of at least 3 experiments. $^*P < .05$. CSC, cancer stem cell; EGCG, epigallocatechin-3-gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA

NSCLC cells in a dose-dependent manner. According to a previous report, it has been indicated that EGCG can inhibit lung cancer cell growth through upregulating miR-210 expression.[42] Additionally, EGCG can improve cisplatin sensitivity in NSCLC cells by regulating hsa-mir-98-5p.[36] Here, we observed that overexpression of hsa-mir-485-5p could inhibit NSCLC cell growth and induce cell apoptosis. Moreover, it was observed that inhibition of hsa-mir-485-5p could enrich the stemness of NSCLC via increasing $CD44^+$ cell ratios, whereas EGCG could reverse this process.

By performing bioinformatics analysis, RXRα was predicted as a downstream target of hsa-mir-485-5p. Recently, many studies have reported that RXRα is implicated in a number of diseases. In gastric carcinoma tissues and cell lines, RXRα expression was markedly elevated at both mRNA and protein levels.[43] RXRα can promote the proliferation of human cholangiocarcinoma through the modulation of Wnt/β-catenin and NF-κB pathways.[44] Sox9-regulated miRNA-574-3p can inhibit the chondrogenic differentiation of mesenchymal stem cells by targeting RXRα.[45] RXRα can be modulated by miR-128-2 in cholesterol homeostasis.[46] In addition, it has been demonstrated that RXRα can inhibit miR-193a expression, which can activate K-ras and mediate distinct aspects of cellular transformation.[47] Previously, we have proven that RXRα was markedly upregulated in HNSCC tissues and could induce stemness in HNSCC cells.[16] However, the role of RXRα in CSCs of NSCLC remains uninvestigated. Here, in our study, by carrying out a dual-luciferase reporter assay, the correlation between hsa-mir-485-5p and RXRα was confirmed. It was found that hsa-mir-485-5p could modulate RXRα expression negatively. Conversely, we found that RXRα was markedly increased in NSCLC serum samples and NSCLC cells. To validate the effect of RXRα on CSCs, loss and gain of RXRα function tests were performed. Knockdown or inactivation of RXRα can inhibit stemness of NSCLC, whereas overexpression or activation of RXRα can induce CSC-like traits in NSCLC cells. 9-*Cis*-RA is recognized as a prime candidate for cancer chemoprevention by serving biological functions through activating RXR. However, in our study, we observed that the indicated dosage can induce NSCLC stemness by activating RXRα. Hence, the mechanism by which RXRα regulates the stemness in NSCLC should be explored further.

To conclude, our findings indicated an anti-CSC role for EGCG in NSCLC both in vitro and in vivo. Our results suggested that EGCG can inhibit CSC-like properties by targeting the hsa-mir-485-5p/RXRα axis in lung cancer. We propose the possibility of developing EGCG as a CSC-targeting agent for NSCLC chemotherapy.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (81472977) and by funding from the Priority Academic Program Development of Jiangsu Higher Education Institutions.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

ORCID

Qing Feng http://orcid.org/0000-0001-7686-2713

REFERENCES

1. Mahdavifar N, Ghoncheh M, Pakzad R, Momenimovahed Z, Salehiniya H. Epidemiology, incidence and mortality of bladder cancer and their relationship with the development index in the world. *Asian Pac J Cancer Prev*. 2016;17:381-386.
2. Wang F, Meng F, Wang L, Wong SCC, Cho WCS, Chan LWC. Associations of mRNA:microRNA for the shared downstream molecules of EGFR and alternative tyrosine kinase receptors in non-small cell lung cancer. *Front Genet*. 2016;7:173.
3. Souza MC, Cruz OG, Vasconcelos AGG. Factors associated with disease-specific survival of patients with non-small cell lung cancer. *J Bras Pneumol*. 2016;42:317-325.
4. Ortholan C, Puissegur MP, Ilie M, Barbry P, Mari B, Hofman P. MicroRNAs and lung cancer: new oncogenes and tumor suppressors, new prognostic factors and potential therapeutic targets. *Curr Med Chem*. 2009;16:1047-1061.
5. Rusek A, Abba M, Eljaszewicz A, Moniuszko M, Niklinski J, Allgayer H. MicroRNA modulators of epigenetic regulation, the tumor microenvironment and the immune system in lung cancer. *Mol Cancer*. 2015;14:34.
6. Tsai LM, Yu D. MicroRNAs in common diseases and potential therapeutic applications. *Clin Exp Pharmacol Physiol*. 2010;37:102-107.
7. Yu J, Wu SW, Wu WP. A tumor-suppressive microRNA, miRNA-485-5p, inhibits glioma cell proliferation and invasion by down-regulating TPD52L2. *Am J Transl Res*. 2017;9:3336-3344.
8. Jing LL, Mo XM. Reduced miR-485-5p expression predicts poor prognosis in patients with gastric cancer. *Eur Rev Med Pharmacol Sci*. 2016;20:1516-1520.
9. Guo GX, Li QY, Ma WL, Shi ZH, Ren XQ. MicroRNA-485-5p suppresses cell proliferation and invasion in hepatocellular carcinoma by targeting stanniocalcin 2. *Int J Clin Exp Pathol*. 2015;8:12292-12299.
10. le Maire A, Alvarez S, Shankaranarayanan P, Lera AR, Bourguet W, Gronemeyer H. Retinoid receptors and therapeutic applications of RAR/RXR modulators. *Curr Top Med Chem*. 2012;12:505-527.
11. Gronemeyer H, Gustafsson JÅ, Laudet V. Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov*. 2004;3:950-964.
12. Zhong C, Yang S, Huang J, Cohen MB, Roy-Burman P. Aberration in the expression of the retinoid receptor, RXRalpha, in prostate cancer. *Cancer Biol Ther*. 2003;2:179-184.
13. Crowe DL, Chandraratna RA. A retinoid X receptor (RXR)-selective retinoid reveals that RXR-alpha is potentially a therapeutic target in breast cancer cell lines, and that it potentiates antiproliferative and apoptotic responses to peroxisome proliferator-activated receptor ligands. *Breast Cancer Res*. 2004;6:R546-R555.
14. Takiyama Y, Miyokawa N, Sugawara A, et al. Decreased expression of retinoid X receptor isoforms in human thyroid carcinomas. *J Clin Endocrinol Metab*. 2004;89:5851-5861.
15. Wang L, Chen G, Chen K, et al. Dual targeting of retinoid X receptor and histone deacetylase with DW22 as a novel antitumor approach. *Oncotarget*. 2015;6:9740-9755.
16. Jiang P, Xu C, Zhou M, et al. RXRalpha enriched cancer stem cell-like properties triggered by CDDP in head and neck squamous cell carcinoma (HNSCC). *Carcinogenesis*. 2018;39(2):252-262.
17. Bakhshinyan D, Adile AA, Qazi MA, et al. Introduction to cancer stem cells: past, present, and future. *Methods Mol Biol*. 2018;1692:1-16.
18. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat Med*. 2011;17:313-319.
19. Pan Y, Shu X, Sun L, et al. miR196a5p modulates gastric cancer stem cell characteristics by targeting Smad4. *Int J Oncol*. 2017;50:1965-1976.
20. Guo X, Yu L, Zhang Z, Dai G, Gao T, Guo W. miR-335 negatively regulates osteosarcoma stem cell-like properties by targeting POU5F1. *Cancer Cell Int*. 2017;17:29.
21. Min K, Kwon TK. Anticancer effects and molecular mechanisms of epigallocatechin-3-gallate. *Integr Med Res*. 2014;3:16-24.
22. Meng J, Tong Q, Liu X, Yu Z, Zhang J, Gao B. Epigallocatechin-3-gallate inhibits growth and induces apoptosis in esophageal cancer cells through the demethylation and reactivation of the p16 gene. *Oncol Lett*. 2017;14:1152-1156.
23. Sonoda JI, Ikeda R, Baba Y, et al. Green tea catechin, epigallocatechin-3-gallate, attenuates the cell viability of human non-small-cell lung cancer A549 cells via reducing Bcl-xL expression. *Exp Ther Med*. 2014;8:59-63.
24. Fujiki H, Sueoka E, Rawangkan A, Suganuma M. Human cancer stem cells are a target for cancer prevention using (−)-epigallocatechin gallate. *J Cancer Res Clin Oncol*. 2017;143:2401-2412.
25. Toden S, Tran HM, Tovar-Camargo OA, Okugawa Y, Goel A. Epigallocatechin-3-gallate targets cancer stem-like cells and enhances 5-fluorouracil chemosensitivity in colorectal cancer. *Oncotarget*. 2016;7:16158-16171.
26. Li YJ, Wu SL, Lu SM, et al. (−)-Epigallocatechin-3-gallate inhibits nasopharyngeal cancer stem cell self-renewal and migration and reverses the epithelial-mesenchymal transition via NF-kappaB p65 inactivation. *Tumour Biol*. 2015;36:2747-2761.
27. Nassar D, Blanpain C. Cancer stem cells: basic concepts and therapeutic implications. *Annu Rev Pathol*. 2016;11:47-76.
28. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res*. 2007;67:4827-4833.

29. Bertolini G, Roz L, Perego P, et al. Highly tumorigenic lung cancer CD133+cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci U S A*. 2009;106:16281-16286.
30. Chen YC, Hsu HS, Chen YW, et al. Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. *PLoS One*. 2008;3:e2637.
31. Pistollato F, Calderón iglesias R, Ruiz R, et al. The use of natural compounds for the targeting and chemoprevention of ovarian cancer. *Cancer Lett*. 2017;411:191-200.
32. Chung SS, Vadgama JV. Curcumin and epigallocatechin gallate inhibit the cancer stem cell phenotype via down-regulation of STAT3-NFkappaB signaling. *Anticancer Res*. 2015;35:39-46.
33. Lin CH, Chao LK, Hung PH, Chen YJ. EGCG inhibits the growth and tumorigenicity of nasopharyngeal tumor-initiating cells through attenuation of STAT3 activation. *Int J Clin Exp Pathol*. 2014;7:2372-2381.
34. Zhang Y, Wang SX, Ma JW, et al. EGCG inhibits properties of glioma stem-like cells and synergizes with temozolomide through downregulation of P-glycoprotein inhibition. *J Neurooncol*. 2015;121:41-52.
35. Zhu J, Jiang Y, Yang X, et al. Wnt/beta-catenin pathway mediates (−)-epigallocatechin-3-gallate (EGCG) inhibition of lung cancer stem cells. *Biochem Biophys Res Commun*. 2017;482:15-21.
36. Jiang P, Wu X, Wang X, Huang W, Feng Q. NEAT1 upregulates EGCG-induced CTR1 to enhance cisplatin sensitivity in lung cancer cells. *Oncotarget*. 2016;7:43337-43351.
37. Kang M, Ren MP, Zhao L, Li CP, Deng MM. miR-485-5p acts as a negative regulator in gastric cancer progression by targeting flotillin-1. *Am J Transl Res*. 2015;7:2212-2222.
38. Kim TH, Kim YK, Kwon Y, et al. Deregulation of miR-519a, 153, and 485-5p and its clinicopathological relevance in ovarian epithelial tumours. *Histopathology*. 2010;57:734-743.
39. Lou C, Xiao M, Cheng S, et al. MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1alpha expression. *Cell Death Dis*. 2016;7:e2159-e2159.
40. Lin XJ, He CL, Sun T, Duan XJ, Sun Y, Xiong SJ. hsa-miR-485-5p reverses epithelial to mesenchymal transition and promotes cisplatin-induced cell death by targeting PAK1 in oral tongue squamous cell carcinoma. *Int J Mol Med*. 2017;40:83-89.
41. He N, Zheng H, Li P, et al. miR-485-5p binding site SNP rs8752 in HPGD gene is associated with breast cancer risk. *PLoS One*. 2014;9:e102093.
42. Wang H, Bian S, Yang CS. Green tea polyphenol EGCG suppresses lung cancer cell growth through upregulating miR-210 expression caused by stabilizing HIF-1alpha. *Carcinogenesis*. 2011;32:1881-1889.
43. Ren HY, Huang GL, Liu WM, et al. IL-1beta induced RXRalpha overexpression through activation of NF-kappaB signaling in gastric carcinoma. *Biomed Pharmacother*. 2016;78:329-334.
44. Huang GL, Zhang W, Ren HY, Shen XY, Chen QX, Shen DY. Retinoid X receptor alpha enhances human cholangiocarcinoma growth through simultaneous activation of Wnt/beta-catenin and nuclear factor-kappaB pathways. *Cancer Sci*. 2015;106:1515-1523.
45. Guérit D, Philipot D, Chuchana P, et al. Sox9-regulated miRNA-574-3p inhibits chondrogenic differentiation of mesenchymal stem cells. *PLoS One*. 2013;8:e62582.
46. Adlakha YK, Khanna S, Singh R, Singh VP, Agrawal A, Saini N. Pro-apoptotic miRNA-128-2 modulates ABCA1, ABCG1 and RXRalpha expression and cholesterol homeostasis. *Cell Death Dis*. 2013;4:e780-e780.
47. Iliopoulos D, Rotem A, Struhl K. Inhibition of miR-193a expression by Max and RXRalpha activates K-Ras and PLAU to mediate distinct aspects of cellular transformation. *Cancer Res*. 2011;71:5144-5153.

How to cite this article: Jiang P, Xu C, Chen L, et al. Epigallocatechin-3-gallate inhibited cancer stem cell–like properties by targeting hsa-mir-485-5p/RXRα in lung cancer. *J Cell Biochem*. 2018;1-13. https://doi.org/10.1002/jcb.27117