KDM4 Inhibition Targets Breast Cancer Stem-like Cells

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Abstract

Traditional treatments for breast cancer fail to address therapy-resistant cancer stem–like cells that have been characterized by changes in epigenetic regulators such as the lysine demethylase KDM4. Here, we describe an orally available, selective and potent KDM4 inhibitor (QC6352) with unique preclinical characteristics. To assess the antitumor properties of QC6352, we established a method to isolate and propagate breast cancer stem–like cells (BCSC) from individual triple-negative tumors resected from patients after neoadjuvant chemotherapy. Limiting-dilution orthotopic xenografts of these BCSCs regenerated original patient tumor histology and gene expression. QC6352 blocked BCSC proliferation, sphere formation, and xenograft tumor formation. QC6352 also abrogated expression of EGFR, which drives the growth of therapy-resistant triple-negative breast cancer cells. Our findings validate a unique BCSC culture system for drug screening and offer preclinical proof of concept for KDM4 inhibition as a new strategy to treat triple-negative breast cancer. Cancer Res; 77(21); 5900–12. © 2017 AACR.

Introduction

Breast cancer is the leading cause of cancer-related death among women worldwide (1). Among the subtypes of breast cancer, triple-negative disease is associated with a particularly poor prognosis and limited therapeutic options (2). During breast cancer treatment, therapy resistance and metastatic dissemination are the main problems that have to be faced (1). Of note, breast cancer stem cells (BCSC) have been suggested to be responsible for both therapy resistance and metastatic dissemination (3, 4). Until now, these resistant cancer stem cell populations have only been poorly characterized and targeted therapeutics have yet to be identified.

It has been shown that alterations of epigenetic regulators such as the KDM4 family members control tumor cell proliferation particularly in aggressive breast cancers (5) and dysregulation of KDM4 demethylases has been documented in a variety of cancers including breast cancer (6). The KDM4 subfamily is comprised of KDM4A, B, C, and D and belongs to the Jumonji C (JmjC) domain-containing family of histone demethylases (7). KDM4 demethylases has been documented in a variety of cancers including breast cancer (6). The KDM4 subfamily is comprised of KDM4A, B, C, and D and belongs to the Jumonji C (JmjC) domain-containing family of histone demethylases (7). KDM4A, B, C, and D have been shown to have a role in the regulation of cell proliferation and differentiation (8).

Materials and Methods

Tissue specimens

All patients were operated at the Department of Obstetrics and Gynecology at the University Medical Centre Freiburg. Tumor

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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All patients were operated at the Department of Obstetrics and Gynecology at the University Medical Centre Freiburg.
tissue specimens for BCSC isolation and paraffin embedding were obtained from pathologists of the tumor bank of the Comprehensive Cancer Centre Freiburg. All experiments were performed in accordance with the Declaration of Helsinki. We confirm that all experimental protocols were approved by the Institutional Review Board in the Ethics vote 307/13 (Independent Ethics Committee University of Freiburg). Written informed consent was obtained from each patient.

**BCSC isolation method**

All primary breast cancer tumors were collected from individuals who had received chemotherapy and were classified as triple-negative. Primary BCSC lines were isolated by mechanical dissociation of the tumor material followed by enzymatic digestion in 5 mL DPBS (Gibco) supplemented with 6 units DNase I (Machery-Nagel) and 1 mg liberase (Roche) for 1 hour at 37°C. Afterward, the digestion medium was diluted with 10 mL DPBS and filtered through a cell strainer (40 μm, BD). Following centrifugation at 200 × g for 5 minutes, the supernatant was discarded and the cell pellet was washed with MEBM (Gibco). Subsequently, if red blood cells were visible in the pellet, 2 mL ACK Lysis-buffer (Gibco) was added to the cell pellet. After 1 minute of incubation at room temperature, the suspension was filled up to 6 mL with MEBM and centrifuged at 200 × g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL MEBM and filtered through a 40-μm strainer. Following centrifugation at 200 × g for 5 minutes, the supernatant was discarded and the remaining cell pellet suspended in MSC medium. Isolated cells were counted and 2 × 10^5 cells in 200 μL of a 1:1 mixture of MSC medium and Matrigel (ice cold, Corning, 354230) were plated per well in a 24-well low attachment plate (Corning). After solidification of the Matrigel at 37°C for 30 minutes, each well was topped up with 500 μL of MSC medium. The cells were cultured at 37°C under low oxygen conditions (3% O2, 5% CO2, 92% N2). 3D cells stably proliferating cells were cultured and expanded in 2D. All primary BCSC lines were isolated in 2014 and authenticated by the high-throughput Multiplex human cell authentication test (MCA) developed at the DKFZ in 2016 (9). Mycoplasma tests were conducted every 3 months via PCR detection utilizing the positive control provided free of charge by the Leibniz-Institute DSMZ. Experiments with cells were conducted in a passaging window of 15 passages.

**MSC medium**

The mammary stem cell (MSC) medium is composed of mammary epithelial basal medium (Gibco, 31331-028), supplemented with 1 × B27 (Gibco, 17504-044), 1 × amphotericin B (Sigma-Aldrich, A2942), and 1 × penicillin-streptomycin (Gibco, 15140122). Furthermore, epidermal growth factor (20 ng/mL, PeproTech, AF-100-15), heparin (4 μg/mL, Sigma-Aldrich, H3149), fibroblast growth factor (20 ng/mL, PeproTech, AF-100-18B), gentamicin (35 μg/mL, Gibco, 15750-045), and rhino kinase inhibitor (500 nmol/L, Calbiochem, 555552) were added.

**Cell culture**

BCSCs were cultured as spheres in a 3D environment as described above. One milliliter medium was added after 2 days. Cells were split once a week using Dispase (Corning) to resolve Matrigel and Accutase for sphere dissociation. To expand BCSCs in a 2D environment, 4 × 10^5 cells were seeded in 2 mL MSC medium containing 2% Matrigel (ice-cold) in a 10 cm culture dish. After solidification of the Matrigel at 37°C for 30 minutes, the dish was topped up with 8 mL of MSC medium. Cells were grown under low oxygen conditions as described above. Medium was changed after 3 days. Cells were split once a week.

**Anchorage-independent cancer stem cell sphere assay in methylcellulose**

Cells were detached by Accutase and counted. A total of 3 × 10^5 single BCSC1 and 1 × 10^5 single BCSC2 cells were seeded into individual wells of 96-well ultra-low attachment plates (Corning, 3474) in serum-free MSC medium containing 1% methylcellulose (Sigma, M0512). After 7 days, spheres were counted to evaluate the sphere-forming capacity except for experiments including QC6352 treatment, where only spheres over 50-μm diameter were counted.

**Cancer stem cell sphere assay in Matrigel**

Cells were detached by Accutase and counted. Single BCSC1 and BCSC2 cells (1 × 10^5) were seeded as triplicates in 50% Matrigel into individual wells of 24-well ultra-low attachment plates (Corning) in serum-free MSC medium. QC6352 and QC6688 were dissolved in DMSO and paclitaxel in 0.9% saline solution. After 7 days, spheres over 50-μm diameter were counted for QC6352- and QC6688-treated and control cells and spheres over 20-μm diameter were counted for paclitaxel-treated and control cells. For the assessment of secondary sphere formation, wells with 4 × 10^3 single BCSC1 and BCSC2 spheres were seeded and treated as described above. After 7 days, BCSC1 and BCSC2 cells were split and counted as described above. From these, 1 × 10^3 single BCSC1 and BCSC2 cells were seeded in triplicates as described above to assess secondary sphere formation in the absence of QC6352, QC6688 or paclitaxel. After 7 days, spheres over 50-μm diameter were counted for QC6352- and QC6688-treated and control cells, and spheres over 20-μm diameter were counted for paclitaxel-treated and control cells.

**Cell proliferation assay**

High titer lentiviral stock (CMV-NL5-mCherry) was obtained from the Sanford Burnham Prebys Medical Discovery Institute. Lentiviral particles were added at a multiplicity of infection (MOI) of 5 to BCSC1 and BCSC2 in MSC medium. Cells were cultured as described. For cell proliferation assay, BCSC1-mCherry and BCSC2-mCherry were detached by Accutase and counted. 384-well plates (Greiner, 781091) were coated with 10 μL of MSC medium containing 2% of Matrigel (Corning, 354230). After incubation at 37°C for 30 minutes to solidify the Matrigel, 1 × 10^3 single cells were seeded per well in 384-well. Inhibitor was added 24 hours later to the indicated final concentrations. Subsequently, daily cell number assessment for the indicated number of days was performed utilizing the ScanR microscope-based imaging platform (Olympus) and ScanR software 6. At each time point, mCherry-positive cell nuclei in 9 sectors of each well were assessed with a 10× lens.

**Adenoviral knockdown of KDM4 isoforms**

High titer adenoviral stocks (shRNA Ctrl (Ad-GFP-U6-scrambled-shRNA; #1122N), shRNA KDM4A (Ad-GFP-U6-h-KDM4A-shRNA; #shADV-212841), shRNA KDM4B (Ad-GFP-U6-h-KDM4B-shRNA; #shADV-212842), shRNA KDM4C (Ad-GFP-U6-h-KDM4C-shRNA; #shADV-212844), and shRNA...
BCSC1 were obtained from Vector BioLabs. Adenoviral particles were KDM4D [Ad-GFP-U6-hKDM4A-shRNA(#70); #shADV-212848). 

Metzger et al. 105 cells transplanted into immunocompromised mice as and reinfected with an MOI of 300. To generate xenografts, of 300. After 5 days, BCSC1 were seeded into 384-well plates matin immunoprecipitation (ChIP) and ChIP-seq analyses. For (4 described in the animal protocol G13/114. NOD/SCID females dance with an Institutional Animal Care and Use Committee as dance with German Animal Welfare regulations and in accor-

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KDM4D [Ad-GFP-U6-h-KDM4A-shRNA(#70); #shADV-212848] were obtained from Vector BioLabs. Adenoviral particles were added at an MOI of 300 to BCSC1 cells and MOI of 150 to BCSC2 cells in MSC medium. Cells were harvested 3 days after infection for Western blot analyses and after 5 days for Chromatin immunoprecipitation (ChIP) and ChIP-seq analyses. For cell proliferation assays, BCSC1 were infected with an MOI of 300. After 5 days, BCSC1 were seeded into 384-well plates and reinfected with an MOI of 300. To generate xenografts, BCSC1 were infected with an MOI of 300. After 24 hours, BCSC1 were detached, reinfected with an MOI of 300 and 1 × 10^5 cells transplanted into immunocompromised mice as described below.

ChIP

ChIP experiments were performed essentially as previously described (10). BCSC1 cells were either cultured for 18 hours in the absence or presence of 5 × 10^-8 mol/L QC6352 or cells were infected 5 days before harvesting with adenoovirus expressing either shRNA against KDM4A or scrambled control shRNA (Ad-GFP-U6-hKDM4A-shRNA and Ad-U6-RNAi-GFP, respectively, Vector Biolabs) according to the manufacturer’s instructions. Immunoprecipitation was performed with specific antibodies (anti-KDM4A (#5766, lot 021110, Schule Laboratory), anti-H3K9me3 (Diagenode, #C15410056, lot A1675-001P), anti-H3 (#ab1791, lot GR300976-1, Abcam), spike-in antibody (#61686, Active Motif), rIgG (#C15410206, lot RIG001L, Abcam) in the presence of spike-in chromatin (#53083. Active Motif) on GenoDx GenoSeparator (GE-Healthcare). For PCR, 2 μL out of 70 μL isolated DNA were used. Primer sequences were as follows: EGFR 5’-cagagctcatcctggccaac-3’ and 5’-ttctgtctgcacacttggca-3’. Libraries were prepared from immunoprecipitated DNA according to standard methods. ChIP-seq libraries were sequenced using a HiSeq 2000 (Illumina) and mapped to the hg19 reference genome using bowtie 2 (11). Data were further analyzed with the peak finding algorithm MACS 1.42 (12) using input as control. Normalization to spike-in chromatin was performed according to Ref (13). All peaks with FDR greater than 0.5% were excluded from further analysis. The uniquely mapped reads were used to generate the genome-wide intensity profiles, which were visualized using the IGV genome browser (14). HOMER (15) was used to annotate peaks, to calculate overlaps between different peak files, and for motif searches. The genomic features (promot-

Orthotopic breast cancer xenografts

All animal studies and experiments were performed in accordance with German Animal Welfare regulations and in accordance with an Institutional Animal Care and Use Committee as described in the animal protocol G13/114. NOD/SCID females (4–5 weeks old) were anesthetized using an isoflurane inhalator. A small sagittal incision (no longer than 1.0 cm) on the shaved back of the animal was made. The skin was incised with a scalpel and the underlying tissue was gently separated from the skin. The subcutaneous tissue was carefully dissected from the underlying muscle, and a small incision was made in the muscle to facilitate entry into the tissue. The muscle was then gently separated from the underlying bone. The bone was then gently separated from the underlying skin. The skin was then closed with suture material.

Each transplant was localized distal to the lymph node in the gland. Surgical incisions were sealed by suturing with a 5/0 thread (Ethicon, Z995). Animals were monitored twice weekly for weight and tumor growth, which was determined by caliper measurement. Tumor volumes were calculated using the formula 4/3 × π × r^3.

Ultrasound 3D tumor model

Ultrasound measurements of xenograft tumors in NOD/SCID mice were performed using a small animal high-resolution ultrasound system (Veo3100) and transducer (MX550D) with 40 MHz (VisualSonics). For 3D tumor modeling, the transducer was moved along the tumor automatically with a step size of 0.076 mm. Tumors were visualized with Vevo LAB (Version 1.7.1) at start and end of treatment.

In vivo treatment with QC6352

Immediately before treatment, QC6352 was dissolved in 50% polyethylene glycol (Sigma-Aldrich)/50% DPBS (pH 9, Gibco) with sonication (Diagenode bioruptor) until a clear solution was formed. When tumors reached a palpable size of 3 mm^3, mice were treated with vehicle (control) or QC6352. The inhibitor was administered daily to mice via oral gavage at 10 mg/kg. Control animals received vehicle only. Animals were monitored twice weekly for weight and tumor growth.

Dose–response assay

Cells were detached by Accutase, dissociated and counted. The wells of a black 384-well plate (Greiner) were coated with 10 μL of MSC medium containing 2% of Matrigel (354230, Corning). After incubation at 37°C for 30 minutes, to solidify the Matrigel, 1 × 10^5 single cells were seeded as described above. The inhibitor was added 24 hours later. Following 96 hours of incubation, cells were washed with DPBS and fixed with ice-cold methanol for 15 minutes at −20°C. Cells were washed with DPBS, stained with DAPI, and counted using the ScanR microscope-based imaging platform (Olympus).

Microarray analysis

Total RNA was isolated from patient tumor material, xenografts, and cells using the Universal RNA Purification Kit (Robok- lon) according to the manufacturer’s instructions. Isolated RNAs were processed with the Ambion WT Expression Kit (Ambion) as described by the manufacturer and hybridized to Illumina HT-12 v4 Expression Bead Chips following the standard Illumina protocols. Expression data were processed and quantile normalized using the R Bioconductor Beadarray package (16) in version 2.22. Only probesets mapping to an Entrez ID applying the Biocon-
ductor package illuminaHumanv4.db (Version 1.26) were con-
sidered for further downstream analysis. In case of multiple probesets matching the same Entrez ID, we selected the probeset having the highest interquantile range across all samples. The dendrogram depicts a complete-linkage hierarchical clustering based on the Euclidean distance between the samples. Data are deposited under GSE95042.

Flow cytometry

To analyze the expression of established CSC markers, cells were detached and counted as described above. Cells (1 × 10^6) were washed with staining buffer (DPBS + 1% BSA) and stained for 20 minutes at room temperature in the dark with the following antibodies diluted in staining buffer: anti-CD24 (eBioscience,
46-0247; 1:100), anti-CD44 (eBioscience, 12-0441-81; 1:1000), anti-EpCAM (eBioscience, 660 50-9326; 1:100), and anti-CD49f (eBioscience, 46-0495; 1:200). Cells were analyzed using BD LSR Fortessa and Flowjo software (Version 6).

Immunohistochemistry

Patient tumor tissue specimens were fixed in 10% formalin and embedded in paraffin. Two-μm-thick paraffin-embedded tissue sections were mounted onto glass slides. All slides were stored for two days at 58°C in a drying chamber, subsequently deparaffinized using xylene and hydrated with ethanol. Human and corresponding xenograft tumor tissue were stained using the following antibodies: anti-ER (clone EP1, code IR084, Dako); anti-PR (clone Fg8 636, code IR068, Dako); anti-HER2 (code A0485, Dako); anti-Ki67 (clone MIB-1, code IR626, Dako); antivimentin (clone V9, code IR630, Dako); and anti-E-cadherin (clone NCH-38, code IR059, Dako), and anti-tyroskinase 8/18 (clone EP17/EP30, code IR094, Dako). For the horseradish-based peroxidase detection EnVision Flex Peroxidase-Blocking Reagent (Dako, K8019) or EnVision Flex+ Rabbit (LINKER, Dako, K8021) and EnVision Flex/HRP (Dako, SM802) were used. Counterstaining was performed with hemalum before adding a coverslip. As internal control for vimentin. For HER2, tissue specimens corresponding xenograft tumor tissue were stained using the following antibodies: anti-KDM4A (#5766, lot 021110, Schule Laboratory), anti-KDM4B (#4662, lot 962009, Schule Laboratory), anti-KDM4C (#23855, lot 23062015, Schule Laboratory), anti-KDM4D (#ARP35946, lot 001, Aviva Systems Biology), anti-EGFR (#2232S, lot 16, Cell Signaling Technology).

RNA preparation and analysis

Cells were cultured in the presence of vehicle or 5 × 10⁻⁸ mol/L QC6352. RNA was isolated as described above. RNA samples were sequenced by the standard illumina protocol to create raw sequence files (fastq files) at the sequencing core facility of the DKFZ. Reads were aligned to the hg19 build of the human genome using TopHat version 2 (21). The aligned reads were counted with the homer software (analyzeRNA) and DEG's were identified using edgeR (22). Data are deposited under GSE95294.

Pharmacokinetic analyses

An intravenous dosing solution was made by dissolving QC6352 into phosphate buffered saline. The pH was adjusted to 9 by dropwise addition of 1 N NaOH. An oral dosing suspension was made by dissolving QC6352 into 0.5% methylcellulose. The intravenous and oral dosing solutions were administered at 5 and 10 mg/kg, respectively, to female CD-1 mice. Pharmacokinetic parameters were calculated as the average of groups consisting of three animals.

Statistical analyses

Data are represented as mean and SD or SEM as indicated. Significance was calculated by a two-tailed Student t test or one-way ANOVA as indicated with GraphPad Prism Version 6. P values below 0.05, 0.01, and 0.001 are indicated in figures as *, **, and *** respectively.

Determination of the QC6352 IC₅₀

The ability of QC6352 to inhibit the activity of different KDM family members was determined in 384-well plate format under the following reaction conditions: 2 nmol/L enzyme, 300 nmol/L H3K9me3, H3K4me3, or H3K36me2 biotin-labeled peptides (Anaspec), 100 μmol/L alpha-ketoglutaric acid in assay buffer containing 50 mmol/L HEPES, pH 7.3, 0.005% Brij35, 0.5 mmol/L TCEP, 0.2 mg/mL BSA, 50 μmol/L sodium L-ascorbate, and 2 μmol/L ammonium iron(II) sulfate. Reaction product was determined quantitatively by TR-FRET after the addition of detection reagent phycolink streptavidin-allophycocyanin (Prozyme) and Europium-anti-H3K9me2, H4K4me2, or H3K36me1 antibody (PerkinElmer) in the presence of 5 mmol/L EDTA in LANCE detection buffer (PerkinElmer) at a final concentration of 50 mmol/L and 1 mmol/L, respectively. To initiate the assay reaction, 2 μL of 6 mmol/L enzyme and 2 μL of 11-point serially diluted QC6352 in 3% DMSO were added to each well for 60 minutes, followed by the addition of 2 μL of a mixture of 900 nmol/L biotin labeled peptide and 300 μmol/L L-α-ketoglutaric acid. After incubation at room temperature for 20 minutes, the reaction was terminated by addition of 6 μL of 5 mmol/L EDTA in LANCE detection buffer containing 100 nmol/L Phycolink streptavidin-allophycocyanin and 2 μmol/L europium-labeled antibody. After 60 minutes of incubation at room temperature, plates were read by EnVisionMultilabel Reader in TR-FRET mode (excitation at 320 nm, emission at 615 and 665 nm). A ratio was calculated (665/615) for each well and fitted to determine inhibition constant (IC₅₀).

Results

BCSC xenografts recapitulate original tumors of patients

As shown in Table 1, we established four BCSC lines (BCSC1-4), which originate from four independent breast tumor samples lacking estrogen receptor (ER), progesterone receptor...
We performed adenoviral CRISPR–Cas9-mediated KDM4A, B, C, and D knockdown in BCSC1 and BCSC2. In contrast, expression levels of KDM4B, C, and D were more heterogeneous (Fig. 2A). To see whether both BCSC1 and BCSC2 harbor a stem cell population. To further corroborate the stem cell potential of the BCSCs we performed limiting dilution orthotopic xenografts in immunocompromised NOD/SCID mice, the current gold standard assay for self-renewing CSCs. As few as $1 \times 10^3$ BCSC1 and BCSC2 were able to form tumors demonstrating that both cell lines contain tumorigenic CSCs (Fig. 1E and F; Supplementary Fig. S1E). In summary, we used three independent methods namely sphere formation, flow cytometry analysis, and xenografts to demonstrate stemness, self-renewal capacity, and tumorigenicity of BCSC1 and BCSC2.

To further address the ability of these cells to regenerate the original patient tumor in a xenograft tumor model we analyzed tumors derived from BCSC1 and BCSC2 transplants histologically and genetically. Immunohistochemical analyses of BCSC1 and BCSC2 xenograft tumors using the mammary epithelial marker cytokeratin 8/18, E-cadherin, and vimentin as well as the proliferation marker Ki67 indicated that the xenograft tumors share a similar pattern with their parental patient tumors (Fig. 1G and Supplementary Fig. S1F). Matching the parental tumor, the BCSC xenografts were devoid of ER, PR, and HER2 protein expression (Table 1, Fig. 1H, and Supplementary Fig. S1G). Unsupervised hierarchical clustering analysis of RNA microarray data showed that the BCSC tumor xenografts share a close expression profile with the parental patient tumors, indicating preservation of the respective molecular tumor subtype (Fig. 1I). BCSC lines clustered within the corresponding host tumor and xenograft subtype depicting a close correlation between the three entities (Fig. 1I). Taken together, our data demonstrate that BCSCs can be isolated from TNBC patient tissue using optimized culture conditions and faithfully recapitulate the patient tumor in xenografts.

**KDM4A controls proliferation and xenograft tumor growth of BCSC1**

to evaluate the expression levels of the KDM4 family members in BCSC1 and BCSC2 we performed Western blot analysis. As shown in Fig. 2A, we detected robust expression of KDM4A in both BCSC1 and BCSC2. In contrast, expression levels of KDM4B, C, and D were more heterogeneous (Fig. 2A). To see whether KDM4s control proliferation of BCSC1 we performed adenoviral shRNA-mediated knockdown of KDM4A, B, C, and D in BCSC1 and monitored proliferation in real time. As shown in Fig. 2B,
depletion of KDM4A impaired proliferation of BCSC1 cells. In contrast, knockdown of KDM4B, C, or D did not influence proliferation of BCSC1 (Supplemental Fig. S2A–S2E).

Next, we wondered whether knockdown of KDM4A affects growth of BCSC1 xenografts. Therefore, BCSC1 were infected with adenovirus encoding either shRNA control (shRNA Ctrl) or...
shRNA against KDM4A (shRNA KDM4A) and implanted into the fat pads of immunocompromised NOD/SCID mice. Importantly, upon knockdown of KDM4A, tumor growth, and final tumor weight of the BCSC1-derived xenografts was strongly reduced (Fig. 2C–E). In contrast, the control knockdown of KDM4D (shRNA KDM4D) in BCSC1 did not significantly affect tumor growth and final tumor weight of the xenografts (Supplementary Fig. S2F–S2H). Together, our data demonstrate that the histone demethylase KDM4A controls proliferation and xenograft tumor growth of BCSC1. Furthermore, our bioinformatic analyses indicate that relapse-free survival over time decreases for patients with TNBC that express high levels of KDM4A in comparison to patients with TNBC that express low levels of KDM4A (Supplementary Fig. S2I). These findings indicate that targeting KDM4 might be a therapeutic option to limit expansion of BCSC populations.

QC6352 is a potent KDM4 inhibitor that blocks proliferation of BCSCs

Based on our observations that KDM4A controls proliferation and xenograft tumor growth of BCSC1 we decided to test whether the drug-like KDM4 inhibitor QC6352 (also called compound 6; ref. 26) might qualify for the treatment of BCSC-originating tumors (Fig. 3A). As a first step, prior to any investigation of either biological function or underpinning mechanism of action, we assessed the selectivity profile of the inhibitor. QC6352 was
evaluated against the KDM4 subfamily and other JmjC domain-containing histone demethylases such as KDM2A, 2B, 5B, 6A, and 6B. QC6352 inhibited the demethylase activity of KDM4A, B, C, and D at nanomolar concentrations with IC_{50} values between 35 and 104 nmol/L (Fig. 3B). QC6352 showed 100-to 300-fold selectivity over KDM2 and KDM6 demethylases and only weak inhibition of KDM5B (Fig. 3B). Finally, QC6352's favorable pharmacokinetic properties warranted further in vivo investigation (Fig. 3C). In summary, QC6352 presented as an orally available, potent, and selective KDM4 inhibitor.

When investigating the in vitro efficacy of QC6352 in BCSCs we observed that concentrations as low as 10 nmol/L inhibited...
BCSC1 and BCSC2 cell proliferation (Fig. 3D and E; Supplementary Fig. S3A and S3B). To characterize and define the effect of QC6352 on stem cell potential (self-renewal and differentiation) we tested whether QC6352 treatment might interfere with the sphere-forming capacity of both BCSCs. Consequently, BCSCs were plated as single cells to ensure clonality and inspected for one week. In a concentration-dependent manner, QC6352 dramatically reduced the anchorage-independent sphere-forming capacity of BCSC1 and BCSC2 (Fig. 3F and Supplementary Fig. S3C). We then isolated single cells from 1 week QC6352-treated spheres and evaluated for anchorage-independent sphere formation in a secondary assay in the absence of QC6352. Of note, the secondary sphere-forming capacity was blocked even in the absence of inhibitor (Fig. 3G and Supplementary Fig. S3D). In contrast, the differentiation-inducing LSD1 inhibitor QC6688 (27) neither affected primary nor secondary sphere formation (Supplementary Fig. S3E and S3F). In addition, chemotherapeutics such as the taxane paclitaxel impaired proliferation of BCSC1 in primary sphere formation but did not interfere with stem cell potential and thus allowed secondary sphere formation (Supplementary Fig. S3G). Taken together, our data demonstrate that QC6352 blocks proliferation and self-renewal of BCSCs.

Figure 4. QC6352 targets BCSCs through EGFR regulation. A, Pie chart displaying the number of genes that are differentially regulated in BCSC1 upon treatment with QC6352. B, Pie chart displaying genomic distribution of KDM4A in BCSC1 as determined by ChIP-seq analysis. C, Venn diagram showing the intersection and number of genes where KDM4A is present on the promoter region with genes that are differentially regulated in BCSC1 upon treatment with QC6352. A hypergeometric test was performed to calculate the significance of the overlap (P < 10−5). D, mRNA level analysis. Heatmap representing the mRNA levels detected in BCSC1 cultured in the presence of vehicle (−) or QC6352. The 30 represented genes are direct target genes of KDM4A that belong to the “EGF receptor signaling pathway.” Verification of transcriptome data by qRT-PCR analysis showing relative mRNA levels of indicated genes in BCSC1 cultured in the presence of vehicle (−) or QC6352 (n = 4). F and G, Anti-EGFR, anti-KDM4A, and anti-tubulin Western blots. Samples are lysates from BCSC1 cultured in the presence of vehicle (−) or QC6352 (F) or treated with control shRNA (shRNA Ctrl) or an shRNA against KDM4A (shRNA KDM4A; G). Data represent means ± SD (E); *, P < 0.01; **, P < 0.001 by two-tailed Student’s t test.
QC6352 targets BCSCs through *EGFR* regulation

To unravel the molecular mechanism underlying the action of the inhibitor, we performed transcriptome analyses to identify genes that were differentially regulated upon QC6352 treatment. BCSC1 were cultivated in the presence or absence of QC6352 and subjected to RNA-seq. Our analysis identified a total of 580 differentially regulated genes (Fig. 4A). Among them, 254 were upregulated and 326 were downregulated (Fig. 4A). To see whether these genes are direct KDM4A targets, we performed ChIP-seq in BCSC1 cells with anti-KDM4A antibody. The analysis shown in Fig. 4B identified 46,822 high confidence KDM4A peaks. Motif analysis using HOMER indicates enrichment of FOXA3, FOXL2, FOXA1, FOXP1, or FOXF1 motifs at KDM4A locations (Supplementary Fig. S4A). Only 3,221 (6.9%) KDM4A locations were observed in BCSC1 treated with shRNA against KDM4A, thus confirming specificity of the KDM4A antibody (Supplementary Fig. S4B). This finding prompted us to intersect the KDM4A cistrome with the QC6352 transcriptome. Among the 580 differentially regulated genes, KDM4A was present at the promoter of 258 genes (44%; Fig. 4C). Pathway analysis for these 258 genes revealed that 30 of these genes, including *EGFR*, belong to the "EGF receptor signaling pathway" (Fig. 4D). We verified by qRT-PCR analysis that treatment with QC6352 reduced the expression levels of genes such as *versican* (*VCAN*), *proline rich 5* (*PRR5*), *activating transcription factor 4* (*ATF4*), *early growth response 1* (*EGR1*), *follistatin* (*FST*), and importantly *EGFR* (Fig. 4E). EGFR is an emerging therapeutic target that is associated with poor clinical outcome of TNBC (28). To unravel the importance of *EGFR* signaling in growth of BCSCs we treated BCSC1 and BCSC2 with erlotinib, a specific *EGFR* inhibitor. Of note, treatment with erlotinib blocked proliferation of both BCSC1 and BCSC2 (Supplementary Fig. S4C–S4F). Furthermore, stem cell potential measured by anchorage-independent sphere-forming capacity of both BCSC1 and BCSC2 was dramatically reduced upon treatment with erlotinib (Supplementary Fig. S4G and S4H). Together, these data demonstrate that *EGFR*, at least in part, controls growth and stem cell potential of BCSCs. As shown by Western blot analysis, the protein levels of *EGFR* were reduced in both BCSC1 and BCSC2 upon treatment with QC6352 (Fig. 4F and Supplementary Fig. S4I). Because *EGFR* is a direct KDM4A target, we tested whether knockdown of KDM4A affects *EGFR* protein levels. As depicted in Fig. 4G and Supplementary Fig. S4J, shRNA-mediated knockdown of KDM4A led to reduced levels of *EGFR* in both BCSC1 and BCSC2. Of note, knockdown of KDM4B, C, or D in BCSC1 did not affect the levels of *EGFR* (Supplementary Fig. S4K–S4M). Taken together, the data show that *EGFR* expression in BCSCs is blocked by QC6352 via inhibition of KDM4A.

Because KDM4A is a demethylase that erases the repressive H3K9me3 mark, we hypothesized that an increase in H3K9me3 should be observed upon inactivation of KDM4A by QC6352. To this end, we performed genome-wide ChIP-seq with H3K9me3 antibody in BCSC1 cells that were treated with either vehicle or QC6352. To allow for normalization of H3K9me3 ChIP-seq tags, we added spike-in chromatin during the ChIP procedure (13). The analysis shown in Supplementary Fig. S5A identified 141,722 high confidence H3K9me3 peaks in vehicle-treated cells and

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**Figure 5.**

Levels of H3K9me3 increase upon treatment with QC6352. **A,** Meta-analysis of sequencing read density based on H3K9me3 ChIP-seq around KDM4A peaks in BCSC1 cultured in the presence of vehicle or QC6352. **B,** ChIP-seq tracks. Normalized levels of H3K9me3 and KDM4A tracks at the *EGFR* promoter. **C,** ChIP analyses performed with anti-KDM4A, anti-H3, anti-H3K9me3, and rigG. Samples originate from BCSC1 infected with an adenovirus coding either for shRNA Ctrl or shRNA KDM4A. The precipitated chromatin was quantified by qPCR using primers in the promoter region of the *EGFR* gene. Data represent means ± SD (C); *, *P* < 0.001 by two-tailed Student t test.
144,266 peaks in cells treated with QC6352. Importantly, we observed a global increase of the H3K9me3 reads over the KDM4A peaks (Fig. 5A). Similarly, on the EGFR promoter we also observed increased levels of the repressive H3K9me3 mark over the KDM4A peak subsequent to inactivation by QC6352 (Fig. 5B). To demonstrate that the increase of H3K9me3 levels at the EGFR promoter upon treatment with QC6352 is due to inhibition of KDM4A, we infected BCSC1 cells with adenovirus encoding either shRNA control (shRNA Ctrl) or shRNA against KDM4A (shRNA KDM4A) and performed ChIP assays. ChIP-qPCR analyses indicate that in BCSC1 infected with shRNA KDM4A the levels of H3K9me3 at the EGFR promoter increase concomitantly to a decrease in KDM4A when compared to control cells (Fig. 5C). In summary, these results demonstrate that treatment of BCSCs with QC6352 targets EGFR via inhibition of the KDM4A demethylase activity.

**QC6352 inhibits BCSC-derived xenograft tumor growth**

Next, we wondered whether QC6352 might affect growth of BCSC1 and BCSC2 xenografts. Therefore, $1 \times 10^5$ cells were implanted into the fat pads of immunocompromised NOD/SCID mice. To mimic the clinical treatment situation of a preexisting tumor we allowed growth of the xenografts to a size of 3 mm$^3$ before starting treatment. Mice carrying tumors were then treated with QC6352 for 21 days at 10 mg/kg per os. Importantly, QC6352 strongly affected tumor growth and final tumor weight of both BCSC1 and BCSC2 xenografts (Fig. 6A–E and Supplementary Fig. S6A–S6E). Treatment with QC6352 was well tolerated and did not affect body weight of the mice (Supplementary Fig. S6F and S6G). Furthermore, we analyzed whether treatment with QC6352 affected expression of KDM4A target genes in the BCSC1 xenograft tumors in a similar manner as observed in cell culture (Fig. 4E). As displayed in Fig. 6F, expression of VCAN, PRR5, ATF4, EGR1, FST, and EGFR was similarly affected in BCSC1 xenograft tumors of QC6352-treated mice as in vitro. Taken together, our data demonstrate that treatment with the KDM4 inhibitor QC6352 blocks BCSC xenograft tumor growth.

**Discussion**

In recent years, CSCs from solid tumors were identified using surface marker profiles and subsequent limiting dilution orthotopic xenografts. The first description of BCSCs by Al-Hajj and colleagues identified that the BCSC population is marked by
CD24 low/CD44 high cells (24). In addition, several other surface molecules such as CD61, CD49f, and EpCAM identifying BCSCs were proposed (29, 30). Importantly, due to a lack of appropriate culture conditions BCSCs cannot be cultivated continuously in vitro in a stem cell state (31, 32). To address this issue and to stabilize the CSC phenotype, we used a 3D Matrix and applied a Rho Kinase inhibitor in a low oxygen environment. Thus, our culture conditions allowed cultivation of BCSCs directly from the patient tumor without the need for initial separation of tumor tissue and CSCs by FACS or the expansion of the patient’s tumor tissue in murine xenografts. Limiting dilution transplants of our cultivated BCSCs regenerated the original patient’s tumor. The xenograft tumors had an almost identical immunohistochemical staining of the tissue in murine xenografts. Limiting dilution transplants of our BCSCs regenerated the original patient’s tumor. The xenograft tumors had an almost identical immunohistochemical pattern compared with the tumor of origin, which is comparable to results obtained from direct patient xenografts (24, 33). In addition, transcriptome analyses uncovered a strong clustering of BCSCs with alterations of KDM4 demethylase family members (8, 34) and BCSC xenografts, and are therefore an ideal cellular platform to test novel therapeutics.

Therefore, our models are ideal tools for the identification and validation of novel therapeutics. In line with this idea, we identified the histone demethylase KDM4A as a therapeutic target for BCSC-originating tumors. Consequently, we showed that the orally available, potent, and selective KDM4 inhibitor QC6352 abrogates expression of target genes via inhibition of the KDM4A demethylase activity, thereby blocking proliferation, sphere-forming capacity in vitro, and xenograft tumor growth of BCSCs in vivo. Thus, modulation of KDM4 activity is a promising therapeutic strategy for the treatment of TNBC.

Disclosure of Potential Conflicts of Interest
R. Schule is a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

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KDM4 Inhibition Targets Breast Cancer Stem–like Cells

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