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 BCSC 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.
Introduction

Breast cancer is the leading cause of cancer 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 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 catalyze removal of the repressive H3K9me3 mark and that of H3K36me3, a mark linked to transcriptional elongation (8) thereby regulating a range of crucial pathways. These findings highlight KDM4 demethylases as potential therapeutic targets for breast cancer treatment.

Consequently, we set out to test potent and selective drug-like KDM4 inhibitors. In order to validate inhibitors on cancer stem cells (CSCs) from triple-negative breast cancer, we established an efficient 3 dimensional (3D) cultivation method allowing for growth of CSCs from patient tumor tissue without prior fluorescence-activated cell sorting (FACS) or murine xenografts to enrich for CSCs. We used defined conditions including a serum-free culture medium, a rho kinase inhibitor, Matrigel,
and a low oxygen environment to isolate and enrich for BCSCs from individual patient tumors after neoadjuvant chemotherapy.

Materials and Methods

Tissue specimens

All patients were operated at the Department of Obstetrics and Gynecology at the University Medical Centre Freiburg. Tumor 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 (IRB) 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 breast cancer stem cell (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 h at 37 °C. Afterwards, 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 min, 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 min incubation at room temperature, the suspension was filled up to 6 ml with MEBM and centrifuged at 200 g for 5 min. 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 min, the supernatant was discarded and the remaining cell pellet suspended in MSC medium. Isolated cells were counted and 2 x 10^4 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 min, each well was topped up with 500 µl of MSC medium. The cells were cultured at 37 °C under low oxygen conditions (3 % O_2, 5 % CO_2, 92 % N_2). 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 Authentification 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 x B27 (Gibco, 17504-044), 1 x amphotericin B (Sigma-Aldrich, A2942), and 1 x penicillin-streptomycin (Gibco, 15140-122). 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 rho kinase inhibitor (500 nM, Calbiochem, 555552) were added.

**Cell culture**
BCSCs were cultured as spheres in a 3D environment as described above. One ml medium was added after 2 days. Cells were split once a week using Dispase (Corning) to solve residual Matrigel and Accutase for sphere dissociation. To expand BCSCs in a 2D environment, 4 x 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 min, 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. 3 x 10^3 single BCSC1 and 1 x 10^3 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, all 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. 1 x 10^3 single BCSC1 and BCSC2 cells 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 x
10^4 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 x 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-NLS-mCherry) was obtained from the Sanford Burnham Prebys Medical Discovery Institute. Lentiviral particles were added at an MOI of 5 to BCSC1 and BCSC2 in MSC medium. Cells were cultured as described. For cell proliferation assay, BCSC1mCherry and BCSC2mCherry 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 min to solidify the Matrigel, 1 x 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 10x 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 KDM4D (Ad-GFP-U6-h-KDM4D-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 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 h, BCSC1 were detached, reinfected with an MOI of 300 and 1 x 10^5 cells transplanted into immunocompromised mice as described below.

**Chromatin immunoprecipitation (ChIP)**

ChIP experiments were performed essentially as previously described (10). BCSC1 cells were either cultured for 18 h in the absence or presence of 5 x 10^8 M QC6352 or cells were infected five days before harvesting with adenovirus 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, Schuele 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 presence of spike-in chromatin (#53083, Active Motif) on GammaBind™G-Sepharose™ (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’-tctgtctgacacttgca-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 (promoter, exon, intron, 3’UTR, and intergenic regions) were defined and calculated using Refseq and HOMER. Data are deposited under GSE95294.

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 (IACUC) 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 and sterilized abdomen allowed access to the mammary gland #4 on both sides. Indicated numbers of BCSCs were mixed with 1 x 10^6 irradiated fibroblasts (newborn human foreskin fibroblasts (NuFF), p11, GlobalStem, GSC-3002) and suspended in a 1:1 mixture of Matrigel (Corning, 354230) and MSC medium in a total volume of 40 µl per gland. The mixture was injected into the mammary fat pad of the #4 gland on both sides of the animal. 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 x π x r³.

**Ultrasonic 3D tumor model**

Ultrasound measurements of xenograft tumors in NOD/SCID mice were performed using a small animal high resolution ultrasound system (Vevo3100) 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 mm³, 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 min to solidify the Matrigel, 1 x 10³ single cells were seeded as described above. The inhibitor was added 24 later. Following 96 h of incubation cells were washed with DPBS and fixed
with ice-cold methanol for 15 min 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 (Roboklon) 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 Bioconductor package illuminaHumanv4.db (Version 1.26) were considered for further downstream analysis. In case of multiple probesets matching the same Entrez ID, we selected the probeset having the highest interquartile 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. 1 x 10^5 cells were washed with staining buffer (DPBS + 1 % BSA) and stained for 20 min 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 PgR 636, code IR068, Dako); anti-HER2 (code A0485, Dako); anti-Ki67 (clone MIB-1, code IR626, Dako); anti-vimentin (clone V9, code IR630, Dako); anti-E-cadherin (clone NCH-38, code IR059, Dako), and anti-cytokeratin 8/18 (clone EP17/EP30, code IR094, Dako). For the horseradish-based peroxidase detection EnVision® Flex Peroxidase-Blocking Reagent (Dako, SM801), EnVision® Flex+ Rabbit (LINKER) (Dako, K8019) or EnVision® Flex+ Mouse (LINKER) (Dako, K8021) and EnVision® Flex/HRP (Dako, SM802) were used. Counterstaining was performed with hemalum before adding a coverslip. As internal positive control, patient-derived physiological mammary gland was used for ER, PR, Ki67 (nuclear staining), cytokeratin 8/18 and E-cadherin (membranous - cytoplasmic staining). The mammary gland-surrounding myoepithelial layer was used as internal control for vimentin. For HER2, tissue specimens from HER2 positive breast cancer patients (Score 3 according to Ref(17)) were included for every HER2 staining session as external positive control. Triple-negative breast cancer was defined as ER, PR and HER2 negative (score < 2) (18).
Western blot analysis

Experiments were performed as previously described (10). The following antibodies were used: anti-KDM4A (#5766, lot 021110, Schuele Laboratory), anti-KDM4B (#4662, lot 962009, Schuele Laboratory), anti-KDM4C (#23855, lot 23062015, Schuele 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 $5 \times 10^{-8} \text{ M QC6352}$. RNA was isolated as previously described (19). Quantitative RT-PCR was performed using the Abgene SYBR Green PCR kit (Invitrogen) according to the supplier’s protocol. $HPRT$ was used for normalization. Primer sequences for $HPRT$ were described previously (20). Other primers were as follows: $VCAN$: 5'-ACTGTGGATGGGTTGTTGTT-3’, 5'-CTGCGTCACTGC TCAAAT-3’; $PRR5$: 5’-CGGGACAAAGATCCTCTAC-3’, 5’-AGCGCATCC TCTAGCTTCAC-3’; $ATF4$: 5’-CCAACACAGCAAGGAGGAT-3’, 5’-GTGTC ATCCACGTGGTCAG-3’; $EGRI$: 5’-TGACCGCAGAGTCTTTTCTCTCT-3’, 5’-CACAAGGTGTTGCCACTTG-3’; $FST$: 5’-GGAAAACCTACCGCAATGAA-3’, 5’-GAGCTTGCTGGACAGAAAAC-3’; $EGFR$: 5’-CCAAACAGCTCTCTTGAGA-3’, 5’-GCTTTGGAGATGTTGCTTC-3’

RNA sequencing (RNA-seq)

BCSC1 cells were cultured for 18 h in the absence or presence of $5 \times 10^{-8} \text{ M QC6352}$. 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 mg/kg and 10 mg/kg, respectively, to female CD-1 mice. Pharmacokinetic parameters were calculated as the average of groups consisting of three animal.

Statistical analyses

Data are represented as mean and standard deviation (SD) or standard error of the mean (SEM) as indicated. Significance was calculated by a two-tailed Student’s 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 IC$_{50}$

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 nM enzyme, 300 nM H3K9me3, H3K4me3, or H3K36me2 biotin-labeled peptides (Anaspec), 100 µM alpha-ketoglutaric acid in assay buffer containing 50 mM HEPES, pH 7.3, 0.005 % Brij35, 0.5 mM TCEP, 0.2 mg/ml BSA, 50 µM sodium L-
ascorbate, and 2 µM ammonium iron(II) sulfate. Reaction product was determined quantitatively by TR-FRET after the addition of detection reagent Phycollink Streptavidin-allophycocyanin (Prozyme) and Europium-anti-H3K9me2, H4K4me2, or H3K36me1 antibody (PerkinElmer) in the presence of 5 mM EDTA in LANCE detection buffer (PerkinElmer) at a final concentration of 50 nM and 1 nM, respectively. To initiate the assay reaction, 2 µl of 6 nM enzyme and 2 µl of 11-point serially diluted QC6352 in 3 % DMSO were added to each well for 60 min, followed by the addition of 2 µl of a mixture of 900 nM biotin labeled peptide and 300 µM alpha-ketoglutaric acid. After incubation at room temperature for 20 min, the reaction was terminated by addition of 6 µl of 5 mM EDTA in LANCE detection buffer containing 100 nM Phycollink Streptavidin-allophycocyanin and 2 nM Europium-labeled antibody. After after 60 min incubation at room temperature, plates were read by EnVisionMultilabel Reader in TR-FRET mode (excitation at 320 nm, emission at 615 nm and 665 nm). A ratio was calculated (665/615) for each well and fitted to determine inhibition constant (IC₅₀).

Results

Breast cancer stem cell xenografts recapitulate original tumors of patients

As shown in Table 1, we established four breast cancer stem cell lines (BCSC1-4), which originate from four independent breast tumor samples lacking estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) proteins. BCSCs could be cultivated in a 2D and 3D environment and grew as epithelial clusters and spheres, respectively (Figure 1A and Supplementary Fig. S1A). In vitro clonogenic assays such as sphere formation assays
have been developed to study proliferation, self-renewal, and differentiation of cell populations at the single-cell level (23). Thus, to verify their stem cell potential BCSC1 and BCSC2 were challenged in an anchorage-independent growth assay. BCSC1 and BCSC2 demonstrated a sphere-forming capacity of 10.8% and 16.2%, respectively, providing evidence for the potential of BCSC1 and BCSC2 to self-renew (Figure 1B and Supplementary Fig. S1B). CSCs from breast cancer have been described to express specific surface markers such as CD49f, EpCAM, and CD44 while lacking expression of CD24 (24,25). We analyzed BCSC1 and BCSC2 for these stem cell markers and found 58% CD24−/CD44+ and 96% EpCAM+/CD49f− cells in BCSC1 and 5% CD24−/CD44+ and 97% EpCAM+/CD49f+ cells in BCSC2 (Figure 1C, 1D, Supplementary Fig. S1C, and S1D). The flow cytometric analysis clearly showed that 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 1x10^3 BCSC1 and BCSC2 were able to form tumors demonstrating that both cell lines contain tumorigenic CSCs (Figure 1E, 1F, and 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 (Figure 1G and Supplementary Fig. S1F). Matching the parental tumor, the BCSC xenografts were devoid of ER, PR and HER2 protein expression (Table 1, Figure 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 (Figure 1I). BCSC lines clustered within the corresponding host tumor and xenograft subtype depicting a close correlation between the three entities (Figure 1I). Taken together, our data demonstrate that BCSCs can be isolated from triple-negative breast cancer 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 Figure 2A, we detected robust expression of KDM4A in both BCSC1 and BCSC2. In contrast, expression levels of KDM4B, C and D were more heterogeneous (Figure 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 Figure 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 (Figure 2C-2E). 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 (RFS) 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)(26) might qualify for the treatment of BCSC-originating tumors (Figure 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 to 104 nM (Figure 3B). QC6352 showed 100-to 300-fold selectivity over KDM2 and KDM6 demethylases and only weak inhibition of KDM5B (Figure 3B). Finally, QC6352’s favorable pharmacokinetic properties warranted further *in vivo* investigation (Figure 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 nM inhibited BCSC1 and BCSC2 cell proliferation (Figure 3D, 3E, and 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 (Figure 3F and Supplementary Fig. S3C). We then isolated single cells from one 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 (Figure 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.

**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 (Figure 4A). Among them, 254 were upregulated and 326 were downregulated (Figure 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 Figure 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 3221 (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%) (Figure 4C). Pathway analysis for these 258 genes revealed that 30 of these genes, including EGFR, belong to the ‘EGF receptor signaling pathway’ (Figure 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 (Figure 4E). EGFR is an emerging therapeutic target that is associated with poor clinical outcome of triple-negative breast cancer (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 (Figure 4F and Supplementary Fig. S4I). Since EGFR is a direct KDM4A target, we tested whether knockdown of KDM4A affects EGFR protein levels. As depicted in Figure 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.

Since 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 Figure S5A identified 141,722 high confidence H3K9me3 peaks in vehicle-treated cells and 144,266 peaks in cells treated with QC6352. Importantly, we observed a global increase of the H3K9me3 reads over the KDM4A peaks (Figure 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 (Figure 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 (Figure 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 x 10^5 cells were implanted into the fat pads of immunocompromised NOD/SCID mice. To mimic the clinical treatment situation of a pre-existing 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 (Figure 6A-6E 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 (Figure 4E). As displayed in Figure 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 cancer stem cells (CSCs) from solid tumors were identified using surface marker profiles and subsequent limiting dilution orthotopic xenografts. The first description of BCSC 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 BCSC regenerated the original patient’s tumor. The xenograft tumors had an almost identical immunohistochemical pattern compared to 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 primary tumors, xenografts and cell lines. Thus, BCSCs faithfully reproduce the original patient’s tumor and are therefore an ideal cellular platform to test novel therapeutics.

Since breast cancer progression was shown to be associated with alterations of KDM4 demethylase family members (8,34-36) we set out to characterize the expression profiles of the four KDM4 family members, namely KDM4A, B, C, and D in BCSC1 and BCSC2. BCSC1 and BCSC2 are characterized by a robust expression of KDM4A. In contrast, expression levels of KDM4B, C, and D were more heterogeneous. While depletion of KDM4A impaired proliferation of BCSC1 and blocked growth of BCSC1 tumor xenografts knockdown of the other KDM4 family member neither affected cell proliferation nor xenograft tumor growth. These findings are in accordance with previous studies suggesting that KDM4A controls proliferation of breast cancer cells (37,38). Furthermore, by showing for the first time that...
KDM4A controls proliferation and self-renewal of BCSCs isolated from chemotherapy-resistant triple-negative breast tumors we validate KDM4A as a therapeutic target.

In recent years diverse KDM4 inhibitors have been identified, which act either as α-ketoglutarate mimics, a cofactor essential for the enzymatic function of KDM4s, or as inhibitors of the catalytic site (8). These molecules showed inhibitory effects on KDM4s in vitro and in cell culture models (8). However, these KDM4 inhibitors did not qualify as therapeutic agents. In contrast, QC6352 is a drug-like KDM4 inhibitor that is potent, selective, orally available, and presents favorable pharmacokinetic properties.

In summary, we established an advanced culture method that allows isolation and growth of BCSC lines isolated from individual patient tumors after neoadjuvant chemotherapy. We demonstrate that BCSC xenografts faithfully recapitulate parental patient tumors and that BCSCs, BCSC xenografts and the parental tumors share a highly similar transcriptome and phenotype profile. 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 triple-negative breast cancer.

Acknowledgments
We thank Hauke Busch for expert advice and help with bioinformatics analyses. Furthermore, we thank Robert Oshima and Pedro Aza-Blanc for providing reagents and the ZTZ Core Facility for expert advice and technical support. We are obliged to Astrid Rieder, Jessica Pfannstiel, Lisa Füner and Bettina Herde for providing excellent technical assistance.
References


Table 1

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ID = Identifier; BCSC1-4 = breast cancer stem cell lines 1-4; IC = invasive carcinoma; IDC = invasive ductal carcinoma; MC = metaplastic carcinoma; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; FEC = 5FU/epirubicin/cyclophosphamide; FAC = 5FU/doxorubicin/cyclophosphamide; TAC = docetaxel/doxorubicin/cyclophosphamide; TC = docetaxel/cyclophosphamide; EC = epirubicin/cyclophosphamide; AC = doxorubicin/cyclophosphamide; GemCa = gemcitabine/carboplatin.
Tables

Table 1. Table representing data of original patient tumor, patient-derived cell lines BCSC1-4, and BCSC1-4-derived xenografts. ID, Identifier; BCSC1-4, breast cancer stem cell lines 1-4; IC, invasive carcinoma; IDC, invasive ductal carcinoma; MC, metaplastic carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; FEC, 5FU/epirubicin/cyclophosphamide; FAC, 5FU/doxorubicin/cyclophosphamide; TAC, docetaxel/doxorubicin/cyclophosphamide; TC, docetaxel/cyclophosphamide; EC, epirubicin/cyclophosphamide; AC, doxorubicin/cyclophosphamide; GemCa, gemcitabine/carboplatin.
Figure Legends

**Figure 1.** Breast cancer stem cell xenografts recapitulate original tumors of patients.  
A, Representative pictures of BCSC1 cells cultured in 3D and 2D conditions. Scale bar, 100 µm. B, Sphere-forming capacity of BCSC1 cells in an anchorage-independent assay (n=3). Data represent means ± s.e.m. C, D, Expression pattern of CD24, CD44 (C), EpCAM and CD49f (D) in BCSC1 cells analyzed by flow cytometry. E, Limiting dilution xenografts of BCSC1-4. F, Representative growth curves for limiting dilution assay of BCSC1 cell xenografts in immunocompromised mice. G, H, Hematoxylin and eosin (H&E) staining (G) and immunohistochemical detection of CK8/18, Ki67, E-cadherin, and vimentin (G) and ER, PR and HER2 (H) on representative sections of the original BCSC1 patient tumor and the BCSC1 xenograft tumor. Scale bar, 100 µm. I, Unsupervised hierarchical cluster analysis of RNA microarray data. Samples are original patient tumors, the tumor derived BCSC1-4 lines, and the BCSC1-4 xenograft tumors derived from the BCSC lines 1-4.

**Figure 2.** KDM4A controls proliferation and xenograft tumor growth of BCSC1. A, Western blot analyses performed with anti-KDM4A, anti-KDM4B, anti-KDM4C, anti-KDM4D, and anti-Tubulin antibodies. The samples are lysates from HEK293T, BCSC1, or BCSC2. HEK293T were transfected with expression plasmids for KDM4A, KDM4B, KDM4C, or KDM4D as indicated. B, Cell proliferation assay. BCSC1 were infected with adenoviruses coding for a control shRNA (shRNA Control) or an shRNA against KDM4A (shRNA KDM4A) (n=3). Data represent means ± s.d.; ** p<0.01, *** p<0.001 by one-way ANOVA. C-E, BCSC1 derived xenograft tumors grown for 43 days in mice. BCSC1 were infected with adenoviruses...
encoding either shRNA Ctrl or shRNA KDM4A. C, Representative BCSC1 xenograft tumors isolated from individual animals. D, Increase in tumor volume over time. Data represent means ± s.e.m. *** p<0.001 by one-way ANOVA. E, Final tumor weights of the BCSC1 xenografts. Data represent means ± s.e.m.; ** p<0.01 by one-way ANOVA. For C-E: n=9 (shRNA Ctrl), n=15 (shRNA KDM4A).

Figure 3. QC6352 is a potent KDM4 inhibitor that blocks KDM4A-dependent proliferation of BCSC1. A, Structure of QC6352. B, Table depicting the half-maximal inhibitory concentration (IC\textsubscript{50}) of QC6352 on different KDM family members. C, Pharmacokinetic properties of QC6352. iv, intravenous; AUC, Area Under the concentration-time Curve; po, oral route; Vz, Volume of distribution during terminal phase after intravenous administration; F, absolute bioavailability. D, Cell proliferation assay. BCSC1 were cultured in presence of vehicle or the indicated concentration of QC6352 (n=3). E, Dose-response curve of QC6352 (n=3). F, BCSC1 sphere-formation in an anchorage-independent growth assay in presence of vehicle or the indicated concentrations of QC6352 (n=3). G, Primary and secondary sphere-formation of BCSC1 in Matrigel in presence of vehicle or the indicated concentrations of QC6352 (n=3). Data represent means ± s.d. (D, E), or means ± s.e.m; ** p<0.01, *** p<0.001 by one-way ANOVA (F, G).

Figure 4. QC6352 targets BCSCs through \textit{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^{-50}). **D**, mRNA level analysis. Heatmap representing the mRNA levels detected in BCSC1 cultured in presence of vehicle (-) or QC6352. The 30 represented genes are direct target genes of KDM4A that belong to the ‘EGF receptor signaling pathway’. **E**, Verification of transcriptome data. QRT-PCR analysis showing relative mRNA levels of indicated genes in BCSC1 cultured in the presence of vehicle (-) or QC6352 (n=4). **F, 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 ± s.d. (E); ** p<0.01, *** p<0.001 by two-tailed Student’s test.

**Figure 5.** Levels of H3K9me3 increase upon treatment with QC6352. **A**, Meta-analysis of sequencing read density-based on H3K9me3 ChIP-seqs 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 ± s.d. (C); *** p<0.001 by two-tailed Student’s test.

**Figure 6.** QC6352 inhibits BCSC1-derived xenograft tumor growth. **A-E**, Mice bearing BCSC1 xenograft tumors were treated for 21 consecutive days with either vehicle or QC6352. **A**, Representative BCSC1 xenograft tumors isolated from
individual animals after 21 days of treatment with either vehicle or QC6352. B, Increase in tumor volume was measured over time. C, Tumor weights after 21 days of treatment with vehicle or QC6352. D, E, Representative images of tumors (D) and volume quantification of all tumors (E) obtained by ultrasound imagery at the start (Day 0) and after 21 days of treatment (Day 21) with either vehicle or QC6352. For A-E: n=11 (vehicle), n=12 (QC6352). F, QRT-PCR analysis showing relative mRNA levels of indicated genes in BCSC1 xenograft tumors of mice treated with either vehicle or QC6352 (n=4). Data represent means ± s.e.m. (B), means + s.e.m. (C, E), or means + s.d. (F); * p<0.05, ** p<0.01, *** p<0.001 by one-way ANOVA (B, C, E) or two-tailed Student’s test (F).
Figure 1

A. Cellular phenotype / BCSC1 cells

B. 3D sphere-forming capacity / BCSC1 cells

C. CSC marker profile / BCSC1 cells

D. CSC marker profile / BCSC1 cells

E. Limiting dilution xenograft / tumor formation

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F. Limiting dilution xenograft / BCSC1 tumor growth curves

G. Immunohistochemical analysis / BCSC1

H. Immunohistochemical analysis / BCSC1

I. Cluster analysis

Cluster dendrogram

Patient tumor

Xenograft tumor

anti-ER  anti-PR  anti-HER2

Positive control ER/PR/HER2 breast tumor

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**A** QC6352 Structure

**B**

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**C** Pharmacokinetic data on QC6352

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**D** Proliferation assay / BCSC1 cells

**E** QC6352 dose-response curve / BCSC1 cells

**F** 3D sphere-forming capacity / BCSC1 cells

**G** Secondary sphere-forming capacity / BCSC1 cells
KDM4 inhibition targets breast cancer stem-like cells

Eric Metzger, Stella S Steputtis, Juliane Strietz, et al.

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