MCF7 Side Population Cells with Characteristics of Cancer Stem/Progenitor Cells Express the Tumor Antigen MUC1

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Abstract
Chemotherapy, radiation, and growth inhibitory drugs preferentially eliminate actively growing cancer cells. Cancer recurrence is currently thought to be due to nondividing cancer stem/progenitor cells that are resistant to these therapies. Different therapeutic approaches need to be considered for the elimination of the cancer stem cell population. Immunotherapy is one such approach. In addition to specificity and lack of toxicity, immunotherapy targets cancer cells irrespective of their state of proliferation, as long as they express particular tumor antigens. For that reason, it is important to examine if the tumor antigens that are currently being tested as immunotherapeutic agents are also present on cancer stem cells. This study aimed to determine if one well-known tumor antigen, MUC1, which is being tested as an immunotherapy target on tumor cells, is also expressed on the quiescent cancer stem/progenitor cells. We used the so-called side population (SP) cells found in the MCF7 breast cancer cell line, which we first confirmed by cell surface markers and gene profiling to be highly enriched in cells that fulfill specific functional, phenotypic, and molecular criteria for being tumor stem/progenitor cells. We show that these cells express MUC1 and give rise to MUC1+ tumors in vivo, which maintain the MUC1+ SP population. MUC1 on SP cells is hypoglycosylated and heavily sialylated; the characteristics of the tumor-specific form were expressed on mature cancer cells and recognized by tumor-specific T cells and antibodies. This suggests that stem/progenitor cells, like mature tumor cells, would be targets of MUC1-directed immunotherapy. [Cancer Res 2008;68(7):2419–26]

Introduction
Most current therapies for cancer preferentially target rapidly cycling cells and thus are not expected to have a cytotoxic effect on nondividing cells. If the hypothesis is correct that tumor recurrence is driven by a small population of resting, therapy-resistant stem cells (1), this has enormous implications for designing new tumor therapies that would target these cells as well. Immunotherapy is one type of therapy that could be expected to target both the mature dividing tumor cells and the quiescent tumor stem cells, dependent primarily on their expression of the target antigens.

One of the well-known tumor antigens is the epithelial cell mucin MUC1, a transmembrane glycoprotein that is differentially expressed on tumor cells compared with normal epithelial cells (2, 3). MUC1 is expressed either not at all or in small amounts on various normal epithelia but aberrantly or neoexpressed at high levels on the majority of adenocarcinomas. Tumor-associated alterations of MUC1 are characterized by hypoglycosylation, increased sialylation, and altered carbohydrate core-type expression (2, 4, 5). These differences are responsible for its antigenicity and its recognition by the immune system as a tumor-specific antigen and have been targeted by various forms of immunotherapy.

Stem cells are a minor population of mostly resting cells defined by their long life, high clonogenicity, self-replicating potential, plasticity, and drug resistance (1, 6). Cells with these properties have been identified in various normal and cancerous human tissues (7–14), as well as in several long-term tumor cell lines (7, 14–18). Normal stem cells are dependent on their so-called “niche,” the tissue microenvironment that regulates their survival and controls their division. Cancer stem cells, especially those found in long-term cell lines, do not require a specialized niche, providing an in vitro system useful for studying some aspects of their biology (19–21).

We have used the MUC1+ breast cancer cell line MCF7 as a source of a minor population of cells with characteristics of tumor stem/progenitor cells to show for the first time that these cells also express the hypoglycosylated (tumor) form of MUC1, previously described only on mature MCF7 cells and other tumors and tumor cell lines. We show that these cells give rise to MUC1+ tumors in vivo and that these tumors maintain a small population of MUC1+ cells with the stem/progenitor characteristics.

Materials and Methods
Cell Culture
Human breast cancer cell line MCF7 was obtained from American Type Culture Collection (ATCC) and cultured, according to the manufacturer’s instructions, in MEM (ATCC) supplemented with 10% fetal bovine serum (FBS; ATCC), 10 ng/mL insulin (Sigma) at 37 °C in the presence of 5% CO2.

Mammosphere culture. Cells (1,000/mL) were grown in suspension culture using ultralow attachment plates (Corning) and serum-free ES-DMEM (ATCC) supplemented with 2 mmol/L l-glutamine (ATCC), nonessential amino acids and B27 supplement (Invitrogen), 20 ng/mL human recombinant epidermal growth factor and basic fibroblast growth factor (PreproTech), and 4 μg/mL heparin and 5 μg/mL insulin (Sigma; refs. 21, 22).

Cytospins and H&E Staining
MCF7 cells were grown for 24 h under serum-free condition to synchronize them and for additional 16 h in complete media. The breast cancer cells were fluorescence-activated cell sorting (FACS)-sorted into side population (SP) and non-SP cells, and 104 cells were cytocentrifuged at 700 rpm for 7 min in a Cytospin3 centrifuge (Shandon) onto glass slides. Slides were air-dried, fixed in 95% acetone, and H&E stained: 10 min

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

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hematoxylin (Sigma), 10 min rinsing in tape water, several dips in EosinY (Sigma), increasing ethanol series, air dry, and mount in vectashield (Vector Labs).

**FACS and Analysis of SP and Non-SP Cells**

Cells were stained with Hoechst 33342 (Sigma) as previously described (23). Briefly, 10^6 cells/mL were resuspended in DMEM supplemented with 2% FBS and 10 mmol/L HEPES (Invitrogen). Incubation after addition of Hoechst (5 μg/mL) and 10 μmol/L Fumitremorgen C (Axxora) to control tubes was at 37°C for 90 min after immunostaining (30 min on ice) and FACS analysis (FACS Aria, BD Biosciences). Intracellular protein expression (CK18, CK19, MUC1) was performed after cell fixation for 20 min on ice using BD Cytofix/Cytopherm after antibody staining for 30 min on ice. Propidium iodiode staining (2 μg/mL, BD Biosciences) ensured dead cell exclusion. Antibodies used to detect proposed stem cell markers were antihuman CD133 (Miltenyi), CD117, CD90, CD44, CD24, CD49f, EpCAM, CD10, ATP-binding cassette subfamily G member 2 (ABC2G2; Biosciences or eBioscience), and CK18/19 (StemCell Technologies). Anti-MUC1 antibodies used were HMPV (BD Biosciences), VU-3C6 (purified from hybridoma supernatant), E29 (DAKO), VU-4H5 (Santa Cruz), and My1E12 (gift from T. Irimura, University of Tokyo). These monoclonal antibodies recognize well-characterized epitopes on MUC1 (24). Antibodies recognizing carbohydrate epitopes were anti-CD176/TF (DAKO), CD175/Tn (GeneTex), and CD15s/Le^a^-catenin were calculated. In addition, a universal human reference RNA (Stratagene) was used for normalization purposes.

**Real-Time Reverse Transcription–PCR**

Total RNA (100 ng) was reverse transcribed using the high-capacity cDNA archive kit (Ambion). cDNA (6.25 ng) was amplified using TaqMan Universal PCR Master Mix and an Assay on Demand (MUC1: Hs00159357_m1; Applied Biosystems) in an ABI Prism 7900HT instrument. The Sequence Detection Software v2.1 has been used to determine the relative amount of PCR product based on the cycle threshold (Ct) value. For each sample, Ct values for β-actin gene (Hs99999903_m1) were determined for normalization purposes and the ΔΔCt between MUC1 and β-actin were calculated. In addition, a universal human reference RNA (Stratagene) was used for normalization purposes.

**In vivo Tumor Growth and Analysis**

10^5 MUC1^bright or MUC1^low SP and non-SP cells were resuspended in PBS/Matrigel (BD Biosciences) and orthotopically injected into abdominal mammary fat pads of 7-wk-old virgin NOD/scid mice (n = 10). All procedures were conducted in accordance with an approved Institutional Animal Care and Use Committee protocol. Resulting tumors were dissected and either embedded in paraffin for H&E and MUC1 immunohistochemical staining or digested in collagenase/hyaluronidase cocktail (StemCell Technologies) for 1.5 h at 37°C to analyze single cells using flow cytometry.

**Immunohistochemistry**

Paraffin sections (5μm) were deparaffinized by baking overnight at 59°C for antigen-retrieval in citrate buffer (at 100°C for 10 min). Endogenous peroxidase activity was eliminated by treatment with 3% H2O2 for 10 min at room temperature. Nonspecific binding sites were blocked with protein blocking agent (Thermo-Shandon) after incubation with anti-MUC1 antibodies HMPV [recognizes all forms of MUC1 by binding the epitope APDTRPAP in the variable number of tandem repeats (VNTR) region in a glycosylation-independent manner] or VU–H5 (recognizes the epitope APDTRPAP in the VNTR region of the hypoglycosylated MUC1) or isotype control for 1 h at room temperature in a humified chamber. Staining was revealed by treatment with an avidin-biotin-peroxidase complex (Vectorstain ABC kit; Vector Laboratories) according to manufacturer's direction, and color development was achieved using a 3,3-diaminobenzidine kit (BD Phar-Mingen) according to manufacturer's direction.
Results

MCF7 breast cancer cell line SP cells have multiple characteristics of stem/progenitor cells. We began our studies by testing a large number of human long-term epithelial cancer cell lines for the presence of a cell subpopulation with the proposed characteristics of somatic stem/progenitor cells (1, 6, 25). Within the limitations of our highly sensitive flow cytometry–based analysis, only MCF7 cell line contained this type of cells (Supplementary Table S1). We detected this minor population of cells by flow cytometry based on their capacity to efflux the fluorescent DNA-binding dye Hoechst 33342. This is due to their overexpression of ABCG2 drug resistance protein, one of the important characteristics of cancer stem/progenitor cells (26, 27), and thus, a generally accepted cell marker–independent method to identify and enrich stem/progenitor cells (17). When Hoechst blue and red fluorescence signals are plotted against one another, cells that have effluxed the dye (negative label) form a tail-like structure called SP, which separates these cells from the majority of brightly staining mature tumor cells (non-SP cells; ref. 23). An example is shown in Fig. 1A. MCF7 cells after Hoechst staining can be clearly separated into a fluorescence-negative tail of SP cells (7.5% ± 1.19 SD) and the brightly stained mature non-SP cells. Treatment with Fumitremorgin C, an inhibitor of the drug resistance protein ABCG2 and other ABC transporters, allows SP cells to retain the dye and therefore prevents SP tail formation (Fig. 1B).

We were able to confirm that the vast majority of MCF7 SP cells are CD44+/CD24−/low (80.1%; Fig. 1C), a cell phenotype previously reported for breast cancer stem/progenitor cells (9). Furthermore, nearly all MCF7 SP cells express the luminal and epithelial marker CK18, EpCAM, and the stem/progenitor marker CK19 (Fig. 2C). A small fraction of those cells is positive for the stem/progenitor marker CD49f (Fig. 2C). They do not express CD10, CD90, CD117, and CD133. This phenotype is consistent with the breast cancer stem-like and progenitor cell phenotype noted above.

Morphologic differences between SP and non-SP cells were also consistent with SP cells being highly enriched in stem/progenitor cells. Approximately, 57.5% (±2.73 SD) of SP cells have a large nucleus-to-cytoplasm ratio. In contrast, the non-SP cells are composed of only 19.5% (±1.95 SD) of such cells (Fig. 2A). Moreover, when single MCF7 SP cells were grown in suspension culture, they readily developed into floating mammospheres (0.04%; Fig. 2B), a behavior observed very rarely in non-SP cell cultures. This property was reported to be restricted to stem/progenitor cells and verifies a high developmental and proliferative potency of SP cell (22).

Differential gene expression between MCF7 SP and non-SP cells: detection of “stemness” genes in MCF7 SP cells. We used gene array analysis to define differences in gene expression between MCF7 SP and non-SP cells and to confirm also by specific gene expression the stem cell nature of the SP cells defined by flow
Among the genes known to be preferentially expressed in stem cells, we found the gene for Wiskott-Aldrich syndrome protein interacting protein (NM_003387.3) involved in actin cytoskeleton and therefore control of cell shape and size, up-regulated 4.7-fold ($P < 0.00001$). Insulin-like growth factor binding protein 3 (NM_000598.2), involved in growth regulation and apoptosis, was up-regulated 3.7-fold ($P < 0.0000001$). We expected to find and we found the ABCG2 (NM_004827.1) integral membrane component that contributes to multidrug resistance, up-regulated 4.6-fold ($P < 0.00001$). The complete list of differentially expressed genes between MUC1+ SP and MUC1+ non-SP cells is provided as Supplementary Table S2.

MUC1 expression on MCF7 SP cells. The results shown above confirmed that MCF7 SP cells fulfilled specific criteria for being tumor stem/progenitor cells, and thus, we used them for further analysis of MUC1 tumor antigen expression. SP cells were sorted and stained with a monoclonal antibody HMPV that binds to MUC1 irrespective of its glycosylation state. We found that 77.8% ($\pm 6.9$ SD) of SP cells had a high level of cell surface expression (MUC1$^{bright}$) and the other 22% were either negative or expressed very low levels (MUC1$^{dim}$/C0$^{dim}$; Fig. 3A). This parallels almost exactly the staining observed on the mature MCF7 non-SP cells (Supplementary Fig. S1).

We further subdivided the cell population in the tail into cells located in the tail tip (most Hoechst-label negative), those in the middle of the tail, and those at the top of the tail to determine if MUC1 staining might be confined to a particular subset of SP cells. We observed an equal distribution of MUC1-expressing cells through the entire tail structure (Fig. 3A). Assaying MUC1 expression in SP cells selected by their CD44$^+/CD24^−/CD0^−$ phenotype, we found >74% of those cells strongly positive for MUC1 and the remaining cells expressing very low levels of the antigen (Fig. 3A).

Tumor-associated changes in glycosylation of MUC1 in breast cancer comprise core-type switching from extended core2 (GlcNAc$^b$-6][Gal$^b$-3][GalNAc$^a$-O-S/T])–based glycans to shorter core1 (Gal$^b$-3GalNAc$^a$-O-S/T)–based forms with an increased level of sialic acid. The Tn antigen (GalNAc$^a$-O-S/T), Thomsen-Friedenreich (TF) antigen (Gal$^b$-4-3GalNAc$^a$-O-S/T), and sLe$^\alpha$ antigen (NeuAc$^a$-2-3Gal$^b$-$[4-Fuc$^c$-1-3]GlcNAc$^a$(1-R)) are not only well known tumor-associated carbohydrate epitopes in numerous cancers, they are also structures typically expressed on tumor forms of MUC1 (4, 28). We investigated the glycosylation of MUC1 on SP cells using glycosylation-sensitive anti-MUC1 monoclonal antibodies, as well as carbohydrate-specific antibodies. We observed that sialyl-core1 (NeuAc$^a$-2-6Gal$^b$-1-3GalNAc$^a$-O-S/T) antigen represents the dominant carbohydrate structure on MUC1 expressed on >71.3% ($\pm 13.6$ SD) of the SP cells (Fig. 3B). Approximately, 17.2% ($\pm 3.1$ SD) of the cells express MUC1 with either unglycosylated tandem repeats or containing only GalNAc residues (Tn antigen; Fig. 3B). Because other glycoproteins on SP cells could also be aberrantly glycosylated, we costained with...
antibodies specific for those carbohydrates and anti-MUC1 antibody. We confirmed that the tumor carbohydrates Tn, Tf, and sialyl-Le\textsuperscript{a} were indeed present on the MUC1\textsuperscript{+} SP cells by detecting coexpression of MUC1 with Tn antigen on 14.4% (±5.4 SD), Tf antigen on 64.9% (±7.9 SD), and sialyl-Le\textsuperscript{a} on 6.5% (±0.3 SD) of MCF7 SP cells (Fig. 3C). MUC1 glycosylation pattern on non-SP cells was similar (not shown).

**MUC1\textsuperscript{-/dim} versus MUC1\textsuperscript{bright} SP cells analyzed by gene array.** Although we were gratified to find that the majority of SP cells expressed high levels of surface MUC1 suggesting that they could be targeted by MUC1-specific immunotherapy, we were concerned about the minor population of SP cells that were MUC1 negative or dim and questioned if there were additional differences between these two populations. Gene expression profile of MUC1\textsuperscript{+} SP cells and MUC1\textsuperscript{-/dim} SP cells showed that only two genes were differentially expressed in MUC1\textsuperscript{+} SP cells in comparison with MUC1\textsuperscript{-/dim} SP cells: MUC1 (NM_002456.3) was expressed 2.89-fold (P < 0.004) higher and sperm protein associated with the nucleus

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**Figure 4.** MUC1 RNA expression and protein localization in different populations of SP and non-SP cells. A, quantitative real-time PCR of duplicates of two samples per cell population were run and averaged. Bars represent the relative expression \( \Delta C_t \) as a result from the following calculation: \( \Delta C_t = C_t \text{ MUC1 expression} - C_t \text{ \( \beta \)-actin expression of the same sample} \). Error bars correspond to SD from the total of four quantitative reverse transcription–PCR reactions per cell population. The height of the bar is reversely correlated with the amount of RNA. B, MUC1\textsuperscript{-/dim} SP and non-SP cells, as well as MUC1\textsuperscript{bright} SP and non-SP cells, underwent fixation, and intracellular accumulated MUC1 protein was detected using the same anti-MUC1 antibody clone but with a different fluorescent label.
on the X chromosome E (SPANXE; NM_145665.1) was down-regulated 3.84-fold \((P < 0.00001)\). SPANXE belongs to the SPANX gene family, and its specific function has not yet been determined. Expression of other SPANX family members has been detected in melanoma and ovarian cancer cell lines, but not in normal cell counterparts.

Whereas there was a difference in MUC1 mRNA levels by gene array between the MUC1\textsuperscript{bright} SP and MUC1\textsuperscript{−/dim} SP cells, that difference was not as large as could be expected from the FACS data, and thus, we further analyzed the difference in MUC1 expression by quantitative real-time PCR. We confirmed that MUC1 RNA expression was 2-fold higher in MUC1\textsuperscript{bright} SP cells, but we also saw a lot of MUC1 message in MUC1\textsuperscript{−/dim} SP cells (Fig. 4A). We hypothesized that the big difference between the two SP cell populations may be in the cellular location of the MUC1 protein rather than levels of expression. We thus stained both MUC1\textsuperscript{bright} and MUC1\textsuperscript{−/dim} SP cells after membrane permeabilization and found that overall MUC1 protein expression was comparable (Fig. 4B).

**MUC1\textsuperscript{bright} and MUC1\textsuperscript{−/dim} SP cells give rise to MUC1\textsuperscript{+} tumors with a persisting MUC1\textsuperscript{bright} SP cell population.** MUC1\textsuperscript{bright} or MUC1\textsuperscript{−/dim} SP cells were injected orthotopically into mammary fat pads of NOD/scid mice. Tumors became palpable around 6 weeks postinjection. Tumors were removed when they reached the size of 10 mm and analyzed for SP population and MUC1 expression. Both MUC1\textsuperscript{bright} and MUC1\textsuperscript{−/dim} SP cells gave rise to MUC1\textsuperscript{+} tumors by histology (Fig. 5). Tumors grown from either MUC1\textsuperscript{bright} or MUC1\textsuperscript{−/dim} SP cells maintained a stable SP population, indicating persistent expression of the SP phenotype.

![Figure 5](image_url). High level of MUC1 expression on tumor xenografts. Tumor sections were stained with anti-MUC1 antibody HMPV that recognizes MUC1 regardless of glycosylation (MUC1 all forms), anti-MUC1 antibody 4H5 that recognizes MUC1 that is unglycosylated or GalNAc-restricted (MUC1 underglycosylated), or appropriate isotype control. Bar, 200 \(\mu\)m.
minor population of SP cells (MUC1\(^{\text{bright}}\) SP 1.4%, ±1.2 SD; MUC1\(^{-\text{dim}}\) SP 2.6%, ±1.9 SD; Fig. 6A). All of these SP cells had the MUC1\(^{\text{bright}}\) phenotype (Fig. 6B).

The MUC1\(^{\text{bright}}\) and MUC1\(^{-\text{dim}}\) non-SP cells grew in NOD/scid mice slightly slower in the beginning (palpable around 8 weeks postinjection versus 10 weeks for non-SP cells), but reached the size of about 10 mm around the same time. The tumors derived from both populations were strongly MUC1 positive by immunohistology (Fig. 5). Interestingly, compared with tumors grown from SP cells, the non–SP-derived tumors had a much smaller population of SP cells (Fig. 6A), which was strongly positive for MUC1 as well (Fig. 6B).

**Discussion**

Our study reports for the first time that MUC1 molecule is not only expressed on mature cancer cells, but also on tumor cells that have multiple characteristics of stem and progenitor cells. MUC1, which is overexpressed on most premalignant lesions that are precursors to cancer and other adenocarcinomas (29, 30) is a well known target in immunotherapeutic strategies against cancer. Characterization of its expression on cancer stem and progenitor cells is important for future application of MUC1-based therapies for complete cancer eradication.

Our results show that MUC1 is overexpressed and hypoglycosylated on SP cells, cancer stem-like cells, in the same way as it has been described for mature cancer cells. Analysis of SP cells is a widely accepted flow cytometry–based approach for the isolation of a highly enriched stem/progenitor cell population that can then be further interrogated for other markers and behaviors (14–16, 18, 31).

Considering various documented functions of MUC1 in cell adhesion, cell proliferation, cell survival, and signaling (32–35), we postulate that abnormal expression of this molecule on cancer stem/progenitor cells may interfere with their normal cellular functions and thus play an important role in their progression from stem to progenitor to mature tumor cells. We are not sure at this time what the significance is in our detection of two SP populations, one that expresses MUC1 on the surface and the other, albeit minor population, that sequesters the protein intracellularly. Certainly, the signals received from their microenvironment in the presence or the absence of this molecule on the surface must be different, and we will be exploring these differences in future studies. Our *in vivo* results show that both MUC1\(^{\text{bright}}\) and MUC1\(^{-\text{dim}}\) SP cells give rise to MUC1\(^{+}\) tumors. This might suggest that during tumor progression MUC1\(^{-}\) SP cells become MUC1\(^{+}\) SP cells and then MUC1\(^{+}\) mature tumor cells and that surface expression of MUC1 may mark the transition from a quiescent cancer stem-like cell (MUC1\(^{-\text{dim}}\)) to a more active progenitor cell (MUC1\(^{\text{bright}}\)). We also observed tumor growth from the non-SP cells, which is not unexpected from cells grown as long-term cell lines. The evidence of *in vivo* tumorigenicity of non-SP cells has been observed in other recently performed studies as well (14, 15, 18) and is considered to be derived from ABCG2\(^{-\text{dim}}\) cancer stem-like cells (7) that reside among the non-SP cells. This would be consistent with our finding that the tumor xenografts derived from non-SP cells contained a small but clearly evident SP fraction.

The hypothesis that cancer is propagated and sustained by a rare cell population with stem cell properties has led to rethinking of various approaches to cancer therapy. MUC1-based immunotherapy and immunotherapies based on many other

![Figure 6](https://www.aacrjournals.org) SP and non-SP derived tumor xenografts. A, tumors from MUC1\(^{-\text{dim}}\) or MUC1\(^{\text{bright}}\) SP cells retain a significant population of MUC1\(^{\text{bright}}\) GP cells, whereas tumors from non-SP cells contain only a negligible population of these cells. Fumitremorgin C treatment that blocks SP tail formation is shown in the inserts. B, SP cells from tumors derived from MUC1\(^{\text{bright}}\) or MUC1\(^{-\text{dim}}\) SP and non-SP cells express MUC1. Antibody isotype controls are shown in the inserts.
tumor antigens are currently being evaluated solely by their short-term effects against mature tumor cells. This form of evaluation of efficacy has been adopted from standard cytotoxic therapies and it may be inappropriate for either one, but especially for immunotherapy. Presence of tumor antigens on cancer stem cells fulfills the most important requirement for their susceptibility to immune attack. The fact that the vast majority of MCF7 stem cell enriched SP cells express MUC1 suggests that epithelial cancer stem cells would also be targets of various immunotherapy approaches based on the MUC1 tumor antigen that have been designed with mature tumor cells in mind (36). A recent report described candidate human pancreatic cancer stem cells (32, 37), which if interrogated for tumor antigen expression are likely to be further characterized by aberrant MUC1 expression. In this regard a larger variety of epithelial cancer cell lines and patient-derived tumor samples will be examined in the future to validate the importance of MUC1 expression on epithelial cancer stem cells.

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