M. Margarida Bernardo, Alexander Kaplun, Sijana H. Dzinic, Xiaohua Li, Jonathan Irish, Adelina Mujagic, Benjamin Jakupovic, Jessica B. Back, Eric Van Buren, Xiang Han, Ivory Dean, Yong Q. Chen, Elisabeth Heath, Wael Sakr, and Shijie Sheng

Introduction

Prostate cancer is the most frequently noncutaneous diagnosed tumor and the second leading cause of death among American men (1). Although patients with prostate cancer are initially responsive to androgen deprivation therapy, 80% to 90% of the patients ultimately develop recurrent metastatic castration-resistant to androgen deprivation therapy, 80% to 90% of the patients ultimately develop recurrent metastatic prostate cancer (1). A significant proportion of patients with prostate cancer are initially responsive to androgen deprivation therapy, 80% to 90% of the patients ultimately develop recurrent metastatic castration-resistant prostate cancer (1). This highlights the need for new therapeutic strategies to overcome tumor cell heterogeneity and plasticity. To this end, the drug sensitivity and resistance is thought to be, at least in part, due to a small population of cancer stem cells (2–9) that are capable of self-renewal and undergo plastic phenotypical changes in response to changes of microenvironments (10–12). Epigenetic reprogramming has been shown to determine the specific lineages of tumor cell dedifferentiation (13). The precise histone acetylation that is commonly dysregulated in the progression of many types of cancer may control the hierarchical order of epigenetic changes (14).

We have previously shown that maspin, a 42-kDa tumor-suppressive endogenous histone deacetylase (HDAC) 1 inhibitor (15, 16), plays a predominant role in the maintenance of the epigenetic program for differentiation (17, 18). Consistently, accumulated experimental evidence showed that maspin exerts multifaceted tumor-suppressive effects, including reduces tumor cell–associated uPA/αPAR activity (19, 20), blocks tumor cells detachment from established contacts with the extracellular matrix (21), inhibits tumor cell motility and invasion in vitro (17, 22), and inhibits tumor growth and metastasis in xenograft (18) or syngeneic tumor models (23–25). Ectopic expression of maspin in prostate tumor cells was sufficient to drive the full spectrum of progressive changes leading to acini formation in three-dimensional (3D) collagen I (17) and in a xenograft model for prostate tumor bone metastasis (18). It is important to note that maspin also enhances the sensitivity of tumor cells to apoptosis-inducing drugs (26–28). Consistently, clinical evidence...

Abstract

Future curative chemotherapy has to overcome tumor cell heterogeneity and plasticity. To test the hypothesis that the tumor suppressor maspin may reduce microenvironment-dependent prostate tumor cell plasticity and thereby modulate drug sensitivity, we established a new schematic combination of two-dimensional (2D), three-dimensional (3D), and suspension cultures to enrich prostate cancer cell subpopulations with distinct differentiation potentials. We report here that depending on the level of maspin expression, tumor cells in suspension and 3D collagen I manifest the phenotypes of stem-like and dormant tumor cell populations, respectively. In suspension, the surviving maspin-expressing tumor cells lost the self-renewal capacity, underwent senescence, lost the ability to dedifferentiate in vitro, and failed to generate tumors in vivo. Maspin-nonexpressing tumor cells that survived the suspension culture in compact tumorspheres displayed a higher level of stem cell marker expression, maintained the self-renewal capacity, formed tumorspheres in 3D matrices in vitro, and were tumorigenic in vivo. The drug sensitivities of the distinct cell subpopulations depend on the drug target and the differentiation state of the cells. In 2D, docetaxel, MS275, and salinomycin were all cytotoxic. In suspension, while MS275 and salinomycin were toxic, docetaxel showed no effect. Interestingly, cells adapted to 3D collagen I were only responsive to salinomycin. Maspin expression correlated with higher sensitivity to MS275 in both 2D and suspension and to salinomycin in 2D and 3D collagen I. Our data suggest that maspin reduces prostate tumor cell plasticity and enhances tumor sensitivity to salinomycin, which may hold promise in overcoming tumor cell heterogeneity and plasticity. Cancer Res; 75(18): 1–10. ©2015 AACR.
demonstrated the correlation of maspin with better differentiated phenotypes and better prognosis (29).

To test the hypothesis that maspin may control the state of differentiation and dictate the drug sensitivity of prostate tumor cells, we characterized the effects of maspin on prostate tumor cell stemness, differentiation lineage, and drug sensitivity in two-dimensional (2D), 3D, and in the tumorsphere assay suspension culture systems. Our results demonstrate that different microenvironments selectively enriched subpopulations of prostate tumor cells whose distinct phenotypes could be stratified on the basis of maspin expression. Although no single drug was sufficient to effectively eliminate all tumor cells that survive and thrive in different microenvironments, our data point to a novel scheme that may accelerate rational drug screening to target the full spectrum of tumor cell plasticity.

Materials and Methods

Reagents and cell culture

Reagents used were B27 supplement (Life Technologies), Accu-

cum cell detachment solution (EMD Millipore), Cultrex rat col-

lagen I (Trekon), Matrigel (Corning Incorporated), X-Gal (Pro-

mega), collagenase (Clostridium histolyticum), docetaxel, MS275, rapamycin, salinomycin (Sigma-Aldrich), and Hoechst

33342 (Molecular Probes, Life Technologies). The antibodies

used were LC3B (Cell Signaling Technology, 2775S), PARP

(Abcam, 96476), and GAPDH (Abcam, 9484). Embedded cell
culture in 3D collagen I or Matrigel was performed as described
previously (17).

The immortalized human normal prostate epithelial cells
CRL2221 and the human prostate carcinoma cell lines DU145,
PC3, and LNCaP were supplied from ATCC and were cultured
as described (21, 30). LNCaP C4-2B, derived from LNCaP cells
as described (31), was generously provided by Dr. L.K. Chung
(Cedars-Sinai Medical Center, Los Angeles, CA). DU145 cells
stable transfected with maspin (M7) or empty vector (Neo)
were generated and cultured as described (30). Maspin in PC3
were knocked down by transfection with a shRNA against
maspin (PC3-sh) as described (32). A PC3 clone transfected
with an shRNA with a scrambled sequence (PC3scr) was used
as a control (32). These cells were maintained in RPMI-1640
medium containing 5% FBS and 30 g/mL of hygromycin.

Homozygous and heterozygous mouse prostate epithelial cells
were generated and cultured as described (21, 30). Maspin in PC3
stable transfected with maspin (M7) or empty vector (Neo)
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as described (21, 30). LNCaP C4-2B, derived from LNCaP cells
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maspin (PC3-sh) as described (32). A PC3 clone transfected
with an shRNA with a scrambled sequence (PC3scr) was used
as a control (32). These cells were maintained in RPMI-1640
medium containing 5% FBS and 30 g/mL of hygromycin.

Homoygous and heteroygous mouse prostate epithelial cells
derived from mice harboring a specific deletion of PTEN in the
mouse prostate epithelium (PTEN<sup>−/−</sup> and PTEN<sup>+/−</sup>
respective;
refs. 33, 34) were cultured in DMEM supplemented with 5% FBS, 1-glutamine, and antibiotics (penicillin/streptomycin; ref. 35).

Suspension cell culture

Cells harvested from the maintenance culture were seeded in
6-well ultra-low adherence plates (Costar, Corning), at a
density of 2,000 cells/mL/well, in 2.5 mL of serum-free RPMI-1640
supplemented with 2% B27, 1-glutamine, and antibiotics (peni-
cillin/streptomycin). Cell viability was measured with the
WST-1 reagent (Roche) according to the manufacturer’s instruc-
tions. The fraction of viable cells in the total cellular particulate
determined with the Coulter Z1 particle counter (Beckman
Coulter) was calculated on the basis of a working standard
curve of viability versus the number of cells in maintenance
culture.

Fluorescence nuclear staining and confocal imaging

Cells in 3D collagen I or in suspension were stained with
Hoechst dye (0.1 μg/mL). Confocal imaging of the cells was
performed using the Zeiss LSM 510 and 780 confocal micro-
scopes, both equipped with dipping lenses (20× and 40×).

Detection of senescence-associated β-galactosidase activity with X-gal staining

Cells grown in suspension were transferred to 15-mL conical
tubes, centrifuged, dispersed with Accumax, and fixed with a
freshly prepared solution of PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes, at room temperature.
The assay was carried out as described (36). The cell suspensions
were transferred to ultra-low attachment 6-well plates and were
incubated overnight at 37°C. The next day, the cells were photo-
graphed using a Leica fluorescence microscope at 5 different fields,
and the proportion of cells exhibiting a medium to dark blue
stain, indicative of senescence-associated β-galactosidase (SA-
β-gal) activity, was determined by counting the stained and
unstained cells (36).

RNA extraction and mRNA quantification by real-time PCR

The RNA from cells grown in suspension was extracted (RNasey
Mini kit, Qiagen) and reverse-transcribed (iScript cDNA synthesis
kit, Bio-Rad). qRT-PCR was performed as described (20) using a
Bio-Rad iQ5 Multicolor Real-Time PCR Detection System and a
Applied Biosystems StepOnePlus Real-Time PCR system from Life
Technologies. The sequences of the primers are listed in Supple-
mentary Table S1. Normalization of qRT-PCR results was per-
formed using the ΔΔC<sub>T</sub> method (37).

Imaging flow cytometry

Trypsinized cells cultured in 2D as well as cells in suspension
dispersed with Accumax were stained with the following mouse
anti-human fluorescence-labeled antibodies: CD44-PE-CY7
(eBioscience, 25-0441-82), CD29-APC (BD Pharmigen,
561794), CD133/1 AC133-PE (Miltenyi Biotec, 130-080-801),
and CD166-BV421 (BD Horizon, 562936). Mouse FCR Blocking
Reagent (Miltenyi Biotec, 130-093-575) was used for blocking
and unstained cells were used as a control. Imaging flow cyto-
metry was performed on an Amnis ImageStream<sup>®</sup> MK II single
camera imaging cytometer (Amnis) equipped with 405 nm, 488
nm, and 642 nm excitation lasers (to acquire fluorescent images of
each label on single cells) and the multimagnification option.
Poly(styrene beads (BD CompBead Plus anti-mouse Ig, κ and
CompBead Plus negative control, 51-9006274 and 51-900667,
respectively) were used to establish fluorescence compensation
settings for multicolor flow cytometric analyses. The acquired data
were analyzed using the IDEAS (v6.1, Amnis) and FlowJo
(v10.0.7, FlowJo) software.

In vivo tumorigenicity assay

Cells harvested from the suspension culture on day 12 were
dispersed with Accumax enzyme cocktail, resuspended in 1 mL of
serum-free RPMI-1640 medium, and were injected subcutane-
ously at 5,000, 10,000, and 50,000 cells/mL in the left flanks of
athymic nude mice (Harlan Laboratories), as described (38). A
number of 6 animals was used per cell dilution. At 100 days after
injection, the animals were sacrificed using a CO<sub>2</sub> chamber
followed by cervical dislocation.
Drug treatment and drug sensitivity screening

Cells grown to 90% confluency in 2D, suspension, or 3D collagen I culture were treated with the indicated chemotherapeutic agents for 72 hours. Cells were treated with DMSO in parallel as the vehicle treatment control. The cells were harvested by trypsinization (for 2D culture), collagenase digestion (10 units/2 μL/15 min/37°C; for 3D collagen I culture; ref. 17), and centrifugation followed by Accumax cell detachment (for tumorspheres in suspension, according to the manufacturer’s instructions). The isolated cells were resuspended in 100 μL serum-free, phenol red-free RPMI-1640 and transferred to 96-well plates. Cell viability was assessed with the WST assay. The resulting cleaved tetrasolium was quantified by the increase of absorbance at 450 nm using a Bio-Rad iMark microplate reader. The percentage of live cells was plotted versus the concentration of the drug, and the data were nonlinear least-squares fitted to the sigmoidal equation:

\[ E(D) = \frac{E_0 - E_u}{1 + \left(\frac{D}{EC_{50}}\right)^{HS}} \]

where \( D \) is the drug concentration, \( E(D) \) is the cell viability (in percentage), \( E_0 \) and \( E_u \) are the minimum and maximum of the response curve, \( EC_{50} \) is the concentration at half-maximal effect, and \( HS \) is a slope parameter analogous to a Hill coefficient (39) using either Scientist (Micromath) or SigmaPlot (Systat Software Inc.) software.

Miscellaneous procedures

For statistical analyses, Student’s t tests were performed for paired datasets using SigmaPlot software.

Results

Maspin averts human prostate cancer cell stemness

To examine how maspin expression stratifies the self-renewal competency of stem-like cancer cells, the tumorsphere assay was performed using four isogenic pairs of cell lines with significantly different levels of maspin expression between the 2 cell lines in each pair: DU145-derived M7 (moderate level of maspin)/Neo (undetectable maspin; ref. 30); LNCaP (moderate level of maspin)/LNCaP C4-2B (C4-2B, low level of maspin; ref. 17); PC3aI (moderate level of maspin)/PC3D3 (low level of maspin; ref. 32); and mouse prostate cell lines PTEN(+/-)PC3aI (high level of maspin)/PTEN(-/-) (undetectable maspin; Supplementary Fig. S1). Earlier evidence showed that maspin inhibits HDAC1 activity in DU145-derived transfected cells (15). Consistently, in PC3D3s cells, maspin knockdown led to a significant increase of HDAC catalytic activity (Supplementary Fig. S2). Suggesting that this pair of cell lines supports the biochemical and biologic function of maspin and therefore can be used as an additional investigation tool. As shown in Fig. 1A, the cells expressing low maspin (Neo, C4-2B, PC3D3, and PTEN(-/-)) gave rise to tumorspheres. The PTEN(-/-) high maspin-expressing cells did not form tumorspheres. The other 3 cell lines that express higher levels of maspin (M7, LNCaP, and PC3aI) did not form tumorspheres. Instead, they formed aggregates. Consistent with this pattern, parental DU145 cells that do not express maspin formed tumorspheres, whereas immortalized normal prostate epithelial cells CRL2221 that express a high level of maspin formed aggregates (data not shown).

Using the pair of M7 and Neo cells, we next examined whether the differences in their tumorsphere-forming capacities were consistent with the differential expression of markers for prostate stem-like cancer cells: β1-integrin (CD29), CD133, CD166 (40), α2-integrin, α6-integrin, CD151 (41), and Nanog (42). On the basis of the qRT-PCR analysis, in the primary tumorspheres (passage 1), maspin was significantly lower (66-fold) in Neo relative to M7 cells, as expected (Fig. 1B). In addition, significantly higher levels of β1-integrin (2-fold), CD133 (3-fold), CD166 (6-fold), and Nanog (3-fold) were observed in Neo relative to M7 cells. In parallel, no significant difference was detected in the expression levels of α2-integrin, α6-integrin and CD151 at the mRNA level between the two cell lines. After primary tumorspheres (passage 1) were collected, dispersed and grown in suspension as the tumorspheres of passage two, the expression of CD133, CD166, and Nanog remained significantly higher in Neo cells than in M7 cells (13-, 14-, and 6-fold, respectively). Imaging flow cytometry showed that the cell surface expression of CD166 in both Neo and M7 cells were higher when cultured as tumorspheres than in 2D culture (Fig. 1C and D) and was more prominent in the Neo stem cell population than the M7 counterpart. In parallel, the expression levels of CD44 and CD29 decreased significantly in both Neo and M7 cells in suspension relative to the bulk population. The level of surface-expressed CD133 was below the sensitivity of the detection.

To determine whether the tumorspheres of Neo and M7 cells had distinct tumorigenicity capacities, cells enriched by the suspension culture were injected subcutaneously by serial dilution in the flanks of athymic nude mice. As shown in Table 1, none of the mice injected with M7 cells grew tumors over a 100-day period, regardless of the number of cells injected. However, two out of six animals injected with 50,000 Neo cells developed tumors, starting as early as at day 21.

Maspin expression confers a transient increase of anchorage-independent survival

To further investigate whether maspin expression alters the self-renewal properties of prostate cancer cells. Neo and M7 cultured in suspension were collected and passaged consecutively. As shown in Fig. 2A, the growth kinetics of the Neo cells remained steady, as the total number of cells was practically unchanged in between passages for up to seven passages. The number of M7 cells, however, decreased continuously with the passage number, which indicates that the fraction of surviving cells becomes continuously smaller. These data indicate that the Neo cells in tumorsphere inherited the sustaining self-renewal potential, whereas the anchorage-independent survival of the M7 aggregates in suspension culture was transient.

Interestingly, despite the eventual loss of self-renewal capacity, the M7 aggregates in earlier passages appeared to have more cell counts, as compared with the tumorspheres of Neo cells (Fig. 1A). Detailed microscopic evaluations revealed that upon first-passage seeding of the cells in the tumorsphere suspension culture, Neo cells underwent significant cell death, leaving a small percentage of cells uniformly distributed throughout the plate that eventually gave rise to tumorspheres. In contrast, the aggregates of M7 cells clustered in the center of the well and were the only cells visible in the entire well (Fig. 2B). To address whether those cells visible under the microscope were viable, we quantified the number of cells concurrently by two different methods: the total cell count using the Coulter Z1 particle counter and cell viability using the WST assay. As shown in Fig. 2C, the numbers of Neo cells determined by the two methods were in good agreement. It was
noted that the number of live Neo cells dropped by approximately 40% on day 1, suggesting extensive acute cell detachment–induced apoptosis. In the following 15 days, the cells grew steadily into solitary tumorspheres, without losing viability, and plateaued after day 15. In comparison, on day 1, M7 cells in suspension survived in small clusters. In the next 14 days, these cells expanded, but a concurrent continuous decrease in cell viability offset the cell growth, resulting in a significant slowing down of the growth kinetics. After day 15, both the total number and viability of M7 cells decreased significantly.

The apparent “survival” advantage of M7 cells in the first 14 days of suspension culture did not seem to result from increased proliferation or decreased apoptosis, as Neo and M7 cells expressed similar levels of Ki67 (Fig. 1B), and did not undergo the typical apoptotic cleavage of 116-kDa PARP to a 89-kDa fragment (Fig. 2D). However, more extensive autophagy-associated LC3B cleavage (Fig. 2D) was detected in M7 cells than in Neo cells (Fig. 1B), whereas the two cell lines expressed LC3B mRNA at similar levels. In addition, SA-β-gal activity, a marker of senescence, was dramatically increased in M7 cells (Fig. 2E and F), despite the decrease of SA-β-gal mRNA in M7 cells relative to Neo cells (Fig. 1B). These data suggest that M7 cells survived better in suspension culture via the mechanism of autophagy but eventually lost the renewing capacity due to senescence.

### Maspin exerts distinct effects on the phenotypical plasticity lineages of different tumor cell subpopulations

The expression of maspin in the maintenance exponential growth culture gave rise to a better differentiated phenotype when the cells were subsequently grown in 3D collagen I, the most abundant extracellular matrix protein in tumor stroma. As shown in Fig. 3A, all four isogenic pairs of cell lines formed cobblestone-like organizations, which positively correlated with maspin expression. This result extends from our earlier study with DU145-derived transfected cells (17). The 3D cobblestone-like structures differ from the tumorspheres formed by cells that express lower levels of maspin in the tumorsphere suspension assay. Judging from the nuclear staining, the former had hollow lumens aligned by a monolayer of cells, but the

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<th>Injected cells</th>
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Table 1. Tumorigenicity of prostate cancer stem-like cells

Figure 1. Maspin reduces the stemness of prostate tumor cells. A, phase contrast representative images of isogenic pairs of prostate cancer cells expressing low (left) and high maspin (right) in suspension at days 12 to 17 (scale bar, 50 μm). B, qRT-PCR analysis of potential prostate cancer stem cell markers in Neo and M7 primary (passage 1) and secondary (passage 2) tumorspheres cultured in suspension for 15 days, expressed as fold change over the level of the housekeeping gene GAPDH ± SD. *, P < 0.001; **, P < 0.05. C, imaging flow cytometric dot plots of fluorescence-labeled Neo and M7 cells cultured in 2D and in suspension. The outlined area of each plot is the cell population that was enriched through tumorsphere culture. D, representative imaging of fluorescence-labeled single Neo and M7 cells cultured in 2D and in suspension acquired by the Amnis ImageStream™ MK II single camera imaging flow cytometer at ×60 magnification.
Maspin stratifies the drug sensitivity of prostate cancer cell subpopulations

Taking advantage of the new established scheme that combined the suspension, 2D, and 3D collagen I culture to enrich distinct cancer cell populations and helped reveal the role of maspin on tumor cell plasticity, we examined the effects of maspin on the sensitivity of each of these subpopulations toward clinical and experimental therapeutic agents that target different cellular mechanisms, including anti-mitotic agent docetaxel, mTORC1 inhibitor rapamycin, histone deacetylases (HDAC) inhibitor MS275, and the potassium ionophore salinomycin. As summarized in Table 2A and B, the drug response was dependent on whether the drug targets mitosis or survival and was further dependent on the differentiation state of each subpopulation of tumor cells in different microenvironments.

The dose responses of the M7/Neo and PC3scr/PC32D3 pairs of cells to docetaxel, MS275, and salinomycin, as assessed using the WST cell viability assay, are shown in Fig. 5A and B, with the EC50 and Emax values summarized in Table 2A and B. Docetaxel, the last line of treatment for castration-resistant prostate cancer, was equally toxic to M7 and Neo cells in 2D culture, selectively toxic to Neo cells, but not M7 cells, in 3D culture and showed no toxicity to either M7 or Neo cells in suspension culture at concentrations up to 1 μmol/L (Fig. 5A and Table 2A). In parallel, docetaxel showed no toxicity toward normal immortalized prostate cells (CRL2221) at concentrations up to 400 nmol/L (Supplementary Fig. S3A). The EC50 values obtained with 2D culture of M7 or Neo cells are in line with other reports (43). Interestingly, although the heterogeneous M7 and Neo cells in 2D culture had an apparent higher sensitivity to docetaxel, they were less effectively eliminated (maximally 50–60%) as compared with the Neo cells enriched in 3D culture (maximally 90%). Docetaxel showed no toxicity toward the PC3-derived clones under all experimental conditions except the maspin knocked down clone (PC32D3) when embedded in 3D collagen I (Fig. 5B and Table 2). These results are consistent with the lower proliferative capacity exhibited by this prostate cancer bone metastasis–derived cell line.

Salinomycin, an experimental therapeutic agent shown to be specifically toxic to stem-like cancer cells (44, 45), exerted toxicity toward both Neo and M7 cells (Fig. 5A), relative to the CRL2221 cells (Supplementary Fig. S3B) under all experimental conditions tested. Moreover, maspin expression correlated with elevated sensitivity to the drug in 2D and in 3D collagen I. Interestingly, salinomycin was most toxic toward the M7 acini in 3D collagen I and exhibited significantly lower toxicity toward Neo and M7 cells in suspension. Regardless of the differences in the EC50 values, salinomycin was similarly effective in eliminating tumor cells (80%–100%) under all 3 conditions. Contrary to the DU145-derived cells, in 2D, salinomycin showed no effect on the maspin-expressing PC3 clone (Fig. 5B) and only affected 50% of the

latter were solidly packed with cells without distinct cellular polarity (Fig. 3B).
showed no cytotoxicity to the cells in 3D collagen I culture but was remarkably effective toward 100% of the suspension enriched subpopulations. Consistently, these 2 drugs are thought to target different cellular processes (46, 47). Rapamycin, on the other hand, was ineffective against all cells under all experimental conditions used in this study (Supplementary Fig. S4A and S4B).

Discussion

In this study, the schematic combination of 2D, 3D, and suspension culture enabled us to stratify the original heterogeneous prostate cancer cell populations into distinct subpopulations that were enriched in specific microenvironments. We demonstrate that maspin expression averts the transformative process of stem-like cell generation and drives prostate cancer cells toward a more epithelial-like phenotype, in a microenvironment-dependent manner. Under suspension conditions, maspin expression drove the cells into a nonlinear path to cell death (48), which included initial survival with increased cell-cell adhesion and clustering, followed by autophagy stress response and senescence. These cells exhibited restricted self-renewal capacity and reduced plasticity in 3D matrices (collagen I and Matrigel) and were unable to generate tumors in vivo. In contrast, the totality or the vast majority of the cells expressing low or no maspin underwent immediate cell death (anoikis) under these conditions. The surviving cells generated compact tumorspheres that exhibited unlimited self-renewal capacity, expressed increased levels of prostate stem cell markers (CD166 and Nanog), were able to survive embedded in 3D matrices (collagen I and Matrigel) yielding morphologically identical compact tumorspheres and generated tumors in vivo. Interestingly and consistent with a previous report (17), embedding maspin-expressing cells in a 3D collagen I matrix allowed the selection and enrichment of a subpopulation that exhibits epithelial-like morphology, evidenced by a cobblestone assembly that in 3D is organized in polarized, hollow lumen acini. The cells expressing low maspin under these conditions failed to form multicellular structures and spread instead, protruding the surrounding matrix in all directions.

Our in vitro and in vivo analyses confirmed the strategy to enrich the stem-like prostate cancer cells through tumorsphere culture. To date, despite the evidence that ALDH, CD44 and CD24 showed promise as stem-like cancer cell markers for some types of cancer such as breast cancer (49), the markers for prostate stem-like cancer cells remain controversial (50, 51). Consistent with the earlier reports that CD166 may specifically mark prostate cancer stem-like cells (41), tumorsphere culture specifically enriched cells also expressed higher levels of CD166. Interestingly, the level of CD44 expression actually decreased in this cell population, which included initial survival with increased cell-cell adhesion and clustering, followed by autophagy stress response and senescence. These cells exhibited restricted self-renewal capacity and reduced plasticity in 3D matrices (collagen I and Matrigel) and were unable to generate tumors in vivo. In contrast, the totality or the vast majority of the cells expressing low or no maspin underwent immediate cell death (anoikis) under these conditions. The surviving cells generated compact tumorspheres that exhibited unlimited self-renewal capacity, expressed increased levels of prostate stem cell markers (CD166 and Nanog), were able to survive embedded in 3D matrices (collagen I and Matrigel) yielding morphologically identical compact tumorspheres and generated tumors in vivo. Interestingly and consistent with a previous report (17), embedding maspin-expressing cells in a 3D collagen I matrix allowed the selection and enrichment of a subpopulation that exhibits epithelial-like morphology, evidenced by a cobblestone assembly that in 3D is organized in polarized, hollow lumen acini. The cells expressing low maspin under these conditions failed to form multicellular structures and spread instead, protruding the surrounding matrix in all directions.

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Maspin expression drives prostate cancer cell dedifferentiation in 3D collagen I. A, phase contrast representative fields of isogenic pairs of prostate cancer cells expressing low (left) and high maspin (right), embedded in 3D collagen I at days 12 to 17 (scale bar, 50 μm). B, confocal fluorescence imaging of the nuclei of M7 cells in 3D collagen I (top; scale bar, 50 μm) and Neo cells in suspension (bottom; scale bar, 100 μm) stained with Hoechst dye (blue).
currently available therapies. To date, in the clinic, emphasis is placed in debulking the tumor and a variety of antimitotic drugs have been approved by the FDA, including docetaxel (53). Currently, docetaxel is the last resort treatment for castration refractory prostate tumors but the results have been disappointing (54–56). This is consistent with our evidence that docetaxel only targets a fraction of the highly proliferative cells and is likely to be ineffective toward subpopulations with diminished proliferative capacity, which encompasses the stem-like cells and the dedifferentiated quiescent cell subpopulations. However, the stem-like prostate cancer cell subpopulation was found to be highly sensitive to the class I HDAC inhibitor MS275. MS275 showed no toxicity toward the normal-like cell subpopulation in 3D collagen I though. Salinomycin was the only drug that targeted all cell subpopulations albeit with differential potency.

The underlying antitumor mechanisms of action of HDAC inhibitors and salinomycin remain unclear. Both have been shown to induce cell death via apoptotic and autophagic pathways (57–59). Moreover, the HDAC inhibitors have also been shown to induce dedifferentiation and in so doing reduce tumor growth and metastasis (60). Thus, therapeutic application of these drugs may promote survival as opposed to cell death. The interplay between maspin and the pathways targeted by MS275 and salinomycin also requires further investigation. Maspin has been shown to sensitize cells to drug-induced apoptosis (26–28). Consistently, maspin sensitized the stem cell subpopulations to MS275 and salinomycin. Here we show that maspin induces nonlinear cell death via autophagy and senescence pathways under suspension conditions. In 3D collagen I, however, maspin converted poorly differentiated tumor cells to a better differentiated and quiescent phenotype, which has been ascribed to the regulation of a cluster of genes involved in the homeostasis of epithelial differentiation due to endogenous and selective HDAC1 inhibition by maspin (17). Unlike synthetic HDAC-specific inhibitors, however, maspin may also target other serine protease-like molecules in different subcellular compartments, leading to better cancer prognosis and inhibition of tumor growth.

**Figure 4.**
Maspin-expressing cells in suspension lost the capacity to form acini in collagen I. Phase contrast images (top) and confocal fluorescence imaging of the nuclei (bottom) of Neo and M7 cell subpopulations cultured embedded in 3D collagen I (left) and Matrigel (right) after being enriched in suspension (scale bar, 50 μm).

**Table 2.** The effects of maspin on tumor cell drug sensitivity

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<td>Neo</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>MS275</td>
<td>292 ± 55</td>
</tr>
<tr>
<td></td>
<td>NEO</td>
<td>970 ± 272</td>
</tr>
<tr>
<td>Salinomycin</td>
<td></td>
<td>(42 ± 4) × 10³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.3 ± 0.1) × 10³</td>
</tr>
<tr>
<td>B.</td>
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<tr>
<td>Docetaxel</td>
<td></td>
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<tr>
<td>PC3scr</td>
<td>NE</td>
<td>390 ± 272</td>
</tr>
<tr>
<td>PC3203</td>
<td>NE</td>
<td>500 ± 400</td>
</tr>
<tr>
<td>Salinomycin</td>
<td></td>
<td>(1.3 ± 0.2) × 10³</td>
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<td>(10 ± 2) × 10³</td>
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| Abbreviation: NE, no effect.
invasion and metastasis. Overall, this study highlights the challenges to the application of antitumor therapies in the clinic due to tumor heterogeneity and plasticity. It also emphasizes the importance of the tumor microenvironment. On the basis of our evidence, an effective therapeutic regiment needs to include drugs that target the sustaining cellular mechanisms of all cell subpopulations, including the stem-like and the normal-like quiescent cells. For that to happen, the need for new drugs and biomarkers cannot be overstated. Our novel drug testing paradigm exposes the pitfalls of using mixtures of cell populations under stress-free conditions to test drugs and is likely to yield more reliable preclinical leads with greater translational potential.

Our finding that maspin stratifies prostate cancer cells into better differentiated lineages under all our experimental conditions is consistent with the clinical evidence that maspin correlates with better differentiated tumor morphology and better cancer prognosis. Of note, maspin has not been found mutated in cancer and may remain a target of complex transcriptional regulation in different phases of tumor progression. However, maspin has been recently found to be reexpressed in breast cancer cells due to activation of the maspin transcription promoter by myocardin (61). It is intriguing to speculate that the effect of maspin in tumor cell dedifferentiation in collagen I may be the underlying mechanism for the breast cancer

Figure 5.
Maspin modulates prostate cancer cell drug sensitivity in a microenvironment-dependent manner. Dose-dependent effect of docetaxel, MS275, and salinomycin on the cell viability of the Neo/M7 (A) and PC3scr/PC32D3 (B) pairs of cells cultured in tumorsphere suspension assay conditions (△), in 2D exponential growth culture (●), and embedded in 3D collagen I (■). The data were non-least-squares fitted to equation (A) as described in Materials and Methods.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Bernardo, S.H. Dzinic, J. Irish, J.B. Back, E. Van Buren, X. Han, W. Sakr, S. Sheng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.M. Bernardo, A. Kaplun, S.H. Dzinic, X. Li, J. Irish, J.B. Back, E. Van Buren, E. Heath, S. Sheng

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Study supervision: X. Li

Other (project coordinator): M.M. Bernardo, S. S. Sheng

Grant Support

This work was supported by NIH grants (CA127735 and CA084176 to S. Sheng), Fund for Cancer Research (S. Sheng and E. Heath), and the Ruth Sager Memorial Fund (S. Sheng). The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by NIH Center grant P30CA22453 to the Karmanos Cancer Institute, Wayne State University, and the Perinatology Research Branch of the National Institutes of Child Health and Development, Wayne State University.

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Published OnlineFirst July 24, 2015; DOI: 10.1158/0008-5472.CAN-15-0234

Received January 22, 2015; revised June 1, 2015; accepted June 16, 2015; published OnlineFirst July 24, 2015.
Bernardo et al.


Maspin Expression in Prostate Tumor Cells Averts Stemness and Stratifies Drug Sensitivity


Cancer Res Published OnlineFirst July 24, 2015.