Genistein versus ICI 182, 780: An Ally or Enemy in Metastatic Progression of Prostate Cancer

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BACKGROUND. Androgen signalling through the androgen receptor (AR) plays a critical role in prostate cancer (PCa) initiation and progression. Estrogen in synergy with androgen is essential for cell growth of the normal and malignant prostate. However, the exact role that estrogen and the estrogen receptor play in prostate carcinogenesis remains unclear. We have previously demonstrated the metastasis-promoting effect of an estrogen receptor beta (ERβ) agonist (genistein) in a patient-derived PCa xenograft model mimicking localized and metastatic disease.

METHODS. To test the hypothesis that the tumor-promoting activity of genistein was due to its estrogenic properties, we treated the xenograft-bearing mice with genistein and an anti-estrogen compound (ICI 182, 780) and compared the differential gene expression using microarrays.

RESULTS. Using a second xenograft model which was derived from another patient, we showed that genistein promoted disease progression in vivo and ICI 182, 780 inhibited metastatic spread. The microarray analysis revealed that the metallothionein (*MT*) gene family was differentially expressed in tumors treated by these compounds. Using qRT-PCR, the differences in expression levels were validated in the metastatic and non-metastatic LTL313 PCa xenograft tumor lines, both of which were originally derived from the same PCa patient.

CONCLUSIONS. Together our data provide evidence that genistein stimulates and ICI 182, 780 inhibits metastatic progression, suggesting that these effects may be mediated by ERβ signalling. *Prostate 73:1747–1760, 2013.* © 2013 Wiley Periodicals, Inc.

KEY WORDS: **estrogen receptor beta; ICI182,780; metallothionein; prostate cancer; metastasis**

INTRODUCTION

The prostate is a male-specific organ that is dependent on androgen for its growth and development. Classic Noble rat and other animal studies have, however, provided evidence for the requirement of a female hormone, estrogen, in normal prostate development, as well as in prostate cancer (PCa) [1–4]. Estrogen is further implicated in PCa by epidemiological studies that indicate a lower risk of PCa in Japanese

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men who have lower estrogen levels than men in the Western countries [5–7]. Similarly, there is a higher risk in African American males who have higher serum estrogen levels than Caucasians who have similar testosterone levels [5–7]. Although these studies point at the importance of estrogen in prostatic carcinogenesis, very little is known about the exact role that estrogen plays in PCa development and progression.

Estrogen elicits its effects by binding to and activating the alpha and beta estrogen receptors (ERα and ERβ, respectively). The receptor-ligand complex then binds to the estrogen responsive element (ERE) and induces transcription of estrogen-responsive genes [8]. Besides this direct genomic function, ERs can also function as co-activators themselves by binding to other transcription factors such as activator protein 1 (AP1) or specificity protein 1 (SP1) and indirectly activate gene transcription without binding to an ERE [9,10].

The two types of estrogen receptors, ERα and ERβ, are comprised of three major functional domains: activation function domain (AF-1), DNA binding domain (DBD), and ligand-binding, or AF-2, domain (LBD) [11]. The extent of homology between ERα and ERβ varies depending on the domains: 96% homology in amino acid sequence in DBD domain, 53% in AF-2 and 15% in AF-1 domains [8,12–14]. The low conservation of LBD sequences between the two receptors may be crucial for generating distinct functional outcomes in various tissues [8,12–14]. While a proliferative function has been reported for ERα in the prostate, a protective (anti-proliferative) role for ERβ has been suggested by early knockout mice studies [15,16]. However, these results were challenged by subsequent studies with inconsistent data resulting from the use of inadequate βERKO mouse models, which were not completely devoid of this receptor [17–20].

The expression of ERβ during PCa development and progression is very unique and stage-dependent compared to expression in the normal prostate where it is predominantly present in the basal and luminal epithelial cells [21]. Immunohistochemical analyses of clinical specimens show loss of ERβ in high-grade dysplasia, an increase in expression in Gleason grade-3 PCa, low expression or absence of ERβ in grade 4/5 and intense staining reappearance in almost all bone metastases [22,23]. Taken together with early knockout studies and clinical expression patterns of ERβ, the exact role that estrogen and ERβ play in PCa development and progression remains unresolved.

Previously, we demonstrated that ERβ activation by the agonist, genistein, stimulated PCa progression [24]. In the present study, we confirmed the results of our previous work using a second xenograft model (LTL313h) derived from a different patient, thus demonstrating that the metastasis-promoting effect of genistein was not patient specific. Furthermore, we observed that this effect on metastasis could be inhibited by an anti-estrogen compound, ICI 182,780 (hereafter abbreviated ICI). As a first step in elucidating the underlying mechanisms, we carried out gene expression analysis of tumors treated with each agent. This revealed that a unique set of genes were reciprocally up-regulated by genistein and down-regulated by ICI. Interestingly, five of the six genes in this group belonged to the metallothionein (*MT*) gene family.

Using qRT-PCR, the changes in expression levels were validated in metastatic and non-metastatic tumor lines of LTL313, both of which had been derived from the same PCa patient. Together our results suggest that signalling through ERβ regulates expression of *MT* genes, and possibly others, which may play a key role in PCa metastasis. This raises the interesting possibility that anti-estrogen treatment may inhibit expression of such estrogen-linked genes and prevent cancer progression.

MATERIALS AND METHODS

Xenograft Model System

The establishment of a distinct human tumor line, LTL313, has been described previously [25–27]. Briefly, they were derived from primary biopsy PCa specimens from a patient, which were grafted under the renal capsule of NOD-SCID male mice, where the tumor tissue receives sufficient amounts of oxygen and nutrients [28,29]. After LTL313h metastatic tumor line was established, tissue was grafted into twenty-NOD-SCID male mice 6–8 weeks old with each kidney having one tumor graft (2 grafts/mouse). Since genistein-treatment has been shown to decrease serum testosterone levels via the hypothalamic/pituitary/gonadal axis, testosterone pellets (10 mg) were implanted subcutaneously in all animals at the time of grafting to maintain adequate serum testosterone levels in all groups. The LTL313h tumor line grows relatively slowly and thus treatments were not started until the tumor grew to approximately 50 mm^3 in size (6 weeks after grafting).

Genistein and ICI (182, 780) Treatment

The animals with tumor grafts were randomly divided into four groups containing five mice per group. The mice in the "low-dose" group were given genistein dissolved in peanut oil by gavage at 5 mg/day (200 mg/kg body weight/day). Mice in the high-dose

group were given 10 mg/day (400 mg/kg/day) of genistein. Mice in the control and anti-estrogen groups received 0.1 ml of the oil-only vehicle by gavage. ICI 182, 780 (Astra Zeneca, Mississauga, ON) was administered to mice in the anti-estrogen group as a single weekly subcutaneous injection of 5 mg. All groups were fed the same standard rodent diet (PicoLab Rodent Diet 20) and given autoclaved drinking water. Treatments continued for 4 weeks and at the end of the fourth week, animals were sacrificed, and blood samples and organs were collected for analyses.

Local Invasion and Metastasis Analyses

Metastatic incidence was assessed in lung tissue collected from each mouse. Half of the organ was fixed and processed for immunohistochemical (IHC) analysis. Sections were stained with an anti-human specific mitochondria antibody-clone 113-1 (Millipore, Billerica, MA) and screened for the presence of metastatic cells. Images of IHC-stained sections from each organ were captured using an AxioCam HR CCD mounted on an Axioplan 2 microscope and Axiovision 3.1 software (Carl Zeiss, Toronto, ON), with final magnifications of 400×. The number of positively stained (human) cells in the lung was counted within three randomly-selected microscopic fields per specimen. The proliferation and apoptotic index were calculated as previously described [24].

Vector Construction

A luciferase reporter gene vector was constructed in which an estrogen response element (ERE) was inserted upstream of the luciferase gene. While several variations of ERE binding motifs are known to exist in the human genome [30], a consensus ERE sequence, EREc38, was selected as it was recently reported to induce high transcriptional activity upon human ERβ binding [30]. The following two oligonucleotides complementary to the consensus sequence (underlined) were obtained from Invitrogen (Burlington, ON):

ERE-F: 5′-CCAGGTCAGAGTGACCTGAGCTAAAA-TAACACATTCAG-3′
ERE-R: 5′-GGTCCAGTCTCACTGGACTCGATTT-TATTGTGTAAGTC-3′

Briefly, the ERE-encoding oligos were suspended in water to 10 μM and equal amounts of each were mixed. Next, the oligonucleotide mixture was placed in a boiling water bath for 5 min then allowed to cool to room temperature. Subsequently, the hybridized oligos were cleaved by the restriction enzymes, SacI and XhoI (Invitrogen), followed by purification using a PCR purification kit (Qiagen, Toronto, ON). The insert was ligated into the pGL4 Basic luciferase vector (Promega, Madison, WI), digested using the same enzymes and purified in the same manner as used for the insert.

The derived pGL4-ERE vector was amplified in DH5α *Eschirichia coli* and purified using a published mini-prep protocol [31]. The isolated pGL4-ERE plasmids were sequenced at the McGill Sequencing Centre to verify their fidelity, prior to final amplification/purification using the Invitrogen maxi-prep kit (Supplementary Fig. 1).

Luciferase Assay

To investigate whether genistein has estrogenic transcriptional activity, primary cultured cells from the tumor line were transiently transfected with a reporter construct containing an ERE in a promoter region upstream of a luciferase gene. The pGL4-ERE-Luciferase reporter was used for the assay.

Cells were seeded in 24-well plates for 24 hr prior to transfection in phenol red-free RPMI 1640 media supplemented with 5% charcoal-stripped FBS (Hyclone/Thermo Scientific, Waltham, MA). After a 24-hr-transfection with lipofectamine (Invitrogen), cells were incubated with genistein (50 μM), ICI 182,780 (200 nM), 17β-estradiol (100nM) or vehicle for an additional 24 hr. The luciferase activity was measured using a Steady Glo Luciferase assay kit (Promega, Madison, WI) with a luminometer (Montreal Biotech, Kirkland, PQ). The bicinchoninic acid (BCA) quantification kit (Pierce/Thermo Scientific, Waltham, MA) was used to measure protein concentrations for normalizing the luciferase activity. The experiments are performed in duplicates and repeated three times.

siRNA Knockdown of ERβ

To assess the efficacy of the siRNAs, PC3 cells were plated in 24-well plates in RPMI-1640, supplemented with 10% FBS and 1% antibiotics. Twenty-four hours after seeding, the siRNA was applied to the cultures by changing the growth media to the Accell Delivery media provided by Accell (Thermo/Scientific, Waltham, MA) containing 1 μM siRNA. The cultures were incubated at 37°C with 5% CO_2 for 72 hr, followed by harvesting and transcript expression analysis by qRT-PCR. The four sequences used for *ERβ* silencing target different regions of exon 2 and exon 7, ensuring knockdown of all isoforms and are listed in Supplementary Table I.

RT-PCR

The RT-PCR reaction was carried out using 10 μM gene-specific primer pairs and Platinum Taq DNA Polymerase (Invitrogen) over 35 cycles for *ERβ* and 25 cycles for *GAPDH*.

The following primer sequences were used:

ERβ-F: 5′- CTGTTACTGGTCCAGGTTCAA
ERβ-R: 5′- TCGATTGTACACTGATTTGTAGC
GAPDH-F: 5′CACCAGGGCTGCTTTTAACTC
GAPDH-R: 5′GACAAGCTTCCCGTTCTCAG

Agilent Whole-Genome Gene Expression Microarray and Analysis

Gene expression microarray analysis was performed using total RNA extracted from three snap-frozen LTL313h tumors that were untreated (control) or treated with either 10mg-genistein or ICI. Briefly, 1μg of total RNA per sample was submitted to the Vancouver Prostate Centre, Microarray Core Facility for gene expression microarray analysis. The quality of RNA was analyzed using an Agilent 2100 Bioanalyzer, and samples with a RIN value of greater than or equal to 8.0 were used for analysis (Agilent, Mississauga, ON). Total RNA was converted into cDNA, which was used to generate cyanine-3-labeled cRNA in accordance with the Agilent protocol (Agilent, Mississauga, ON). After quantification, 1.65μg of fluorescently labelled cRNA was hybridized to an Agilent Human GE 4 × 44K v2 Microarray, which targets 34,127 human genes. After hybridization of the microarray at 65°C for 17 hr, the slides were washed twice in Agilent Wash Buffer and acetonitrile and scanned using an Agilent DNA Microarray Scanner (Agilent, Mississauga, ON). The data was processed using the Agilent Feature Extraction 10.5.1 and analyzed in Agilent GeneSpring 7.3.1 (Agilent, Mississauga, ON). To normalize expression data, each measurement was divided by the median expression of all genes on the array, provided that the data followed a normal distribution. Functional and Canonical Pathway Analysis was carried out using Ingenuity Pathway Analysis Software (IPA 8.7). The array data was submitted to GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43146).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

One microgram of total RNA, which was extracted from siRNA-treated cells and LTL 313h treated-tumors was converted to cDNA using a reverse transcriptase by mixing the RNA, the enzyme, reverse transcription (RT) primer mix (Qiagen) and RT buffer. The mixture was then incubated for 15 min at 42°C and 95°C for 3 min. The cDNA was diluted 20-fold for PCR amplification. Quantitation of target gene expression was performed using a 7900HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). The reaction was carried out in 25 μl volume containing cDNA, 10 μM gene-specific primer pair and Platinum SYBR Green qPCR Master Mix (Invitrogen). At least three replicates of each sample per plate were used for the amplification reaction at the cycling parameters of 50°C for 2 min; 95°C for 10 min; 40×[95°C for 15 sec; 60°C for 1 min]. The averaged data were normalized to a housekeeping gene, *hprt*, which is stably expressed across PCa cell lines and the patient-derived tumor tissue lines (data not shown). Gene expression was quantified using the comparative C_T and standard curve methods [32] and presented as average fold-change of triplicates ± SE. Due to the possibility of mouse cell contamination in the xenografts, all primers were designed to be human-specific and to span adjacent exons. The primer sequences are listed in Supplementary Table II.

Statistical Analysis

Quantitative real time PCR data were evaluated using unpaired *t*-test for statistical analysis. For microarray data analysis, Fisher's Exact Test was performed. Differences were considered statistically significant if *P*-values were smaller than 0.05.

Results

Genistein Stimulates and ICI Inhibits Metastasis

In order to confirm and extend our previous study, we studied the effects of genistein and anti-estrogen (ICI) on tumor growth in mice bearing xenografts derived from a different patient tumor. As shown in Figure 1A, the high-dose genistein group had larger tumors (242 mm^3) than the control and ICI- treated groups (157 and 167 mm^3, respectively). We next examined paraffin-embedded sections of organs harvested from the treated mice and immunostained with a human-specific mitochondrial antibody to detect metastatic spread in the mice. Human cancer cells were present in the lungs of all animals regardless of treatment (Figure 1B and C). However, genistein-treated mice had a higher number of invading cells (low-dose: 21 ± 6.9 and high-dose: 33 ± 7.2 cells) compared to control (13 ± 2.6 invading cells) or ICI-treated mice (3 ± 1.4 cells). The invasive-promoting effect of genistein was dose-dependent in our model,

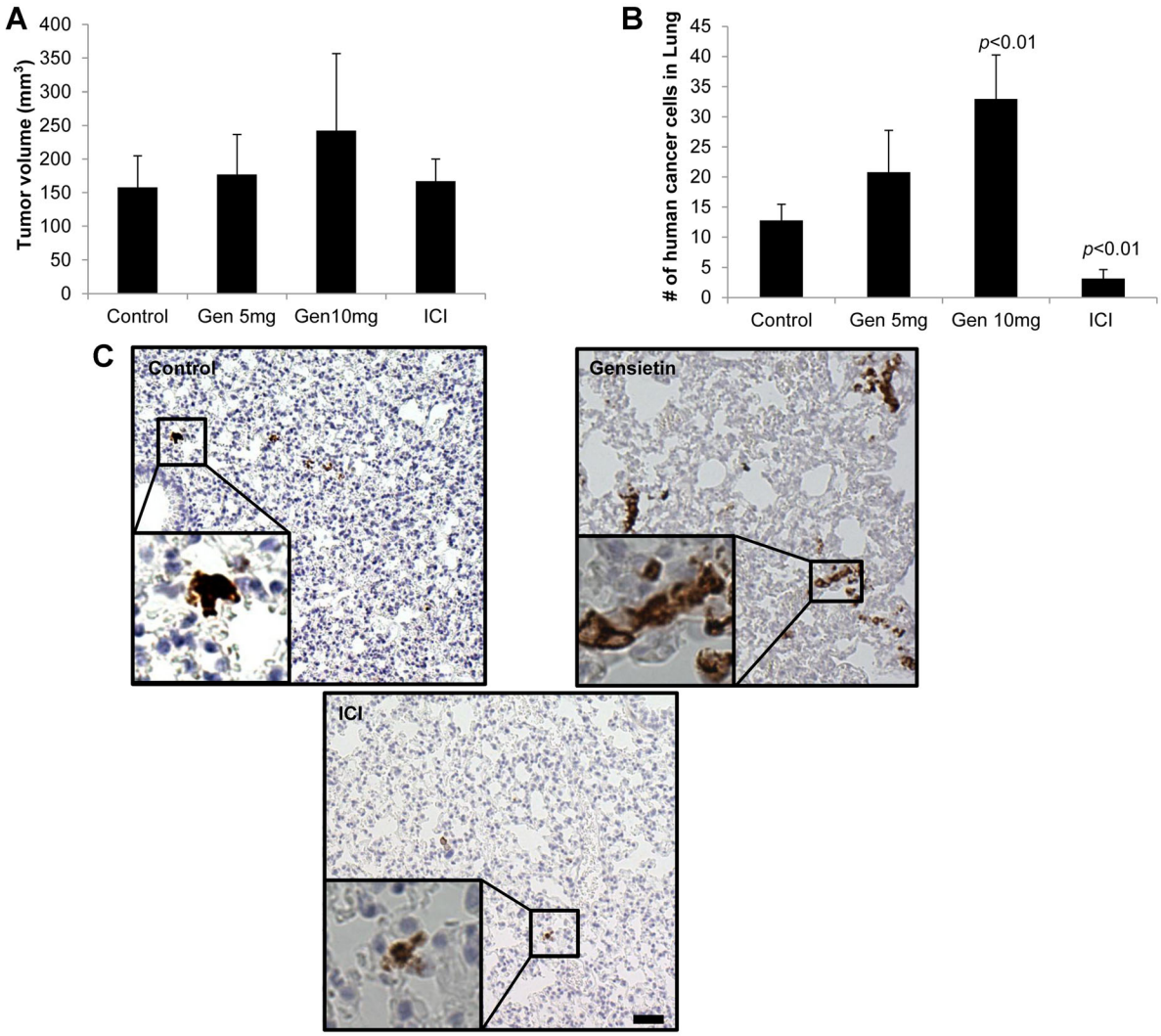

Fig. 1. In vivo effects of genistein and anti-estrogen (ICI 182, 780) in theLTL313h-human PCa xenograft model. Equal sized xenografts were surgically implanted under the renal capsule of NOD-SCID mice, and genistein dissolved in peanut oil was given by gavage to mice in low (5 mg/day) and high-dose (10 mg/day) groups. Mice in the control and ICI groups received 0.1 ml of vehicle only. A single subcutaneous injection of ICI 182,780 (5 mg/week) was given to mice in the anti-estrogen group. Tumor volume and metastatic incidences were measured after 4 weeks of treatment. **A**: Tumor volume of LTL313h xenografts at harvest. After 4 weeks of treatment, tumor grafts were surgically removed from the renal graft sites. For each animal, height, width, and length of the tumors were measured using calipers. Columns: mean tumor volumes (mm^3) $\pm$ SD. n = 5/group for LTL313h. Unpaired *t*-test was used for statistical analysis. **B**: Lung metastatic incidence after treatment of LTL313h tumor-bearing mice with genistein and anti-estrogen (ICI 182, 780). The number of positively stained cells was counted within randomly-selected microscopic fields. Columns: mean number of invading cancer cells/microscopic field observed in the lungs of all animals in each group $\pm$ SD. Results were statistically analyzed by unpaired *t*-test at the 95% confidence level. **C**: Immunohistochemical analysis using an antibody specific to human-mitochondria: representative sections of the lungs from untreated control, genistein (high-dose) and ICI-treated mice. Scale bar: 50 μm.

although only the high-dose genistein treatment was statistically significant ($P < 0.01$) compared to the control. The mice treated with the combination of genistein (5 mg/day) and ICI (5 mg/week) did not show a significant difference in the number of invading cells when compared to the mice treated with the same dose-genistein, thus omitted from our data. Interestingly, ICI-treated animals had significantly lower numbers of metastatic cells when compared to the control ($P < 0.01$) or genistein-treated ($P < 0.01$) groups, which indicates that anti-estrogen treatment effectively inhibited metastatic spread.

Genistein Stimulates Metallothionein (*MT*) Gene Expression

Since the two tumor lines used in this and the previous studies, LTL163 and LTL313h, predominantly

express ERβ, and not ERα (Fig. 2), we hypothesized that ERβ plays an important role in the metastatic progression of PCa [24]. Here, we showed that ICI effectively inhibited expression of ERβ in tumors treated with this compound (Fig. 3). To elucidate which estrogen-linked genes were responsible for such opposing effects of ERβ agonist and antagonist, a genome wide expression array analysis of genistein- and ICI-treated tumors was performed.

For the gene expression analysis, we hypothesized that if activation of ERβ by genistein promotes cancer metastasis and inactivation of ERβ by ICI leads to inhibition of metastasis, genes that have a critical role in PCa progression may be identified as those reciprocally regulated by genistein and ICI.

Figure 4A shows a cross comparison of the genistein-up-regulated and ICI-down-regulated gene populations. Ingenuity Pathway Analysis revealed six common genes in the two populations, five of which belong to the *MT* gene family: *MT1B*, *1E*, *1H*, *1X*, and *2A* (Table I). qRT-PCR was carried out using the RNA extracted from treated tumors to validate altered expression levels of the *MT* genes identified by the microarray analysis. As shown in Figure 4B, all five *MT* genes were up-regulated by genistein relative to the controls, although only the up-regulation of the *MT2A* and *MT1X* genes were statistically significant. There was a slight reduction in expression of the *MT2A*, *MT1B*, *MT1E*, and *MT1X* genes in the ICI-treated xenografts compared to the controls; however, the differences were not statistically significant.

MT Gene Expression in the Metastatic (LTL313h) and Non-Metastatic (LTL313b) Tumor Lines

Because we found that genistein-enhanced metastasis observed in our study was associated with increased *MT* gene expression, it appeared worthwhile to investigate if *MT* gene expression was higher in the metastatic tumor line than in the non-metastatic line, derived from the same PCa patient. Our metastatic tumor line was developed by passaging metastatic cells that have invaded into lymph nodes onto the kidney of NOD-SCID mice, while slow-growing, non-invasive tumors were passaged as non-metastatic tumor line in these mice. Figure 5 shows higher expression of the *MT2A*, *MT1E*, and *MT1X* genes in the metastatic tumors compared to the non-metastatic tumors, suggesting that *MT* genes may be linked to metastatic progression of PCa.

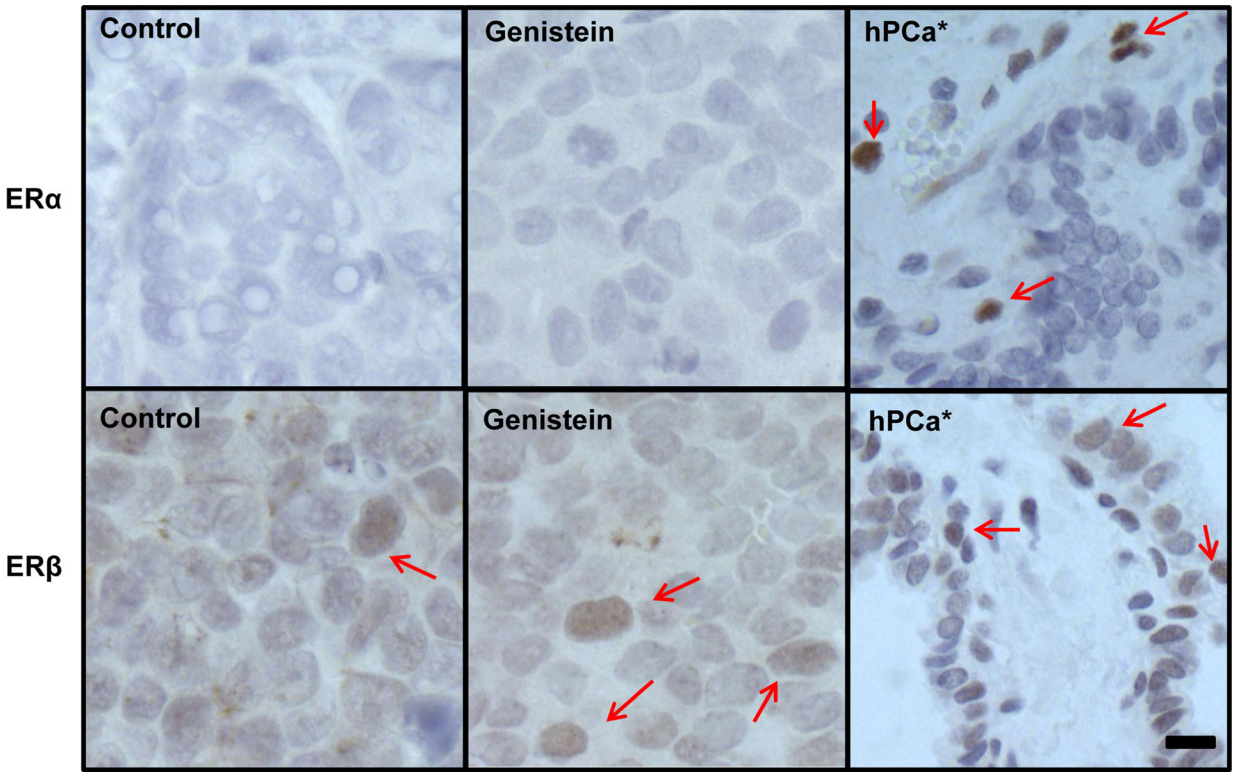

Fig. 2. ERα and ERβ-IHC staining of representative sections of LTL313h xenograft tumors from untreated-control (left panel) and genistein-treated mice (middle panel). Right panels: human prostate cancer tissues as positive controls. All magnifications are 400×. Scale bar: 10 μm.

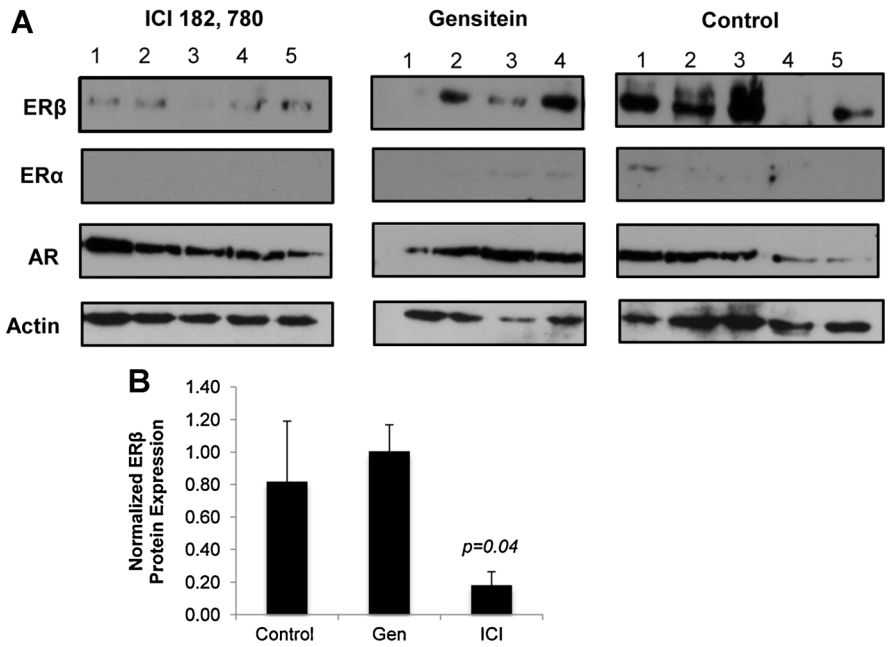

Fig. 3. Western blot analysis of ERα and β expression in LTL313h tumors of untreated, genistein- or ICI 182,780-treated mice. **A**: Expression of ERβ, as exhibited by control and genistein-treated mice, was significantly inhibited by ICI 182,780. Each lane represents protein lysate harvested from tumors of an individual mouse. (One animal in the genistein-treated group died before the end point). **B**: Densitometric quantification of ERβ. Columns: mean ratio of ERβ/actin band intensity ± SD.

ERβ Knockdown Effects on *MT* Gene Expression

The microarray and qRT-PCR studies showed that expression of the *MT* gene family is modulated by genistein and ICI. To determine if *MT* gene expression is regulated via ERβ, siRNA knockdown of ERβ was performed in PC3 cell lines, in which higher expression of *ER*β and *MT* genes is observed compared to other PCa cell lines (data not shown). We were not able to use our tumor lines for this experiment as they are viable only for a few days and do not proliferate in a culture condition.

There are five isoforms of ERβ identified: ERβ-1, ERβ-2, ERβ-3, ERβ-4, and ERβ-5, all of which are encoded by *ESR2* gene on chromosome 14 [33,34]. The full length form, ERβ-1, is translated from eight exons and composed of 530 amino acids. All isoforms share identical sequences from exon 1 to 7 but differ in exon 8, resulting in different amino acid sequences in the ligand binding domain at the C-terminus [34,35]. The Accell delivery system (Thermo/Scientific, Waltham, MA) used in our study contains four siRNA sequences that target different regions of the gene (three siRNA sequences targeting different regions of exon 2 and one targeting exon 7). Since all isoforms contain exon 2 and 7, the combination of four siRNA sequences was mostly likely to silence all isoforms.

After 96 hr of incubation with siRNA, the transcript level of ERβ was markedly reduced (70% reduction in expression) in contrast to vehicle or non-target siRNA controls (Fig. 6A). Expression analysis by qRT-PCR indicated decreased expression of *MT2A*, *MT1E*, *MT1H*, and *MT1X* genes in si-ERβ-treated cells compared to vehicle- or non-target siRNA cultures (Fig. 6B). These results support ERβ-regulation of *MT* genes.

DISCUSSION

Since the introduction of androgen ablation therapy by Huggins and Hodges in the 1940s, hormone manipulation by surgical or chemical castration has been used as the main therapy for advanced PCa and has been proven effective in causing initial tumor regression [36]. Unfortunately, however, the majority of patients will eventually reach castration resistance and succumb to metastasis [37]. Originally, xenoestrogen or diethylstilbestrol (DES) was used as a chemical castration agent to treat patients [38]. Although DES was effective in controlling PCa growth by reducing testosterone production [39], its clinical usage was discontinued due to severe adverse cardiac effects. Moreover, its potential stimulatory effects on prostate cell growth added to the controversy in its use for PCa therapy [3,40–43].

The function of estrogen in the growth and development of normal and malignant prostate cells appears to vary depending on the receptor type that it interacts

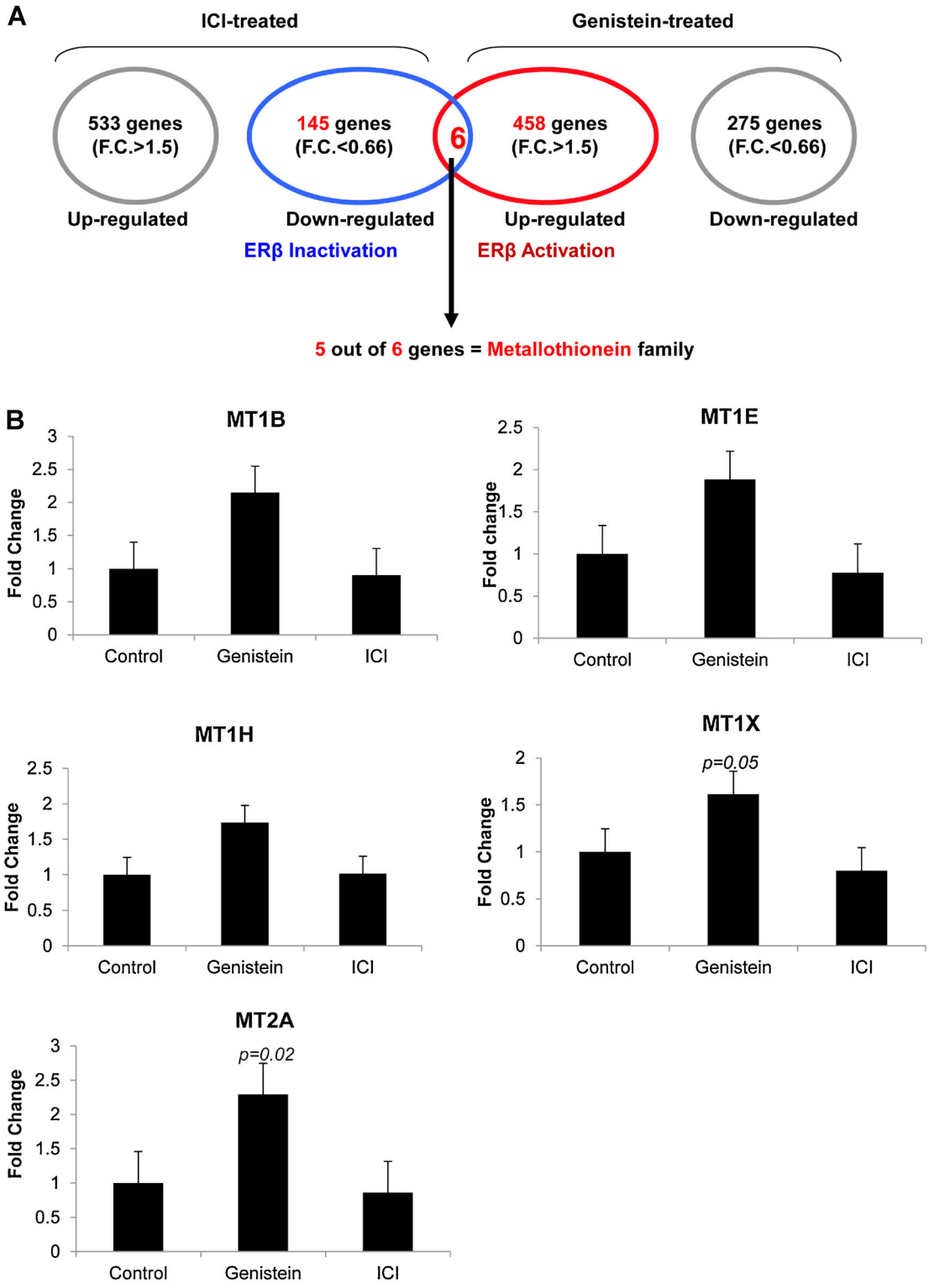

Fig. 4. Cross comparison of the genistein-up-regulated genes versus. ICI-down-regulated genes. **A**: Six commonly shared genes were identified in these populations by Ingenuity Pathway Analysis which are listed in Table I. Genes which exhibited at least 1.5-fold increase or 0.66-fold decrease were categorized as up-regulated and down-regulated genes, respectively. **B**: Expression of metallothionein genes (*MT1B*, *1E*, *1H*, *1X*, and *2A*) in genistein- and ICI 182,780-treated PCa LTL313h xenografts as determined by qRT-PCR. Gene expression is presented as fold change relative to control. Values are normalized to *hprt* levels. Genistein increased expression of all *MT* genes tested. There was only a slight reduction in *MT* expression by ICI treatment. Columns: mean fold change of triplicates ± SE, and the experiment was repeated twice.

TABLE I. List of Genes Shared by Genistein-Up-Regulated and ICI-Down-Regulated Gene Populations. Five of the six commonly shared genes belong to the metallothionein gene family: *MT2A*, *MTIE*, *MTIB*, *MTIH*, and *MTIX*.

Gene name	Description
Metallothionein 2A	Homo sapiens metallothionein 2A (MT2A), mRNA
Metallothionein 1E	Homo sapiens metallothionein 1E (MT1E), mRNA
Metallothionein 1B	Homo sapiens metallothionein 1B (MT1B), mRNA
Metallothionein 1H	Homo sapiens metallothionein 1H (MT1H), mRNA
Metallothionein 1X	Homo sapiens metallothionein 1X (MT1X), mRNA
	AGENCOURT_6543460 NIH_MGC_71 Homo sapiens cDNA clone IMAGE:5549618 5′, mRNA sequence [BM552308]

with. Early studies suggested opposing effects of estrogen elicited in prostatic cells via ERα and ERβ; for example, a proliferative function for ERα versus a protective (anti-proliferative) role for ERβ [15,16]. Due to lack of appropriate models, especially for ERβ, the exact role that estrogen plays in PCa development and progression remains unclear.

In efforts to elucidate the effects of estrogen in PCa, we showed that an ERβ-agonist, genistein, promoted metastasis and an antagonist, ICI, inhibited cancer spread. In this present study, we sought to determine if genomic actions of ERβ modulated by these compounds induced alterations that might contribute to their effects on PCa metastasis.

Our luciferase reporter assay with an ERE vector showed increased activities in the genistein-treated cells of our tumor lines compared to the vehicle control, indicating the ability of genistein to bind to and activate ERβ, which is predominantly expressed in the tumor lines (Supplementary Fig. 2). With the addition of the anti-estrogen, ICI, the genistein-induced luciferase activity was inhibited.

It has been suggested that ER can form heterodimers in cells that co-express two isotypes of the receptor, which can bind to ERE and influence transcriptional activity [44,45]. Transfection experiments performed by Gougelet et al. [46] showed that ERβ inhibited ERα-dependent transcription and that AF-1 domain (the ligand-independent activation domain) of ERβ is necessary for the dominant negative function of the heterodimer, while ERα transfection in breast

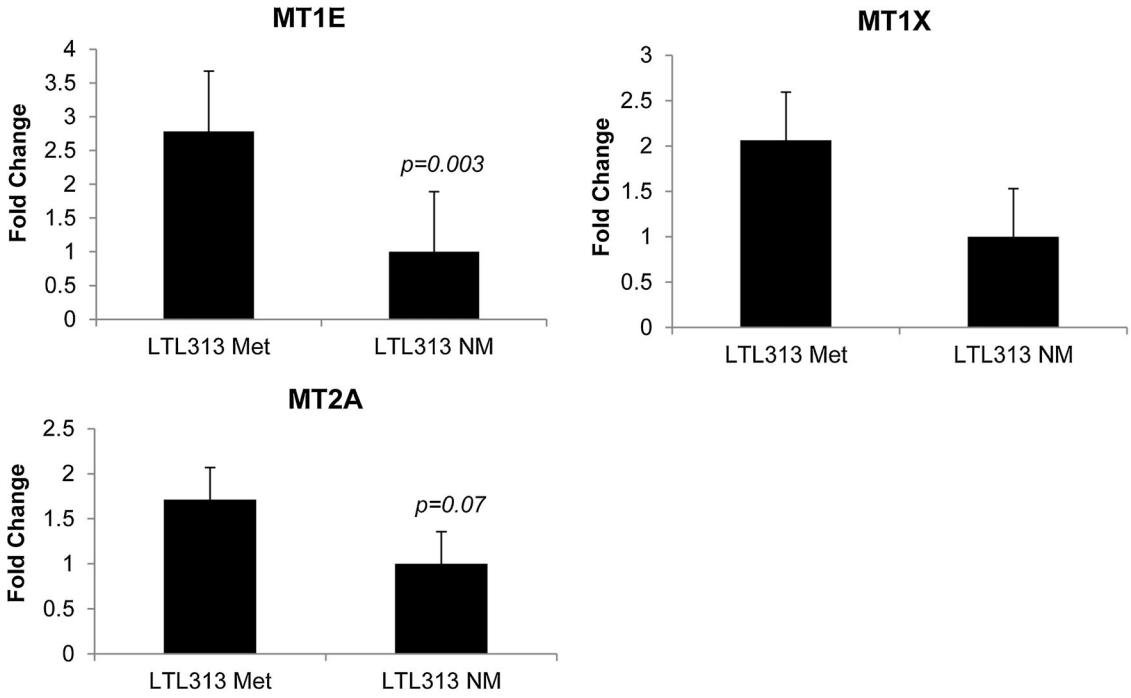

Fig. 5. Metastatic (LTL313h Met) tumor line exhibited higher *MT* gene expression compared to non-metastatic (AB313b NM) tumor line. Gene expression is presented as fold change. Values are normalized to *hprt* levels. Columns: mean fold change of duplicates ± SE, and the experiment was repeated twice.

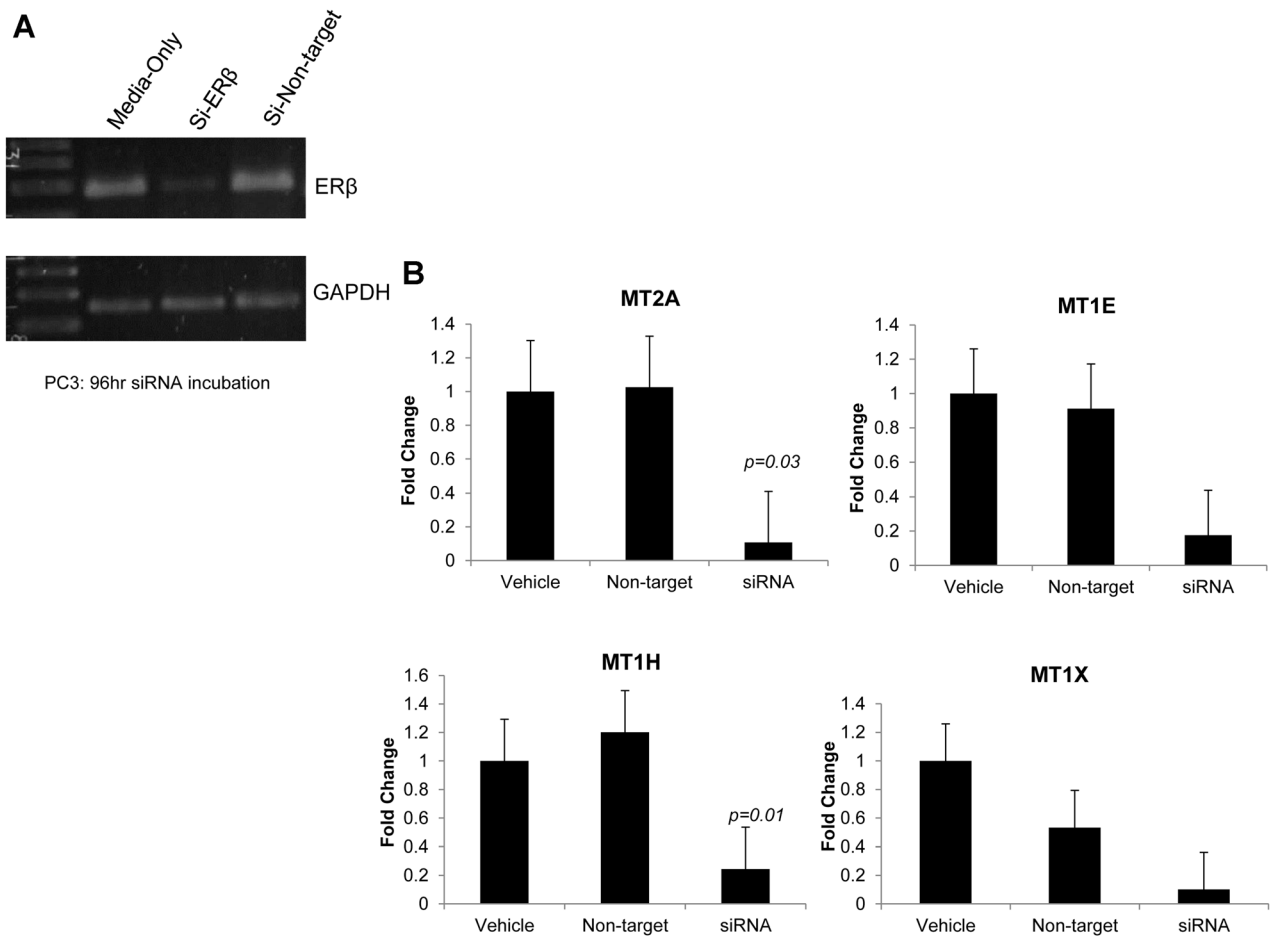

Fig. 6. ERβ regulation of *MT* genes. **A**: siRNA knockdown of ER© in PC3 cells after 96 hr. RT-PCR results show reduced transcriptional expression of ERβ in siRNA-treated PC3 cells compared to non-target siRNA and vehicle controls. **B**: *MT* gene expression following siRNA knockdown of ER© in PC3 cells. Ninenty-six hours after incubation with siRNA, the siRNA-treated cells showed reduced expression of *MT* genes (*MT2A*, *MTIE*, and *MTIH*) in contrast to vehicle-or non-target siRNA controls. Gene expression is represented as fold change. Values are normalized to *hprt* levels. Columns: mean fold change of triplicates ± SE, and the experiment was repeated twice.

cancer cell lines had only minor effects on ERβ-transcriptional capacity. The exclusive expression of ERβ in our tumor lines, however, suggests that the effects observed in our study are highly likely the result of β/β, and not α/β heterodimerization. Chang et al. [47] demonstrated in their CHIP assay that in response to low and high concentrations of genistein, co-expression of ER-α was not necessary for genistein-induced recruitment of ERβ to ERE in MCF7 cells. Also, predominant expression of ERβ in clinical PCa cells (ERα is expressed in stroma: Figure 2) and enhanced expression of ERβ in most metastatic cases [22,23] suggest that in vivo and clinical effects are mainly modulated by β/β dimers.

To delineate which estrogen-linked genes were responsible for the metastasis-stimulatory effects observed in our studies, a genome wide expression array was performed using genistein- and ICI -treated tumors. If activation of ERβ by genistein promoted cancer metastasis and inactivation of ERβ by ICI led to inhibition of metastasis, the genes that were reciprocally regulated by these two compounds may play important roles in PCa progression. A cross-comparison analysis of the array data revealed six genes that were up-regulated by genistein (F.C. > 1.5) and also were down-regulated by ICI 182,780 (F.C. < 0.66). Of the six genes identified, five of them belonged to the *MT* gene family: *MT1B*, *1E*, *1H*, *1X*, and *2A*. The differences in gene expression levels were validated by qRT-PCR.

Metallothioneins are low-molecular weight, metal-binding proteins, which are known to bind both essential metals (zinc and copper) and toxic metals (cadmium and mercury) in two distinct clusters at the N- and C-terminal domains [48,49]. In humans, there are at least ten functional isoforms of MT (1A, 1B, 1E,

1F, 1G, 1H, 1X, 2A, 3, and 4) expressed in various tissues and organs [50].The major isoforms, MT-1 and MT-2, are known to mainly bind zinc and cadmium [48,50]. Due to this metal-chelating property, it is suggested that they may provide protection against oxidative stress/DNA damage and function as a reservoir for essential metals, which can be donated to transcription factors and metallo-enzymes [50].

During growth and development, MT expression increases as the demand for zinc and copper reaches a peak in the human fetus; however, once reaching adulthood, expression levels decline [51]. In cancer, increased MT expression has been reported in a large number of malignancies, including breast, colon, kidney, prostate, ovary, and lung cancers [50]. It has also been suggested that this gene plays a role in cancer progression. Wu et al. [52] conducted a migration assay and gene expression profiling and identified the *MT1E* gene as one of the key genes associated with invasion in bladder cancer. Similarly, clinical evidence showed that enhanced expression of MT is associated with poor prognosis and recurrence in ductal breast carcinomas and oral squamous carcinomas [53–55]. Yamasaki et al. [56] showed upregulation of *MT1E* and *MT2A* in both PC-3 and LNCap under hypoxia and in clinical tumor specimen of PCa patients who have undergone androgen ablation therapy compared to benign tissues. Their knockdown experiment of *MT2A* in cell lines exhibited reduction in cell growth and enhanced apoptosis.

Consistent with the results reported by others, our study showed enhanced *MT* expression in our PCa metastatic tumor tissue xenograft model compared to non-metastatic tumor, suggesting a significant role for ERβ-regulated *MT* in the metastatic progression of PCa. These data may indicate that genistein binds to and activates ERβ, which increases *MT* gene transcription via its genomic action and in turn promotes metastatic progression. It has been shown that ERβ forms a complex with the transcription factor, Sp1, which then binds to a GC-rich Sp1-binding sequence in the *MT* promoter region, allowing for its expression (Fig. 7) [57]. This gene regulatory effect was specific to ERβ, and not observed with ERα. It can be speculated that Sp1 may preferentially bind to ERβ, and not ERα, due to the unique protein- binding pocket of the β-receptor. There is a subtle difference between the amino acid sequences within the binding cavity of the β- and α-receptors [58], which may favor Sp1 binding to ERβ. Our work showed that siRNA knockdown of ERβ reduced *MT* gene expression in PC3 cells. Together with the evidence provided by others, it confirms ERβ regulation of *MT* gene transcription (Fig. 6).

There are many transcription factors and matrix metalloproteinases (MMPs) that require zinc for their catalytic activity or ECM-degrading function that is important in invasion and migration [59]. Zinc and a zinc-provider are, therefore, very important in nucleic acid and protein synthesis, cell growth and invasion processes, which are all hallmarks of cancer. With the proposed potential to store and donate zinc, MT may potentially increase the catalytic activity of metastasis-linked enzymes, driving cancer progression.

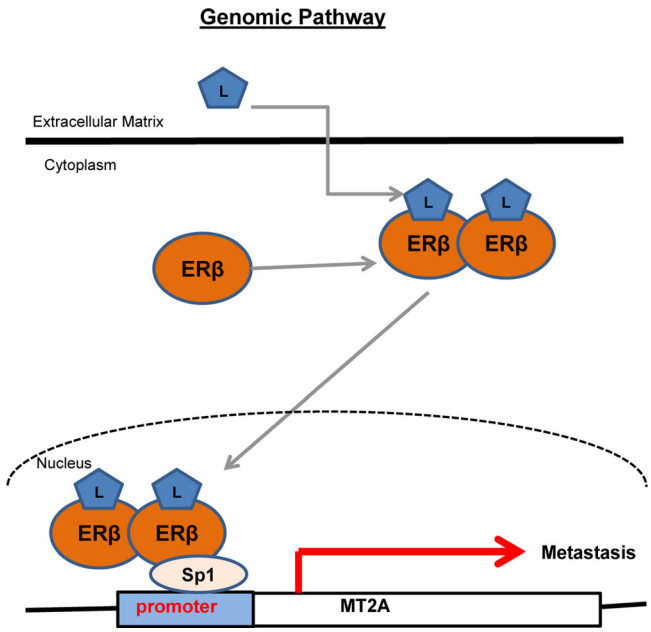

Fig. 7. Model depicting genomic actions of ERβ: induction of *MT* gene. Ligand-activated receptor forms a complex with SpI, which binds to GC-rich SpI-binding sequence of *MT* promoter region, inducing transcription. L: ligand.

CONCLUSION

PCa is known for its heterogeneity, and types and expression levels of nuclear receptors and co-activators vary among patients and within a tumor tissue of a single patient. It is possible that genistein elicits heterogeneous actions through which promotes cancer progression in certain subtypes of cancers while inhibiting other tumors due to differential ERβ expressions among patients. We previously demonstrated that genistein increased metastasis in a patient-derived PCa xenograft model, LTL163. In the subsequent study, we confirmed the metastasis-promoting effect of genistein using a second xenograft model (LTL313h) which was derived from another patient. This provides evidence that this effect is not patient specific, but may be limited to ERβ-positive cancers.

Through genomic actions of ERβ demonstrated in this study and the non-genomic actions on the EGFR [24] and FAK signalling pathway (manuscript in

preparation), this receptor may be responsible for inducing expression of invasion-promoting genes creating favorable in vivo conditions for metastatic progression. Taken together, sub-classification of PCa based on ERβ expression and development of targeted therapy for invasion-promoting genes may help improve the survival of metastatic PCa patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publishers website.

Fig. S1. Map of the constructed ERE luciferase reporter gene vector. Oligos containing an ERE consensus sequence, CCAGGTCAGAGTGACCTGAGCTAAAA-

TAACACATTCAG, were hybridized, cleaved by restriction enzymes, SACI and XhoI, and inserted upstream of the luciferase gene.

Fig. S2 Genistein activates ER transcriptional activity. Primary cultured cells of the tumor line were transfected with pGL4B-ERE luciferase reporter for 24 hr and incubated with genistein and ICI 182,780 for an additional 24 hr. Genistein treatment induced luciferase transcription, while addition of ICI inhibited such activity. *Columns*: mean of fold change in relative light unit (RLU) of duplicates ± SE. The experiment was repeated three times.

Table S1 The sequences used for ERβ siRNAs. ERβ and non-target control siRNAs (Accell) were purchased from Thermo/Scientific.

Table S2 The primer sequences used for real-time QPCR. Due to the possibility of mouse cell contamination in the xenografts, all primers were designed human-specific and to span adjacent exons.