Muscadine grape skin extract can antagonize Snail-cathepsin L-mediated invasion, migration and osteoclastogenesis in prostate and breast cancer cells

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Abstract

To develop new and effective chemopreventive agents against bone metastasis, we assessed the effects of muscadine grape skin extract (MSKE), whose main bioactive component is anthocyanin, on bone turnover, using prostate and breast cancer cell models overexpressing Snail transcription factor. MSKE has been shown previously to promote apoptosis in prostate cancer cells without affecting normal prostate epithelial cells. Snail is overexpressed in prostate and breast cancer, and is associated with increased invasion, migration and bone turnover/osteoclastogenesis. Cathepsin L (CatL) is a cysteine cathepsin protease that is overexpressed in cancer and involved in breast cancer, and is higher in prostate and breast tumor tissue compared to normal tissue. MSKE decreased Snail and pSTAT3 expression, and abrogated Snail-mediated CatL activity, migration and invasion. Additionally, Snail overexpression promoted osteoclastogenesis, which was significantly inhibited by the MSKE as effectively as Z-FY-CHO, a CatL-specific inhibitor, or osteoprotegerin, a receptor activator of nuclear factor kappa B ligand (RANKL) antagonist. Overall, these novel findings suggest that Snail regulation of CatL may occur via STAT-3 signaling and can be antagonized by MSKE, leading to decreased cell invasion, migration and bone turnover. Therefore, inhibition using a natural product such as MSKE could potentially be a promising bioactive compound for bone metastatic cancer.

Introduction

The primary cause of prostate and breast cancer death is metastasis, which is regulated by signaling pathways such as epithelial mesenchymal transition, a dynamic process that promotes cell motility with decreased adhesive ability (1). Snail, a zinc-finger transcription factor, has been found to regulate epithelial mesenchymal transition in part by increasing extracellular matrix degradation via upregulation of matrix metalloproteinase (2). STAT3 signaling has been shown to increase Snail expression through Liv-1 zinc transporter (3). We have shown previously that receptor activator of NFkB ligand (RANKL), a member of the tumor necrosis factor family that is normally expressed on the cell surface of stromal cells and osteoblasts and mediates osteoclast differentiation and osteolysis or bone resorption, can be upregulated by Snail overexpression in ARCaP and LNCaP prostate cancer cells, which was associated with increased osteoclastogenesis in vitro and in vivo (4).
Acidosis of the bone microenvironment results in increased osteoclast resorption pit formation via the release of proteolytic enzymes such as Cathepsins B, D and L which degrade the extracellular matrix and facilitate metastasis (5). Cathepsins are cysteine proteases belonging to the papain family of peptidases and currently 11 cysteine cathepsins have been identified including cathepsins K, L, S and V, which have been implicated in a number of pathological diseases including atherosclerosis (6–9), abdominal aortic aneurysms (9–11), osteoporosis and arthritis (12–14) and colon and breast carcinomas (15,16). Cathepsin L (CatL) is a cysteine cathepsin that is overexpressed in a variety of cancers such as breast, ovary, colon, adrenal, bladder, prostate and thyroid (17), and degrades the extracellular matrix during tumor progression (18). Procathepsin L and processed mature CatL can degrade laminin and fibronectin extracellular matrices (19), while CatL can also degrade collagen in vitro (20). Currently, no drugs that target CatL are in use; however, many are in development.

Studies have suggested that fruit and vegetables can have chemopreventive and therapeutic effects on tumor cells (21). Muscadine grape skin extract (MSKE) is derived from the muscadine grape (Vitis rotundifolia), and has anthocyanin as the main bioactive component, inhibits prostate cancer cell growth and promote apoptosis in vitro without toxicity to normal prostate epithelial cells (22). Although out current study focused on muscadine skin, profiling has been performed to examine the phenolic contents of muscadine seed, skin and pulp (23). In brief, the phytochemical constituents of muscadine grapes differ from most other grape varieties in that they contain a predominance of anthocyanin 3,5-diglucosides, ellagic acid and ellagic acid precursors (23,24). For purple skinned muscadine grapes, the anthocyanins are primarily delphinidin-3,5- diglucoside, cyanidin-3,5-diglucoside and petunidin-3,5-diglucoside (23). Shin et al. (25) have reported that treating human hepatoma cells with anthocyanin 3,5 diglucoside, led to the inhibition of invasion. Anthocyanin 3,5 diglucosides have also been shown to induce apoptosis and inhibit invasion in colorectal cancer cells (26). Currently, MSKE is in Phase II Clinical Trial for treatment of localized prostate cancer (27).

In this study, we show that CatL expression increases with tumor grade in prostate and breast patient tissue. Additionally, Snail overexpression increases CatL activity via STAT3 signaling, associated functionally with increased migration, invasion and osteoclastogenesis, which can be inhibited by MSKE. This is the first study showing that Snail can regulate cathepsins to promote bone turnover in part via CatL, which can be abrogated by MSKE.

### Materials and methods

#### Cell culture, reagents and antibodies

LNCaP prostate and MCF-7 breast human cancer cells were purchased from American Type Culture Collection, Manassas, VA, while ARCaP-epithelial (ARCaP-E) human prostate cancer cells were purchased from Novicure, Birmingham, AL. C4-2 human prostate cancer cells, an aggressive derivative of LNCaP cells were a kind gift from Dr Leland Chung (Cedars Sinai Medical Center, Los Angeles, CA). ARCaP-E and LNCaP cells stably transfected with constitutively active Snai CDNA have been described previously (4,28). The MCF-7 cells stably transfected with empty Neo vector (MCF-7 Neo) or constitutively active Snai (MCF-7 Snai) were kindly provided by Dr Mien-Chie Hung, The University of Texas MD Anderson Cancer Center, Houston TX, and established as described previously (29). C4-2 cells transfused with Snai shRNA for stable Snai knockdown has been described previously (30). All cells were grown in RPMI (Lonza, Alpharetta, GA) supplemented with 10% fetal bovine serum (Atlanta biological, Flowery Branch, GA) and 1x penicillin-streptomycin (Mediatech, Herndon, VA) at 37°C with 5% CO₂ in a humidified incubator. MSKE was kindly provided by Dr Tamaro Hudson, Howard University, Washington, DC, and the preparation of the extract which is composed mainly of anthocyanins has been described previously (22). Anti-mouse α-tubulin antibody and tartrate-resistant acid phosphatase (TRAP) staining kit was from Sigma–Aldrich, St Louis, MO. Rat monoclonal anti-Snai and horseradish peroxidase (HRP)-conjugated goat antirabbit antibodies were from Cell Signaling Technology, Danvers, MA. Goat monoclonal anti-Cat-L antibody, recombinant mouse macrophage colony stimulating factor, Cat-L-specific inhibitor (Z-FPY-CHO), osteoprotegerin (OPG) were purchased from R&D Systems (Minneapolis, MN). The HRP-conjugated donkey antibody and mouse monoclonal anti-STAT-3 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated sheep antimouse and sheep antibovine secondary antibodies were purchased from Amersham Biosciences, Buckinghamshire, UK. Luminata Forte HRP chemiluminescence detection reagent and rabbit monoclonal anti-p-STAT-3 antibody were purchased from EMD Millipore (Billerica, MA). The protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN.

#### Western blot analysis

Cells were lysed in a modified RIPA buffer as described previously (26). Supernatants were collected and quantified using a micro BCA assay (Promega, Madison, WI). Forty micrograms of cell lysate was resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transblotting onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with appropriate secondary and primary antibody, followed by visualization using Luminata Forte ECL reagent. The membranes were stripped using Restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL) prior to reprobing with a different antibody. For treatments, 70% confluent cells were serum-starved in phenol red-free serum-free RPMI containing penicillin/streptomycin for 24h, prior to treatment with MSKE or Z-FPY-CHO in phenol-free serum-free RPMI containing 5% dextran charcoal-stripped serum for 3 days.

#### Zymography

We utilized the cathepsin zymography technique as described previously (31). Briefly, 1 ml of conditioned media containing 0.1 mM leupeptin was concentrated utilizing vivapain tube (GE Health Care, Piscataway, NJ). The concentrated conditioned media was diluted 10-fold in 1X RIPA buffer followed by determination of the protein concentration, electrophoresis using Gelatin substrate (Scholar Chemistry, Rochester, NY) (0.2%), incubation in cathepsin-renaturing buffer (65 mM Tris buffer, pH 7.4 with 20% glycerol) and overnight incubation in pH 6 sodium phosphate assay buffer (0.1M sodium phosphate buffer, 1mM ethylenediaminetetraacetic acid, 2mM dithiothreitol) at 37°C. The gel was stained with Coomassie blue stain (10% acetic acid, 25% isopropanol, 4.5% Coomassie Blue), destained (10% isopropanol and 10% acetic acid) and proteolytic activity visualized as cleared bands. The pH conditions used will show both CatL and CatS activity. Cathepsin activity was subsequently quantified by densitometry using NIH Image f software.

#### Transfection with STAT3 short interfering RNA

About 5×10⁴ ARCaP-Snail or C4-2 cells were plated in six-well plates in RPMI/fetal bovine serum (FBS) and left overnight for attachment. The next day, STAT3 short interfering RNA (siRNA) (Dharmacon, Pittsburgh, PA) transfections were performed according to manufacturer instructions. The STAT3 siRNA are pooled from four On-Target plus SMARTpool siRNA with the following identities and target sequences;

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CatL</td>
<td>cathepsin L</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
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<tr>
<td>MSKE</td>
<td>muscadine grape skin extract</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of NFκB ligand</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
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J-003544-07, target sequence: GAGAUUGACCAGUGUAUA, J-003544-08, target sequence: CCAACAAUCCAAGAAUGU, J-003544-09, target sequence: CGAAAGGUCCGAAUCAA, J-003544-10, target sequence: CAACAGAUUGCCUGCAUUG. Briefly, the cells were washed with sterile phosphate-buffered saline (1× PBS) followed by addition of 200 nM control or STAT-3 siRNA in serum-free RPMI. The cells were then incubated at 37°C, 5% CO₂ for 5 h after which the media was replaced with 2 ml of 5% dextran charcoal-stripped serum followed by incubation for 72 h. Cell lysates were then harvested and western blot analysis performed to probe for STAT-3 and p-STAT, while conditioned media was collected for zymography to determine the CatL activity.

Ethical statement related to the use of human breast tumor samples
Breast tumors with matched normal tissues were obtained from Protein biotechnologies, Ramona, CA. Protein Biotechnologies provides pharmaceutical, biotechnology, government and academic institutions with human clinical specimen derivatives. Tissues are obtained through a global network of participating medical centers that employ IRB approved protocols and strict ethical guidelines to ensure patient confidentiality and safety. Identical procedures are used to prepare all patient samples. Specimens are flash frozen to −120°C within 5 min of removal to minimize autolysis, oxidation and protein degradation. Tissue specimens are homogenized in modified RIPA buffer (PBS, pH 7.4, 1 mM ethylenediaminetetraacetic acid and protease inhibitors) to obtain the soluble proteins, and centrifuged to clarify. The samples were subsequently used for western blot analysis.

Ethics statement for use of animals
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animal procedures were approved and performed in accordance with Morehouse School of Medicine IACUC guidelines.

Animal experiments
Four-week-old male athymic nu/nu mice (National Cancer Institute) were injected subcutaneously with 2 × 10⁶ cells per mouse of Neo or Snail-overexpressing LNCaP cells mixed 1:1 volume with matrigel (BD Biosciences). There were six mice in each group. The mice were killed after 5 weeks by carbon dioxide overdose followed by cervical dislocation, the tumors excised and tumor volume measured with a caliper (tumor

Figure 1. CatL expression increases with tumor progression. (A) Immunohistochemical (IHC) analysis was performed using a 96 core prostate adenocarcinoma tissue microarray. Representative images of CatL in various stages of prostate cancer show that CatL increases with tumor progression. Bar represents 50 µM. (B) Normal/tumor matched invasive ductal carcinoma (IDC) grades 1 and 3, infiltrating carcinoma (IFC) grade 3, adenocarcinoma (grade 3) and metaplastic carcinoma (MPC) grade 3 were analyzed for expression of CatL by western blot analysis. Mature CatL expression was generally higher in tumor as compared to normal tissue. Alpha (α)-tubulin was used as a loading control. Data are representative of at least three independent experiments.
volume was calculated as $\pi/4 \times \text{largest diameter} \times \text{smallest diameter}^2$. The tumors were used for immunofluorescence.

**Immunofluorescence**

The tumor xenograft sections were deparaffinized with xylene, dehydrated with alcohol series from 100% ethanol to 50% ethanol, antigens were retrieved at 125°C for 30 s, peroxidases were blocked using 0.03% hydrogen peroxide and blocked using goat or rabbit sera. Primary antibodies (Snail anti-rabbit or CatL antigoat) were added to the slide and incubated overnight at 4°C. After washing with 1× tris-buffered Saline-T ween, secondary antibodies anti-rabbit Oregon green 488 (Invitrogen, Carlsbad, CA) and antigoat Texas red (Vector Laboratories, Burlingame, CA) were added and incubated for 30 min in darkness. The slide was washed with 1× tris-buffered Saline-T ween, counterstained with 4',6-diamidino-2-phenylindole to detect nuclei, washed briefly with double deionized water and mounted using Fluorogel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Fluorescence microscopy was performed using Zeiss (Axiovision Rel 4.8) and Apotome software.

**Immunohistochemistry**

Examination of the expression and distribution of CatL in human prostate cancer was performed by immunohistochemistry (IHC) using prostate normal and cancer tissue microarray slides obtained from US Biomax (PR956a, Rockville, MD). IHC was performed using the Avidin–biotin immunohistochemical method. The microarray was deparaffinised in xylene and rehydrated using alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After antigen retrieval, sections were incubated with 10% serum to avoid the non-specific binding. Sections were incubated with 1:200 primary antibody against CatL at 4°C overnight followed by biotinylated secondary antibody, and incubation with avidin-biotin complex (Vector, Burlingame, CA). Immunoreactivity was visualized using diaminobenzidine (Sigma–Aldrich, St. Louis, MO). The slide was subsequently counterstained with hematoxylin and mounted with xylene solution. Images were acquired using the Axiovision Rel 4.8.

**In vitro cell migration assay**

We utilized Costar 24-well plates containing a polycarbonate filter insert with an 8-μm pore size, to coat with 4.46 μg/μl rat tail collagen I on the outside for 24h at 4°C. 5 × 10^4 cells were plated in the upper chamber containing RPMI supplemented with 0.1% FBS while the lower chamber contained RPMI supplemented with 10% FBS. After 5h, cells that migrated to the bottom of the insert were fixed, stained with 0.05% crystal violet and counted to obtain the relative cell migration.

**In vitro cell invasion assay**

The invasive properties of the cell lines were measured using the BD BioCoat Matrigel Invasion guidelines. Briefly, Boyden chamber inserts
L.J. Burton et al. | 1023

(Thermo Fisher Scientific, Waltham, MA) were coated with 40 μl of 1:4 matrigel and allowed to solidify at 37°C for 1 h. 5 × 10^4 cells were seeded in triplicate in 0.1% FBS, while the lower chamber contained 10% FBS. Cells were allowed to invade through the porous membrane coated with matrigel at 37°C for 24–72 h. Inserts were fixed, stained with 0.05% crystal violet. Cell counts were performed for the determination of relative cell invasion.

**In vitro osteoclastogenesis assay**

3 × 10^5 ARCaP-Neo/ MCF-7 Neo or ARCaP-Snail/ MCF-7 Snail was cocultured with 40 × 10^4 spleen macrophages in 48-well plates plus 1 ng/ml macrophage-colony stimulating factor plus or minus 5 μg/ml MSKE, 20 μg/ml MSKE, 5 μM Z-FY-CHO CatL inhibitor, or 50 ng/ml OPG. We included treatment with OPG as a control since it is known that OPG blocks the binding of RANKL to RANK receptor and would therefore inhibit osteoclastogenesis. The cells were fed every 3 days by replacing half the media with fresh media plus or minus MSKE, Z-FY-CHO or OPG. Macrophages alone were utilized as a negative control. After 7–14 days, the cells were fixed with 3% formaldehyde and processed for TRAP staining according to manufacturer instructions, to visualize the formation of mature osteoclasts.

**Statistical analysis**

Data were presented as the mean ± SEM from three independent experiments. Data analysis for statistical significance was done using Student’s paired t-test or ANOVA using GraphPad Prism Software. A P value of < 0.05 was considered significant.

**Results**

**CatL is increased in patient prostate and breast tumor tissue**

To confirm previous findings that CatL is increased in patient prostate and breast tissue (17, 32), we stained for CatL by IHC using prostate tumor tissue microarray and analyzed CatL expression by western blot using patient breast tissue. Normal prostate epithelial tissue expressed low levels of CatL in the cytoplasm, while higher levels were detected within prostate adenocarcinoma which increased with tumor grade (Figure 1A). Moreover, CatL expression was predominantly cytoplasmic in stages II and III whereas it was both nuclear and cytoplasmic in stage IV and exclusively nuclear in bone metastatic tissue (Figure 1A). However, CatL staining was low in cancer cells that metastasized to the abdominal wall (Figure 1A).

Using normal/tumor-matched breast cancer lysates (see Supplementary Figure 1 and Supplementary Tables 1 and 2, available at Carcinogenesis Online, for patient tissue H&E staining and patient data), we observed that tumor lysate expressed higher levels of mature CatL as compared to normal tissue (Figure 1B). This shows that CatL expression increases with tumor progression in prostate and breast cancer.

**Snail is correlated with increased CatL and CatS activity in prostate and breast cancer cell lines**

Since Snail and CatL are both involved in bone turnover (4, 33, 34), we speculated that Snail may regulate CatL expression/activity. We therefore examined the expression and activity of CatL by western blot analysis and zymography, respectively. Western blot analysis showed that immature (pre-pro and pro) as well as mature CatL expression were higher in ARCaP (prostate), LNCaP (prostate) and MCF-7 (breast) Snail-overexpressing cells compared to the Neo controls but not significantly altered in C4-2 non-silencing control (C4-2 NS) versus C4-2 Snail shRNA with stable Snail knockdown (Figure 2A). However, C4-2 Snail shRNA cells with Snail knockdown displayed decreased CatL and CatS activity compared to C4-2 NS as shown by zymography, while MCF-7, LNCaP and ARCaP cells overexpressing Snail displayed
increased amounts of active CatL and CatS (Figure 2B). To determine the effects of Snail overexpression in vivo, we injected LNCaP-Neo and LNCaP-Snail subcutaneously into male athymic nu/nu mice. Significantly larger tumor volumes were observed in LNCaP Snail tumor xenografts as compared to LNCaP-Neo after 5 weeks (Figure 2C), while LNCaP-Snail tumor xenograft immunofluorescent staining demonstrated higher expression of Snail and CatL as compared to LNCaP-Neo xenograft tissue (Figure 2D). Therefore, Snail is positively correlated with CatL expression and activity.

**STAT3 regulates CatL activity in prostate cancer cells**

Next, we wanted to examine the signaling pathway by which Snail may regulate CatL activity. Since STAT-3 signaling pathway has been shown to regulate Snail via Liv-1 (3) and also regulate CatL activity (35), we tested the hypothesis that the STAT-3 pathway was involved. STAT-3 knockdown with siRNA in C4-2 and ARCaP-Snail cells decreased Snail and mature CatL expression (Figure 3A) and amounts of active CatL (Figure 3B and C). This shows that JAK/STAT pathway may be involved in Snail activation of CatL.

**MSKE antagonizes Snail signaling**

Next, we examined whether Snail/CatL signaling could be antagonized by natural products. MSKE, a plant product has recently been shown to promote apoptosis of prostate cancer cells, but not normal cells (22), and revert epithelial mesenchymal transition (36). We also utilized Z-FY-CHO, a potent and reversible selective inhibitor of CatL, which has been shown to inhibit bone resorption in rat bone cells by inhibiting collagen degradation (37). C4-2, ARCaP-Snail and MCF-7 Snail cells treated with 5 or 20 µg/ml MSKE, or 5 µM Z-FY-CHO for 72 h led to decreased Snail, mature CatL and pSTAT-3 expression as shown by western blot analysis, as well as decreased CatL activity as shown by zymography (Figure 4A and B). Additionally, MSKE also antagonized Snail-mediated CatS. Therefore, Snail activation of CatL can be antagonized by MSKE as effectively as Z-FY-CHO.

**MSKE and Z-FY-CHO can antagonize cell migration and invasion**

Next, we examined if MSKE and/or Z-FY-CHO can inhibit Snail-mediated cell migration and invasion. ARCaP-Snail and MCF-7 Snail showed increased migration and invasion, as compared to empty vector Neo controls, which could be abrogated upon treatment with MSKE or Z-FY-CHO (Figure 5A–D). Similarly, cell migration in C4-2 cells decreased upon treatment with Z-FY-CHO or MSKE (Supplementary Figure 2, available at Carcinogenesis Online). Therefore, MSKE and Z-FY-CHO can antagonize cell migration and invasion.

**MSKE antagonizes Snail-mediated osteoclastogenesis**

Since we have previously shown that Snail can increase osteoclastogenesis in prostate cancer cells by increasing the
expression of RANKL (4), and CatL has been shown to be involved in bone resorption (33), we investigated whether, CatL similarly to RANKL may be involved in Snail-mediated osteoclastogenesis, and whether this biological function can be antagonized by MSKE. ARCaP-Snail and MCF-7 Snail cells displayed significant increase in the formation of mature osteoclasts as seen by TRAP staining as compared to Neo controls, which was significantly inhibited by 5 μg/ml or 20 μg/ml MSKE, 5 μM Z-FY-CHO or 50 ng/ml OPG (RANKL antagonist) (Figure 6A and B). Of note, although MCF-7 Snail cells displayed a significantly higher number of mature osteoclasts as compared to MCF-7 Neo, there were hardly any cancer cells noted on the MCF-7 Snail plate following TRAP staining (Figure 6B); MCF-7 Snail cells generally attach very loosely and we believe the cells tend to be easily washed off during TRAP staining protocol. Our results suggest that Snail mediates osteoclastogenesis in part via CatL activity, which can be inhibited by MSKE.

**Discussion**

The aim of this report is to show that MSKE, a natural product, can antagonize Snail-mediated signaling and bone turnover. CatL expression increases with prostate and breast cancer progression (17,38). Although these cathepsin proteases are mostly secreted by macrophages in tumors (39), the mechanism(s) by which they are upregulated in prostate or cancer has not been elucidated. We have shown previously by IHC that Snail expression is higher in aggressive and bone metastatic prostate cancer patient tissue and can promote osteoclastogenesis in vitro and in vivo (4). It has also been indicated that Snail-positive breast cancer tends to home into the bone in breast cancer patients (40,41). In our present tissue microarray samples, we show that CatL is highly expressed with advanced stages of prostate cancer and that the expression of CatL shifts from predominantly cytoplasmic in lower grade to nuclear in higher grade tumor tissue. Additionally, we show that mature CatL is more highly expressed in breast tumor lysates as compared to normal tissue. Nuclear localization of CatL has been previously documented using in vitro cultures and has been found to have distinct DNA binding and transcriptional regulatory activities (42). In these studies, a truncated form of CatL cleaves the CUX1 transcription factor and as a result accelerates progression into the S phase of the cell cycle (42). CatL is also localized in the nucleus of breast cancer cells and patients with triple negative breast cancer have a higher levels of nuclear CatL (32). We show also for the first time that with increasing progression of prostate cancer that CatL is expressed in the nucleus (Figure 1A), which may infer that CatL activity in the nucleus is associated with a poor prognosis in prostate cancer. We further show that Snail increases CatL expression and CatL and CatS activity (Figure 2). This is the first report showing that Snail can regulate CatL and CatS expression/activity.

When we knocked down STAT-3 in cells overexpressing Snail there was a decrease in Snail and mature CatL expression, and CatL activity (Figure 3). This indicates that Snail activates CatL in part via the STAT-3 signaling pathway. We also present novel findings that MSKE inhibits the activity of CatL possibly by inhibiting Snail expression (Figure 4). MSKE has been shown previously to promote apoptosis of prostate cancer cells without affecting normal prostate epithelial cells (22). Muscadine grape skin contains abundant and numerous types of phenolic compounds, and health benefits have been attributed largely to these phenolic compounds and their significant interactions (43). The recent growing interest in muscadine grapes stems
from a great diversity of phenolic compounds contained within them, including the presence of anthocyanins and ellagic acid (43). We have shown previously that MSKE can antagonize Snail-mediated epithelial mesenchymal transition (36). In our present study, after treatment with MSKE for 72 h we observed that MSKE decreased Snail expression as well as CatL and STAT-3 activity in C4-2 cells that display high levels of Snail, and ARCaP-Snail cells. MSKE may antagonize Snail-mediated signaling by inhibiting the JAK/STAT pathway but further studies are required to confirm this. Interestingly, we also present novel data that Z-FY-CHO can inhibit STAT-3 activity and Snail expression, which suggests that there is a positive feedback loop between Snail and CatL that involves the STAT-3 signaling pathway. Upon activation of STAT-3, it has been shown that there is an increase in both the transcriptional activity of Snail (44) and cathepsin activity (35). Conversely, we show for the first time that Snail overexpression may be able to increase STAT3 activity (Figure 4). Functionally, MSKE decreased cell migration and invasion as well as Snail-mediated osteoclastogenesis in both prostate and breast cancer cells to levels comparable to CatL and RANKL antagonists (Figures 5 and 6). This is the first report showing that MSKE can inhibit bone turnover.

Therefore, we show here for the first time that Snail may mediate migration, invasion and osteoclastogenesis in part via CatL and this can be antagonized by MSKE. CatL inhibition is already being discussed as a possible therapy for bone metastasis (33), but this is the first study suggesting that MSKE may also be a potential therapy for bone metastatic disease. MSKE is currently in Phase II clinical trials for treatment of localized prostate cancer; our study suggests that it could also be a potential therapy for bone metastatic lesions by antagonizing Snail and CatL signaling.

Overall, this study develops novel roles for bidirectional interactions between Snail transcription factor and CatL that involves STAT-3 signaling. Even though the different compounds of MSKE have been identified such as anthocyanin 3,5-diglucosides, ellagic acid and ellagic acid precursors, further work is warranted to elucidate which of these compounds may be responsible for the effects reported in the current manuscript. Although the underlying mechanisms governing these effects are not yet fully understood, the available evidence collectively indicates that antagonizing CatL activity with MSKE may be of therapeutic benefit in clinical settings, suggesting its potential use as an anticancer agent for prostate and breast cancer that has metastasized to bone.

Supplementary material
Supplementary Tables 1 and 2 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/.

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