Prostate tumor cell growth induced by proliferin secreted from castrated mice adipose stromal cells

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Objectives: Recently, some investigators reported that adipose tissue is an endocrine organ that affects behavior of various cell types through its production of growth factors and cytokines. We hypothesize that adipose tissue may contribute to the development of castration resistant prostate cancer (CRPC).

Methods: Adipose stromal cells (ASC) and the conditioned medium were obtained from epididymal white adipose tissue (WAT) in castrated mice at 12 weeks old. Microarray assay was performed to identify the change in ASC after castration. Cell viability assay, tubule formation assay, and transwell invasion assay were performed to confirm that the effect of ASC on human umbilical vein endothelial cells (HUVEC) or on mouse prostate cancer cell line (RM-9) behavior. Glipr1+/- and Glipr1-/- male mice were used in this research.

Results: Tumor promoting factors including proliferin (PLF) in ASC was induced by castration in the animal model. We confirmed surgical castration enhanced PLF secretion in ASC and the ASC conditioned medium promoted cell proliferation, invasion, and tubule formation of HUVEC and RM-9 in vitro. Neutralization of PLF in the conditioned medium blocked the cell growth, invasion, and tubule formation.

Conclusion: Castration may promote CRPC by releasing factors that promote tumor growth including PLF in ASC isolated from WAT.

Key words: adipose stromal cells, castration, proliferin, prostate cancer cells

Introduction

Androgen-deprivation therapy is a standard treatment for prostate cancer and efficiently controls the growth of androgen-dependent tumors. Unfortunately, the majority of these cancers ultimately becomes refractory to hormone deprivation and emerges as castration resistant prostate cancer (CRPC).1 Such CRPC represents a significant clinical challenge, and a better understanding of the biological mechanisms that contribute to tumor re-growth is of critical importance.

There is now a preponderance of evidence that adipose tissue is an endocrine organ that affects behavior of various cell types through its production of growth factors and cytokines.2 Specifically, adipose tissue is an abundant source of mesenchymal stromal cells, also known as adipose stromal cells (ASC). Numerous studies state that ASC secrete high levels of growth factors (GF), such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and also cytokines.3 In fact, ASC secrete nearly all of the GF that take part in normal wound healing and promote wound healing.4,5 A recent study showed that human ASC secreted CCL5 and significantly enhanced MDA-MB-231 breast cancer cell invasion when co-cultured.6 Similar tumor promoting effects of ASC were reported using murine breast cancer 4T1 cells.7

Floryk et al. reported substantial increase of pro-inflammatory cytokine expressions such as CXCL5 and matrix metalloproteinase-2 in epididymal white adipose tissues (WAT) 14 days after mice were castrated.8 Although the role of castration in CRPC is not fully

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understood, such changes in the level of pro-inflammatory cytokines secreted by ASC after the castration may contribute to the disease. In the present study, we found that ASC secreted proliferin (PLF) as a tumor growth factor and castration may promote CRPC by releasing factors that promote tumor growth such as PLF in ASC isolated from WAT.

Materials and Methods

Cell lines
Mouse prostate cancer cell line RM-9 was generated in Dr. Timothy C. Thompson's laboratory from MD Anderson Cancer Center (MDACC), (Houston, TX, USA) and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (Lonza; Walkersville, MD, USA). Cell lines were validated by short tandem repeat DNA fingerprinting using the AmpFLSTR Identifier kit in the MDACC Cell Line Core.

Animals and analysis of WAT
Glipr1+/+ (Wild type [WT]) and Glipr1−/− (Knockout [KO]) male mice from Dr. Thompson's Laboratory were used in this research. GLIPR1 is a p53 target gene that is downregulated in prostate cancer, partly owing to methylation in the gene's regulatory region.11,12 Enforced GLIPR1 expression in prostate cancer cells led to growth suppression and/or apoptosis,11,13,14 whereas inactivation of the mouse counterpart of GLIPR1, i.e., Glipr1, predisposed mice to tumorigenesis.14 Mice were euthanized at 12 weeks of age to analyze phenotypes of epididymal WAT. Unless noted otherwise, WAT, ASC, and the conditioned mediums were obtained from 12-week-old male mice. Histological analysis of WAT from 4 subgroups (WT ± castration [Cx], KO ± Cx) was performed as described previously to 500 randomly selected adipocytes.9

Mice were housed and all experimental procedures were done in compliance with institutional and governmental requirements and approved by MD Anderson's Animal Care and Use Committee.

Preparation of ASC and conditioned medium
Two weeks after the mice went through either surgical castration or a sham procedure, the mice were euthanized and samples of epididymal WAT were removed. The WAT samples were rinsed with phosphate-buffered saline (PBS), finely minced and digested by collagenase type IA (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 90 minutes with gentle agitation, and filtered through a 100 μm strainer (BD Falcon, Franklin Lakes, NJ, USA). After centrifugation at 300 g for 10 minutes at room temperature, the pellet which contains the stroma vascular fraction was lysed with ACK (ammonium-chloride-potassium) lysing buffer (Lonza) to remove red blood cells. Subsequently, cells were seeded at a density of 1 × 10^6 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Growth medium for ASC from castrated mice contained 1 μM of biculamide (Casodex) to neutralize androgenic substances in FBS. Cells from passages 1−3 were used for the experiments.

A total of 1 × 10⁶ cells of each ASC subgroup were briefly rinsed and incubated for 24 hours with DMEM without phenol red. The ASC conditioned medium (ASC-CM) was collected and concentrated using Ultracel 3K centrifugal filters (Millipore, Billerica, MA, USA) to the final 1 ml. Equal volumes of ASC-CM were obtained from an equal number of ASC subgroups for comparison.

Characterization of ASC
Each ASC subgroup was treated with 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), washed twice with PBS supplemented with 2% FBS and cell aliquots (1 × 10⁶/100 μl) were stained at room temperature for 30 minutes with the following primary antibodies: PE-anti-CD45 I3/2.3 (hematopoietic marker) (Abcom, Cambridge, MA, USA), Alexa fluor 488 anti-CD29 HM B1-1 (stromal/stem cell marker) (BD Biosciences, San Jose, CA, USA), APC-anti-CD31 MEC13.3 (endothelial marker) (BD Biosciences) and PE-Cy-7-anti-CD105 MJ7/18 (stem cell marker) (BD Biosciences). Cell surface antigens on these cells were analyzed by Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA), and the data analysis was performed with Kaluza software (Beckman Coulter). All cells were examined at 1 week of cell culture.

Microarray analysis
Total RNA was isolated from each ASC subgroup using a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Microarray analysis was performed, and the data were normalized and statistical analysis performed as previously described.15

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis
Total RNA was isolated from each ASC subgroup as described above and reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied
Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Appropriate forward and reverse primers to detect transcripts of interest were used in the qRT-PCR for cDNA amplification. qRT-PCR was performed using the following primers (Integrated DNA Technologies, Coralville, IO, USA): Glipr1, PLF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with a StepOne Real Time PCR System (Applied Biosystems). The quantity of each target was normalized against the quantity of GAPDH.

The primer sequences are:

Glipr1: 5’-TTTGACCGAAGCTGGTTGTGA-3’ (Forward) and 5’-CATCCTTGCTGTGATAGTCTGG-3’ (Reverse);

GAPDH: 5’-TGTAGACCATGTAGTTGAGGTCA-3’ (Forward) and 5’- AGGTCGGTGAACGGATTTG-3’ (Reverse);

PLF: 5’-AGCCCCATGAGATGCAATACT-3’ (Forward) and 5’-CACTCACTAGATCGTCCAGAGG-3’ (Reverse).

**Immunofluorescence**

Dual immunofluorescence labeling was performed using primary antibodies to PLF (R&D Systems, Minneapolis, MN, USA) and to Vimentin (stromal marker) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD31 (BD Biosciences), CD11b (macrophage marker) (BD Biosciences), or CD45 (Abcam) on cultured ASC after fixation in acetone-methanol (1:1) for 8 minutes and incubation in non-serum blocking reagent (Dako) for 20 minutes. Alexa-fluor 488 conjugated anti-rabbit IgG or Alexa-fluor 594 conjugated anti-goat IgG were used as secondary antibodies. Slides were mounted in anti-fading medium containing DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride; Invitrogen).

**Western blotting**

ASC were prepared from each subgroup and protein concentration was determined. Conventional western blotting was used to detect protein levels of PLF (Santa Cruz Biotechnology) and Glipr-1. α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control. Computer assisted quantitative analysis was done with Nikon’s NIS-Elements AR 3.0 imaging and quantification software.

**Cell viability assay**

Cells were plated in 96-well plates with growth medium containing 1% FBS at a density of 3 × 10^4 for RM-9 or 5 × 10^4 for HUVEC. The next day, the growth medium from each cell line was aspirated completely, and then ASC-CM was added to each well. After 48 hours incubation, the number of living cells was measured using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One- solution Cell Proliferation assay) (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Absorbance at 490 nm was measured using a multiwell plate reader (Synergy2, BioTech Instruments, Winooski, VT, USA).

To confirm PLF effect in prostate cancer cells, we performed the same cell viability assay with PLF-neutralized ASC-CM by adding anti-PLF antibody. ASC-CM was incubated with anti-PLF antibody (50 mg/ml), normal IgG antibody (50 mg/ml) (Santa Cruz Biotechnology) or PBS and was mixed gently overnight at 4 °C. After the cells were incubated for 48 hours, the number of living cells was measured with an MTS assay as above. Anti-PLF antibody and normal IgG were dialyzed with Slide-A-Lyzer Dialysis Cassette (3.5K MWCO) (Thermo Scientific, Rockford, IL, USA) to remove sodium azide before the addition to ASC-CM.

**Tubule formation assay**

The tubule formation assay was performed as described previously. Briefly, HUVEC were trypsinized counted and plated on Matrigel Matrix (growth factor-reduced Matrigel; BD Biosciences) coated 24-well plates in EGM-2 medium (Lonza) containing 2% FBS. After 16-24 hours of incubation at 37 °C in 5% CO2, images of the tubules formed were captured by a phase contrast microscopy. The tubule lengths of the endothelial network was measured by image analysis of five low-power fields using Nikon's NIS-Elements AR 3.0 imaging and quantification software.

**Transwell invasion assay**

Transwell invasion assay was performed using BD BioCoat Matrigel Invasion Chambers (BD Bioscience) according to the manufacturer's instructions. Briefly, 5.0 × 10^4 of RM-9 cells were seeded in the upper chambers (the inserts with 8.0 μm membranes coated with Matrigel) of 24-well plates and each subtype of ASC-CM was added to the lower chambers. After RM-9 cells were allowed to invade to the bottom of the transwell for 24 hours, the invaded cells on the bottom surface of the membranes were stained with the Hema 3 stain kit (Fisher Scientific, Kalamazoo, MI, USA).
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according to the manufacturer’s instructions. The invading cells were counted under the microscope at $\times 10$ magnification.

Statistical analysis
Statistical analysis was performed using two-tailed Student’s t-tests or ANOVA (analysis of variance) with Statview 5.0 software (SAS Institute, Cary, NC, USA). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

The WAT phenotype in mice
We compared the WAT wet weight and WAT wet weight to body weight ratio between Glipr1 WT and KO mice at the age 12 weeks. We detected a striking reduction in WAT wet weight and WAT wet weight/body weight ratio in Glipr1 KO mice, also their reduction was emphasized by castration (Figure 1A).

Altered WAT response to castration
To detect changes in the size of adipocytes, we evaluated the H&E (hematoxylin and eosin)-stained WAT sections from 5 mice in each subgroup. The adipocytes in KO-

![Figure 1](image)

**Figure 1.** The comparison between Glipr1 WT and KO

A. White adipose tissue (WAT) wet weight and WAT wet weight/body weight ratio of 12-week-old Glipr1 WT and KO mice that either underwent a sham operation (Sh) or were castrated (Cx) as previously described. At the end of the experiment, the mice were euthanized and their body weights and WAT wet weights were recorded. Bars indicate standard error (SE). B. H&E (hematoxylin and eosin) staining of WAT from the Sh and Cx in Glipr1 WT or KO mice was performed as described. Original magnification, $\times 20$. C. Line graphs represent the adipocyte size distribution as assessed by image analyses. For each experimental group, 500 adipocytes were randomly selected from 5 specimens, and the areas of individual adipocytes were measured.
A. qRT-PCR with the primer for specific Glipr1 and PLF. Bar graphs show data of ratios relative to the amount of mRNA for each of these expressions Glipr1 and PLF in ASC from ShWT. The amount of Glipr1 mRNA was detected to be of approximately a 40% reduction in CxWT-ASC compared with that level in ShWT-ASC. The amount of PLF mRNA in the ASC subgroups increased in association with castration or the Glipr1 status. CxKO-ASC showed the highest expression of PLF.

B. Expressions of Glipr1 and PLF protein in the ASC and ASC-CM subgroups by western blotting. α-tubulin was used as a loading control. Glipr1 protein was reduced after castration. PLF protein in the ASC-CM subgroups increased in association with castration and the Glipr1 status. CxKO-ASC showed the highest expression of PLF.

Figure 2

A. Immunostaining for vimentin (stromal marker) and PLF in ASC from CxKO. Nuclei were stained with DAPI. The merged image indicates that PLF was produced by ASC. Mice were castrated at 12 weeks old and euthanized 2 weeks later.
WAT appeared smaller and than those in WT-WAT (Figure 1B). Quantitative image analysis revealed that the average area of the adipocytes was less in KO-WAT (2,239 ± 1,261 mm²; 1,130 ± 703 mm²; WT; KO, respectively) (Figure 1C). The adipocytes in the castration (Cx) group appeared smaller than those in the sham (Sh) group in both genotypes (1,121 ± 953 mm²; 733 ± 492 mm², respectively). Also, we detected a change in the stroma that was characterized by more fibroblast-like stromal cells in the WAT from the Cx group than there were in that from the Sh group.

The ASC phenotype isolated from WAT in mice
To identify the characterization of the ASC phenotype from each subgroup, we performed multicolor flow cytometry. The majority of the cultured cells from each subgroup were negative against either CD45 or CD31 but positive against CD29 and/or CD105, corresponding to ASC as previously established.\(^\text{17,18}\)

Castration induced PLF expression/secrection in the ASC/ASC-CM
To identify the factors secreted in response to castration, we performed microarray assay using isolated RNA from each ASC subgroup. A family of glycoproteins known as PLF was discovered as a promising candidate on the list. Next, we performed qRT-PCR to confirm the induction of transcription of PLF by castration and/or the status of the Glipr1 gene (Figure 2). We detected that PLF were enhanced not only in ASC from ShKO compared to ShWT but also after castration in both genotypes. The amount of PLF mRNA in the ASC subgroup increased in association with castration. PLF mRNA was most expressed in ASC from CxKO. Also

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**Figure 4**

A. Effect of ASC-CM on HUVEC proliferation. CM collected from the cell sources indicated under columns was added to HUVEC. The number of living cells measured using MTS assay. B. Effect of ASC-CM on HUVEC tubule formation in Matrigel. HUVEC tubule formation was measured after 16-24 hours. Images of the tubules formed were captured by phase contrast microscopy. The tubule lengths in each well were measured in 5 low-power fields. C. Neutralization study with PLF-depleted CxKO-CM

A. Proliferation

- ShWT
- ShKO
- CxWT
- CxKO

B. Tubule formation

- ShWT
- ShKO
- CxWT
- CxKO

C. Neutralization study with PLF-depleted CxKO-CM

- PBS
- IgG
- Anti-PLF

- CxKO

Bar graphs represent the values or tubule length ratios relative to those in cells treated with serum free medium. Bars indicate SE.
western blotting with an antibody against PLF demonstrated the most induction of PLF into conditioned medium from CxKO-ASC (Figure 2).

To identify the cells that produced PLF, we performed dual immunostainings using antibodies to PLF and to vimentin, CD31, CD11b, or CD45 on cultured ASC. It was found that PLF was predominantly localized with the vimentin positive stromal cells (Figure 3), and it was not expressed in the CD11b, CD31, or CD45 positive cells.

PLF induced endothelial cell proliferation and tubule formation
We performed endothelial cell proliferation study and tubule formation study to confirm PLF effect (Figure 4A, B). ASC-CM from the CxKO subgroup showed the highest expression of PLF and had a stronger effect on endothelial cell proliferation and tubule formation than did that in the other subgroups.

To confirm that the effect of PLF on endothelial cell behavior is due to secreted PLF, we performed to neutralize PLF in conditioned medium by anti-PLF antibody as described above. PLF-depletion conditioned medium had less effect on HUVEC proliferation and tubule formation (Figure 4Ca, b).

PLF promoted prostate cancer cell growth
Next, to examine whether ASC-CM alters tumor cell

Effect of PLF in RM-9

A. Effect of ASC-CM on prostate cancer cell (RM-9) proliferation. CM collected from the cell sources indicated under columns was added to RM-9. The number of living cells measured using MTS assay. B. Effect of ASC-CM on RM-9 invasion (assayed as describe above). The cells \(3 \times 10^3\) were added to the upper chamber in serum-free medium. ASC-CM was applied as a chemoattractant to the lower compartment of the chamber. C. Neutralizing antibody to PLF decreases RM-9 proliferation. Each subtype of ASC-CM was treated with PBS, normal goat IgG anti-PLF antibody. The addition of anti-PLF antibody significantly inhibited prostate cancer cell proliferation. Bar graphs represent the values or tubule length ratios relative to those in cells treated with serum free medium. Bars indicate SE.
behavior, we performed proliferation and invasion assay on RM-9 (Figure 5A, B). We detected that conditioned medium from CxKO-ASC promoted the highest cancer cell growth and invasion compared to other conditioned media. We also observed that an anti-PLF antibody treatment in conditioned media offsets the effect of PLF in RM-9 as well (Figure 5C).

Discussion
Clinically, CRPC is characterized by a rise in the serum PSA levels despite effective androgen suppression.1 Progression to CRPC is a dynamic process that is currently only partially understood despite extensive research.19,22 Potential mechanisms that contribute to the development of CRPC include selective growth of a pre-existing hormone-insensitive population of cancer cells, as a result of suppression by androgen ablation of the androgen-dependent cell population,19 activation of oncogenes, inactivation of tumor suppression genes,20 interaction of cancer cells with tumor-associated stroma,21 and tumor-associated macrophages.22 There may be multiple pathways for the development of resistance to hormonal therapy. Although such pathways can be described in phenomenological terms, the detailed molecular biology of such a process remains unknown. Prostate cancer frequently metastasizes to bone, an organ that contains a microenvironment rich in extracellular matrix proteins and stromal cells including hematopoietic cells, osteoblasts, osteoclasts fibroblasts, endothelial cells, adipocytes, immune cells, and mesenchymal stromal cells.23

Adipose tissue is an abundant source of mesenchymal stromal cells, termed ASC.2,3,17 Factors secreted from adipose tissue contribute considerably to the regulation of metabolism and inflammatory responses. Different studies have demonstrated the critical influence of adipose tissue-derived factors in cancer cells,24,25 including prostate cancer cells.16 However, there is limited data on androgen and/or castration effects on WAT. 26,27 Understanding of these effects is crucial in characterization of WAT role in the progression of prostate cancer.

Our work demonstrates that morphologic changes on WAT after castration characterized by smaller adipocytes and increased stromal cell compartment. Castration may induce lipolysis in WAT, and increased presence of stromal cells may be a result of the tissue regeneration process induced in WAT by castration.9 We also noticed that numerous cytokines were induced in WAT after castration on the microarray analysis. These results indicate that factors produced by ASC isolated from WAT, especially after castration, may stimulate the progression of cancer cells. However, to our knowledge, the influence of ASC-derived factors after castration on prostate cancer cells has not been reported.

In the current study, PLF was discovered on the list to identify the change in ASC after castration and we detected that PLF was secreted by ASC in the conditioned medium, also surgical castration enhanced PLF secretion in ASC-CM (Figures 2, 3). PLF was originally described as a glycoprotein secreted by mouse embryonic placental tissue during active midgestation period.28,29 PLF belongs to the prolactin/growth hormone/placental lactogen family of polypeptide hormones, which are primarily produced in the pituitary gland and the placenta in most species.28,29 PLF has been associated with angiogenesis in sarcomas, gliomas, progressive fibrosarcoma and many other malignancies.29-31 Yang et al. reported secretion of PLF and binding to the regulatory regions of the PLF is required for signal transducer and activator of transcription 5 (STAT5)-induced mouse endothelial cell migration, invasion, and tubule formation.29 The protein product of the PLF mRNA was the form of PLF that was originally shown to be angiogenic,32 also can mediate human, rat, mice, bovine endothelial cell migration.33,34 Insulin-like growth factor 2/mannose 6-phosphate (IGF2/M6P) receptor is considered as an essential receptor for PLF,12 Although the signaling pathway associated with the IGF2/M6P receptor is poorly understood, binding of either PLF or IGF2 activates a G protein that leads to mitogen-activated protein kinases (MARK) activation and that MARK activation is necessary for the chemotactic response on endothelial cells.35 Thus, secretion of PLF would be expected to contribute to angiogenesis.

In the current study, however, the secretion of PLF contribute to not only angiogenesis with in vitro models using HUVEC as endothelial cells, but also to tumorigenesis with models using RM-9 as mouse prostate cancer cells in vitro (Figures 4, 5). Condition medium of ASC promoted cell proliferation, invasion and tubule formation of RM-9. Neutralization of PLF in the conditioned medium blocked the cell growth, invasion and tubule formation. To our knowledge, this is the first report of surgical castration increasing PLF secretion from ASC and PLF secretion promoting prostate cancer cell growth.

In this research, animal models using GLIPR1+/+ and GLIPR1−/− male mice were used. GLIPR1 expression is downregulated in prostate cancer and other malignant cell lines,36 and shown to have antiangiogenic activity.11
Thus, loss of GLIPR1 may reduce antiangiogenesis activity in the models and enhance castration induced PLF secretion.

Tumor cell progression depends on itself as well as on the surrounding microenvironment, which is able to influence proliferation, migration and metastatic behavior of tumor cells by modulating the extracellular matrix and growth factor production.37 Lin et al. showed that human ASC helps PC-3 tumor growth by increasing tumor vascularity, and which was mediated by FGF2.16 Many malignancies secrete FGF2 which may stimulate angiogenesis directly and/or by promoting endothelial PLF production.20-31 Yang et al. reported that an angiogenesis induced by FGF2 through the STAT5 activation is dependent on activity of PLF.29 These results indicate that ASC, which is the surrounding microenvironment of prostate cells, changed its property by castration and secreted growth factors such as PLF, which can mediate not only endothelial cells, but also directly mediate prostate cancer cell proliferation. The increased production of PLF found in ASC-CM can fuel proliferative and invasive behavior of CRPC. Our findings indicate the effect of PLF on invasion of prostate cancer is mediated by castration in a paracrine and juxtacrine mechanism.

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References
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