

## The Essential Role of Gi $\alpha$ 2 in Prostate Cancer Cell Migration

Miao Zhong, Shineka Clarke, BaoHan T. Vo, and Shafiq A. Khan

### Abstract

Cell- and receptor-specific regulation of cell migration by Gi/o $\alpha$ -proteins remains unknown in prostate cancer cells. In the present study, oxytocin (OXT) receptor was detected at the protein level in total cell lysates from C81 (an androgen-independent subline of LNCaP), DU145 and PC3 prostate cancer cells, but not in immortalized normal prostate luminal epithelial cells (RWPE1), and OXT-induced migration of PC3 cells. This effect of OXT has been shown to be mediated by Gi/o $\alpha$ -dependent signaling. Accordingly, OXT inhibited forskolin-induced luciferase activity in PC3 cells that were transfected with a luciferase reporter for cyclic AMP activity. Although mRNAs for all three Gi $\alpha$  isoforms were present in PC3 cells, Gi $\alpha$ 2 was the most abundant isoform that was detected at the protein level. Pertussis toxin (PTx) inhibited the OXT-induced migration of PC3 cells. Ectopic expression of the PTx-resistant Gi $\alpha$ 2-C352G, but not wild-type Gi $\alpha$ 2, abolished this effect of PTx on OXT-induced cell migration. The Gi $\alpha$ 2-targeting siRNA was shown to specifically reduce Gi $\alpha$ 2 mRNA and protein in prostate cancer cells. The Gi $\alpha$ 2-targeting siRNA eliminated OXT-induced migration of PC3 cells. These data suggest that Gi $\alpha$ 2 plays an important role in the effects of OXT on PC3 cell migration. The Gi $\alpha$ 2-targeting siRNA also inhibited EGF-induced migration of PC3 and DU145 cells. Expression of the siRNA-resistant Gi $\alpha$ 2, but not wild type Gi $\alpha$ 2, restored the effects of EGF in PC3 cells transfected with the Gi $\alpha$ 2-targeting siRNA. In conclusion, Gi $\alpha$ 2 plays an essential role in OXT and EGF signaling to induce prostate cancer cell migration. *Mol Cancer Res*; 10(10): 1380–8. ©2012 AACR.

### Introduction

Heterotrimeric guanine phosphate-binding proteins (G-proteins) are composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. G $\beta$  and G $\gamma$  form a dimer that is associated with inactive G $\alpha$  (1–3). Activated G-protein-coupled receptor (GPCR) catalyzes the exchange of guanosine diphosphate for GTP on G $\alpha$ , resulting in the dissociation of G $\beta\gamma$  from the activated G $\alpha$  (4). G $\alpha$  subunits have intrinsic GTPase activities that hydrolyze the GTP into GDP and terminate the G-protein-coupled signaling (4). The GTP hydrolysis is accelerated by the regulator of G-protein signaling protein (RGS; ref. 4). There are 23 G $\alpha$  isoforms that are divided into 4 subfamilies, including Gs, Gi/o, Gq/11, and G12/13 (4). Activation of Gi/o-proteins inhibits adenylyl cyclases and reduces intracellular cyclic AMP (cAMP) levels (5). Gi $\alpha$ 1, Gi $\alpha$ 2, Gi $\alpha$ 3, Go $\alpha$ A, and Go $\alpha$ B are Gi/o $\alpha$ -proteins that are ubiquitously expressed and sensitive to pertussis toxin (PTx; ref. 4). Go $\alpha$ A and Go $\alpha$ B are derived from splice variants of the *Go $\alpha$*  gene that is preferentially expressed in the central nervous system (6, 7). PTx catalyzes ADP ribosylation of the cysteine (C)

residues at C-terminal ends of the Gi/o $\alpha$ -proteins, which results in the uncoupling of these G-proteins from their cognate GPCRs (8). Substitution of the cysteine for glycine eliminates the PTx ADP-ribosylation site, however, the resulting Gi/o $\alpha$  mutants have been shown to retain abilities to couple to GPCRs and induce biologic responses (9).

Prostate cancer development and progression is androgen dependent, and androgen deprivation therapy is currently among the regular procedures to treat metastatic prostate cancer (10–12). However, disease relapse often ensues from androgen ablation, producing castration resistant prostate cancer that results in increased malignancy and poor prognosis (10, 12, 13). Emerging evidence has suggested that GPCR signaling plays an important role in prostate cancer progression (14). Activation of G12/13-proteins has been shown to increase prostate cancer invasion via activation of RhoA family of small G-proteins (15). Both Gs- and Gq-dependent signaling induce the transactivation of androgen receptor in prostate cancer cells (16, 17), which has been shown to facilitate transition of primary prostate tumor cells to the androgen-independent stage (18). Expression of RGS2, a negative regulator of Gi- as well as Gq-dependent signaling, is significantly reduced in human prostate cancer specimens when compared with the adjacent normal or hyperplastic prostate tissues (19). Therefore, signaling of GPCRs, either Gi or Gq coupled, is potentially amplified in primary prostate tumors. PTx has been shown to inhibit the growth of PC3 prostate cancer cells (20). PTx sensitivity indicates the involvement of Gi/o-dependent signaling (8). Nevertheless, PTx treatment cannot distinguish receptor-

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and cell-specific functions of Gi/o $\alpha$  isoforms. Activated CXCR3 receptor induces migration of T lymphocytes via a Gi $\alpha$ 2-dependent mechanism, which is counteracted by Gi $\alpha$ 3 (21). There is no evidence suggesting the presence of isoform-specific functions of heterotrimeric G-proteins in prostate cancer cells.

Oxytocin (OXT) is a peptide hormone that regulates normal prostate functions. OXT treatment has been shown to increase epithelial cell growth, 5 $\alpha$ -reductase activity, and contractility in normal prostate (22–24). However, the information is very limited about the effect of OXT on prostate cancer progression. OXT receptor (OXTR) has been detected in tissues from hyperplastic and neoplastic prostate (25), but not in the epithelium of normal human prostate (26). Recently, OXTR has been shown to be the primary target of OXT in androgen-independent prostate cancer cell lines (DU145 and PC3; ref. 27). OXTR is a GPCR that promiscuously couples to multiple G-proteins including Gq and Gi (28, 29). Activated OXTR induces migration of PC3 cells via a PTx-sensitive mechanism (27). In the present study, OXTR protein was detected in prostate cancer cell lines (C81, DU145, and PC3), but not in the normal prostate epithelial cell line (RWPE1). Furthermore, we investigated whether specific Gi/o $\alpha$  isoforms were required for the migration of PC3 cells. Gi $\alpha$ 2 was the most abundant isoform of the Gi/o $\alpha$  family members that was detected at the protein level and this isoform was found to play an essential role in prostate cancer cell migration.

## Materials and Methods

### Reagents

Human OXT, pertussis toxin, bovine serum albumin (BSA), rabbit anti-OXTR antibody, anti- $\alpha$ -tubulin antibody, and mouse anti- $\beta$ -actin antibody were obtained from Sigma. Mouse monoclonal anti-Gi $\alpha$ 1 antibody, rabbit anti-Gi $\alpha$ 2, and anti-Gi $\alpha$ 3 antibodies were obtained from EMD-Millipore Total lysates from *Escherichia coli* expressing recombinant rat Gi $\alpha$ 1, Gi $\alpha$ 2, and Gi $\alpha$ 3 proteins and an additional rabbit anti-human Gi $\alpha$ 2 antibody, were purchased from Santa Cruz Biotechnology. Steady glow luciferase reagent, Moloney murine leukemia virus reverse transcriptase, T4-ligase, GoTaq qPCR master mix, and oligo dT primer were purchased from Promega. Taq polymerase was purchased from Lucigen. Phusion high-fidelity PCR kit and restriction enzymes were purchased from New England Biolabs. DNA primers were purchased from IDT. EGF, Trizol, lipofectamine 2000, control and Gi $\alpha$ 2-targeting (siRNA ID#: s5875, Ambion) siRNAs were purchased from Life Technology. TransIT-TKO transfection reagent was obtained from Mirus. Rat-tail collagen and transwell inserts were obtained from BD Biosciences. Cell culture reagents and G418 sulfate were purchased from Mediatech Inc.

### Plasmids

Human Gi $\alpha$ 2 and the Gi $\alpha$ 2-C352G mutant in pcDNA3.1 (Life Technology) were obtained from Missouri S&T cDNA Resource Center. The siRNA-resistant Gi $\alpha$ 2

gene was created by modification of the Gi $\alpha$ 2 siRNA-targeting sequence without any change in the protein sequence. The modified sequence was generated by PCR and ligated into the Gi $\alpha$ 2 gene after digestion with Kpn I and BamH I restriction enzymes. Sequences of the 2 sets of PCR primers were, Forward 1: GCT TGG TAC CAC CAT GGG CTG; Reverse 1: GTG TTC GAA TAC ACT ACG GCT CGG TAC TGC CGG CAT TCC TCC TC, Forward 2: TAC CGA GCC GTA GTG TAT TCG AAC ACC ATC CAG TCC ATC ATG GC; Reverse 2: GCA GTG GAT CCA CTT CTT CCG CT. The siRNA-resistant Gi $\alpha$ 2 clone was verified by DNA sequencing. The pADneo2 C6-BGL plasmid containing luciferase reporter for cAMP was a gift from Dr. Tu-Yi (Creighton University, Omaha, NE).

### Cell culture

Immortalized prostate luminal epithelial cell line (RWPE1), DU145, and PC3 prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Androgen-independent LNCaP subline C81 was kindly provided by Dr. Ming-Fong Lin (University of Nebraska, Omaha, NE). Immortalized pregnant human myometrial smooth muscle cells (PHM1-41) were a gift from Dr. Barbara Sanborn (Colorado State University, Fort Collins, CO). RWPE1 cells were maintained in keratinocyte growth medium (Invitrogen). The C81 cell lines were maintained in RPMI-1640 supplemented with 250  $\mu$ g/mL gentamycin and 10% FBS. DU145 and PC3 cells were maintained in Minimum Essential Media supplemented with 5% FBS. PHM1-41 cells were maintained in Dulbecco's Modified Eagle's Media supplemented with 10% FBS.

### Transfection of siRNAs

DU145 and PC3 cells were seeded in 6-well plates at a density of  $1.5 \times 10^5$  cells per well. The next day, cells were transfected with control siRNA or the Gi $\alpha$ 2-targeting siRNA using the TransIT-TKO transfection reagent. Briefly, Minimum Essential Media (200  $\mu$ L/well) containing 30 nmol/L of siRNA was mixed with the transfection reagent (8  $\mu$ L/well) and incubated for 20 minutes. siRNA mixtures were added drop by drop into 6-well plates. The transfection media were replaced with the regular culture media (2 mL/well) the next day. RNA samples were harvested 72 hours after transfection. Protein samples were collected at 72 and 96 hours after transfection.

### Transfection of plasmid DNAs

Minimum Essential Media (200  $\mu$ L/well) containing plasmid DNA (2  $\mu$ g/well) was mixed with lipofectamine 2000 (6  $\mu$ L/well) and incubated for 30 minutes. Plasmid DNA mixtures were added drop by drop into 6-well plates. Transfected PC3 cells were cultured for up to 48 hours.

### Generation of PC3 cell lines ectopically expressing wild-type and the siRNA-resistant Gi $\alpha$ 2

Transfected PC3 cells were placed in 100 mm<sup>2</sup> culture dishes and were grown in culture medium supplemented with 600  $\mu$ g/ml of G418. A week later, cells were maintained

in culture medium supplemented with 200  $\mu\text{g/ml}$  of G418. Single colonies were picked and grown in 6-well plates. Expression of *Gi $\alpha$ 2* was verified in total cell lysates by Western blot analysis.

#### Western blot analysis

PC3 cell membranes were prepared as described previously (30). Membrane proteins (50  $\mu\text{g}$ ) or total cell proteins (100  $\mu\text{g}$ ) were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes and analyzed for Western blotting as described previously (27). *Gi $\alpha$ 1-3* proteins were detected with isoform-specific antibodies (1:1,000 dilution). OXTR was detected in total cell lysates with anti-OXTR antibody (1:1,000 dilution). Thirty microgram of total cell proteins were used for detection of  $\beta$ -actin with a mouse monoclonal antibody (1:10,000 dilution). The signal was detected by ECL (Thermo Scientific).

#### RNA extraction, reverse transcription-PCR and quantitative real-time PCR

Total RNAs were extracted from cells using Trizol and the RNA content was determined at 260 nm with a UV spectrophotometer. First strand cDNA was synthesized from 2  $\mu\text{g}$  of total RNA as described previously (27). No reverse transcriptase (RT) control samples were prepared in parallel by replacing reverse transcriptase with RNase.

Expression of *Gi $\alpha$ 1*, *Gi $\alpha$ 2*, *Gi $\alpha$ 3*, *Go $\alpha$ A*, *Go $\alpha$ B*, and *L19* genes was detected with the PCR procedure described previously (27). Specific primers are listed in Table 1. PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide. DNA bands were visualized under UV light and images were documented using the Bio-Rad gel imager.

For quantitative real-time PCR, cDNA samples (1  $\mu\text{L}$ ) were mixed with 0.2  $\mu\text{mol/L}$  primers and 2X GoTaq qPCR master mix in a final volume of 25  $\mu\text{L}$  in a 96-well PCR plate. PCR detection was conducted in an iCycler thermal cycler (Bio-Rad) with procedures described previously (27). Melt-

ing curves were examined for the quality of PCR amplification of each sample. Calculations were conducted using the  $\Delta\Delta C_t$  method (27).

#### Cloning *Gi $\alpha$ 1* and *Gi $\alpha$ 3* genes from PC3 and DU145 cell lines

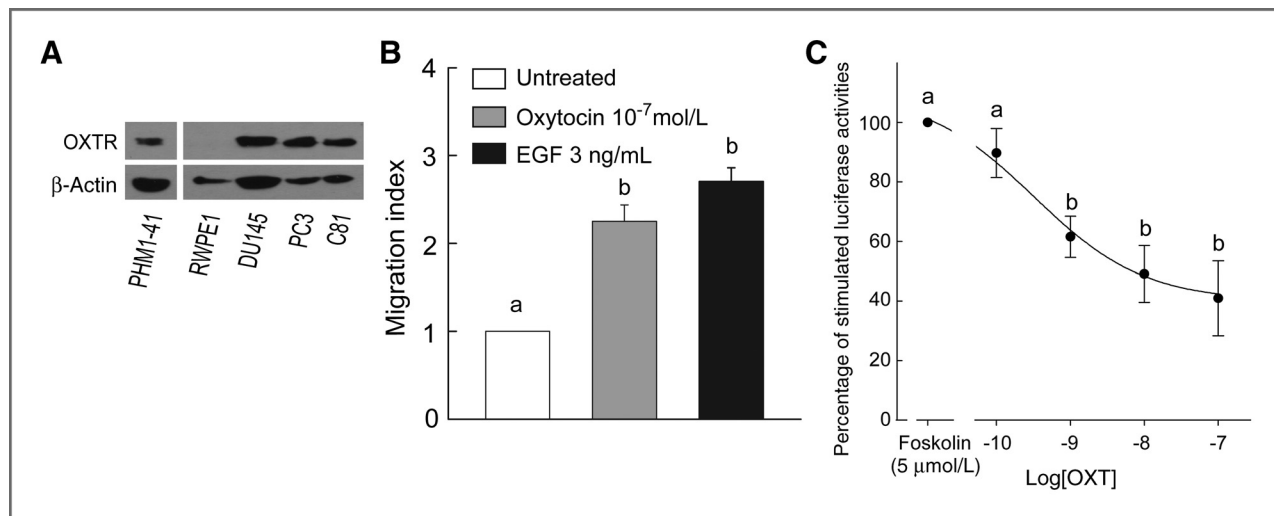
Open reading frames of *Gi $\alpha$ 1* and *Gi $\alpha$ 3* genes were amplified with the Phusion high-fidelity PCR kit. The shared forward primer was, GCT TGG TAC CAT GGG CTG CAC GTT GAG CGC C. The reverse primer for *Gi $\alpha$ 1* was, TAG ACT CGA GAT GCA TTT TAC CAT GAA CTG CAA. The reverse primer for *Gi $\alpha$ 3* was, GCT TGG TAC CAT GGG CTG CAC GTT GAG CGC C. The PCR procedure was programmed as following: 94°C for 2 minutes, 30 cycles of the following steps: denaturation at 94°C for 30 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 1 minute. A final extension of 10 minutes was followed up after the cycles were completed. Purified PCR products were digested with Kpn I and Xho I and ligated into pcDNA3.1 plasmid. After transformation into XL1-Blue competent cells (Stratagene), five *E. coli* colonies containing *Gi $\alpha$ 1*- or *Gi $\alpha$ 3*-expressing plasmid were grown overnight in LB broth supplemented with ampicillin. Plasmids were harvested with QIAprep spin miniprep kit (Qiagen) and sequenced from both ends with the cloning primers.

#### Luciferase reporter assay

The PC3 cells were harvested from the 6-well plates 24 hours after transfection with the pADneo2 C6-BGL plasmid. Aliquots of 30,000 cells were placed in an opaque 96-well plate for different treatments, including control, forskolin (5  $\mu\text{mol/L}$ ) and forskolin plus different concentrations of OXT. After treatment for 5 hours, 100  $\mu\text{L}$  of steady-glow luciferase detection reagent was added to each well of the 96-well plate. The luciferase activities were detected with OPTIMA LUMIstar plate reader (BMG LABTECH, NC). The results are expressed as percentile of the forskolin-stimulated luciferase activity.

**Table 1.** RT-PCR and quantitative PCR primers

Genes	Gene ID	Primers	Sequences (5'-3')	Location	Product size, bp
<i>Gi<math>\alpha</math>1</i>	156071490	Gi1-F	AGCTGCTGAAGAAGGCTTTA	673–692	171
		Gi1-R	GGGATGTAATTTGGTTGAGC	843–824	
<i>Gi<math>\alpha</math>2</i>	49574535	Gi2-F	AGAACAACCTGAAGGACTGC	1,158–1,179	173
		Gi2-R	GGGAGCTGAGAACAAAGAGA	1,330–1,311	
<i>Gi<math>\alpha</math>3</i>	169646784	Gi3-F	TGTTTTAGCTGGCAGTGCT	481–499	169
		Gi3-R	CTGGGATATTCTATCCAGATCA	649–628	
<i>Go<math>\alpha</math>A</i>	162461666	GoA-F	CAAGAAGTCACCTTTGACCA	1,734–1,753	301
		GoA-R	CTACCAGGAGATCAACGTCA	2,034–2,015	
<i>Go<math>\alpha</math>B</i>	162461737	GoB-F	AATCCCTGAAGCTTTTTGAC	1,634–1,653	223
		GoB-R	TAGATCTCTTTGTGGGCTGA	1,856–1,837	
<i>L-19</i>	68216257	L-19-F	GAAATCGCCAATGCCAACTC	306–325	406
		L-19-R	TCTTAGACCTGCGAGCCTCA	711–692	



**Figure 1.** OXTR receptor expression and functions in human prostate cell lines. A, expression of OXTR was detected with rabbit antibody for OXTR in total cell lysates (50  $\mu$ g) of RWPE1, DU145, PC3, and C81 cell lines. PHM1-41 expresses endogenous OXTR and was used as a positive control.  $\beta$ -actin was probed on the same blot as sample loading control. B, treatments with OXT and EGF for 5 hours significantly stimulated PC3 cell migration ( $n = 4$ ). C, twenty-four hours after transfection with the cAMP luciferase reporter (pADneo2 C6-BGL), PC3 cells were pretreated with different concentrations of forskolin for 10 minutes before the treatment with forskolin. Luciferase activities were detected 5 hours after treatments and expressed as percentage of the forskolin-stimulated luciferase activity ( $n = 3$ ). Data were expressed as mean  $\pm$  SEM and analyzed by ANOVA and Duncan's modified range tests. Significant differences between groups in a given category ( $P < 0.05$ ) are designated with different lowercase letters.

### Cell migration assay

*In vitro* cell migration assay was conducted using 24-well transwell inserts (8  $\mu$ m) as described previously (27). Briefly, cells were harvested and centrifuged at 500 g for 10 minutes at room temperature. The pellets were resuspended into Minimum Essential Media supplemented with 0.2% BSA at a cell density of  $3 \times 10^5$  cells/mL. Transwell inserts were treated with rat-tail collagen (50  $\mu$ g/mL) as described previously (27). Chemoattractant solutions were made by diluting OXT (100 nmol/L) or EGF (3 ng/mL) into Minimum Essential Media supplemented with 0.2% BSA. Minimum Essential Media containing 0.2% BSA served as a control medium. Aliquots of 100  $\mu$ L cell suspension were loaded into transwell inserts and placed into 24-well plates that contained control and chemoattractant solutions (300  $\mu$ L/well). The plates were incubated at 37°C for 5 hours. Cells inside the transwell inserts were removed by cotton swabs. The cleaned inserts were fixed in 4% paraformaldehyde (pH 7.5). Cells on the outside of the transwell insert membranes were stained using HEMA 3 staining kit (Fisher Scientific Inc). The number of stained cells was counted as described previously (27). Results were expressed as migration index defined as: the average number of cells per field for test substance/the average number of cells per field for the medium control.

### Statistical analysis

Data from multiple independent experiments ( $n = 3-5$ ) are expressed as mean  $\pm$  SEM. ANOVA and Duncan's modified multiple range test were used to examine significance between multiple treatments.

### Results

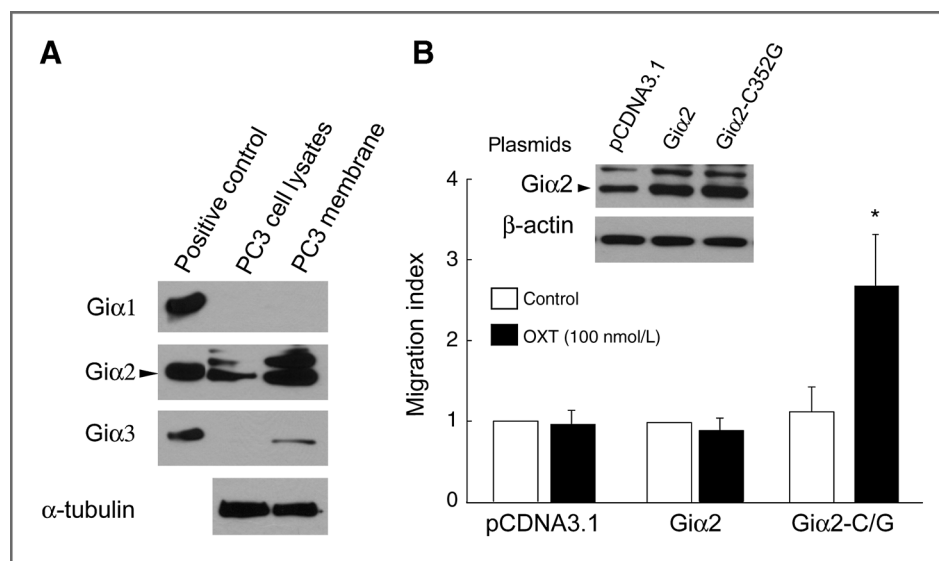
#### OXT activates the Gi/ $\alpha$ -dependent pathway and induces PC3 cell migration

Expression of OXTR is elevated at the mRNA levels in androgen-independent DU145 and PC3 prostate cancer cell lines, compared with normal prostate epithelial cells (27). In the present study, OXTR protein ( $\sim 70$  kDa) was detected in total cell lysates of DU145, PC3, and the androgen-independent subline of LNCaP (C81) prostate cancer cell lines, but not in the cell lysates of immortalized human prostate luminal epithelial cell line (RWPE1; Fig. 1A). Immortalized pregnant human myometrial smooth muscle cell line (PHM1-41) expresses endogenous OXTR and was used as a positive control (Fig. 1A).

OXT (100 nmol/L) and EGF (3 ng/mL) induced PC3 cell migration (Fig. 1B). This effect of OXT has been shown to be sensitive to PTx pretreatment, suggesting the involvement of Gi/ $\alpha$ -proteins (27). Inhibiting cAMP accumulation is the canonical function of Gi/ $\alpha$  proteins. PC3 cells were transfected with the pADneo2 C6-BGL plasmid containing a cAMP-responsive luciferase reporter. Forskolin (5  $\mu$ mol/L) induced a 3-fold increase in luciferase activities in the transfected PC3 cells. Treatment with OXT caused a dose-dependent inhibition of forskolin-stimulated luciferase activity (Fig. 1C), which supports the involvement of Gi/ $\alpha$  in OXTR signaling in PC3 cells.

#### Gi $\alpha$ 2 is important for the OXT-induced migration of PC3 cells

Expression of Gi/ $\alpha$  isoforms was detected by RT-PCR with specific primers listed in Table 1. Gi $\alpha$ 1, Gi $\alpha$ 2, and



**Figure 2.**  $Gi\alpha 2$  is potentially important for the OXT-induced migration of PC3 cells. A, total cell lysates and membrane fractions from PC3 cells were separated on 10% SDS-PAGE and transferred onto PVDF membrane. Recombinant rat  $Gi\alpha 1$ -3 were used as positive controls.  $\alpha$ -tubulin was detected as a sample loading control for both cell lysates and membranes. Similar results were observed with two separate membrane preparations. B, PC3 cells were treated with PTx (200 ng/mL) 24 hours after transfection with pCDNA3.1, wild type  $Gi\alpha 2$  and the  $Gi\alpha 2$ -C352G mutant. The next day, transfected PC3 cells were treated with OXT in a transwell migration assay. OXT induced cell migration only in the PC3 cells that were transfected with the  $Gi\alpha 2$ -C352G mutant. Protein levels of  $Gi\alpha 2$  in cells transfected with empty vector, in  $Gi\alpha 2$  and the  $Gi\alpha 2$ -C352G mutant in total cell lysates are shown on the top. Data were expressed as mean  $\pm$  SEM ( $n = 3$ ) and analyzed by  $t$  test (\*,  $P < 0.05$ ).

$Gi\alpha 3$  were the predominant  $Gi/\alpha$  family members that are expressed at the mRNA levels in PC3 cells (data not shown). Because G proteins are enriched in the cell membranes (5), the membrane fractions were prepared from PC3 cells to determine the expression of  $Gi\alpha 1$ -3 proteins. Total lysates (100 ng) of *E. coli* expressing recombinant rat  $Gi\alpha 1$ -3 proteins were used as positive controls. As shown in Fig. 2A, specific antibodies for  $Gi\alpha 1$ -3 proteins were able to detect respective recombinant proteins.  $Gi\alpha 2$  was the only  $Gi\alpha$  family member that was detected in PC3 total cell lysates and membrane fractions (Fig. 2A). In comparison, trace levels of  $Gi\alpha 3$  protein were detected in only membrane fraction whereas  $Gi\alpha 1$  was undetectable in both total cell lysates and membrane fractions (Fig. 2A).

Mutations causing codon shift might occur in open reading frames of *Giα1* and *Giα3* genes, which could account for the absence of  $Gi\alpha 1$  and  $Gi\alpha 3$  proteins in PC3 cells. Open reading frames of *Giα1* and *Giα3* genes were cloned by RT-PCR from total RNA samples of PC3 and DU145 cells. DNA sequencing did not identify any mutations in protein coding regions of both genes, indicating that wild-type  $Gi\alpha 1$  and  $Gi\alpha 3$  are expressed in DU145 and PC3 cell lines.

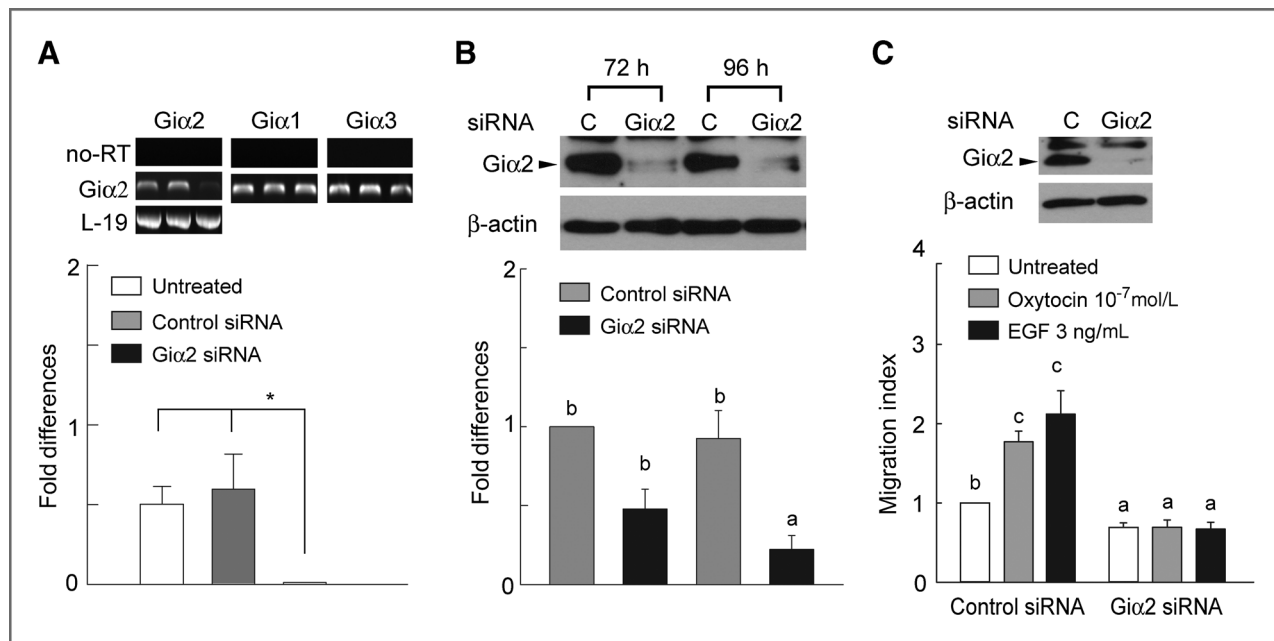
Substitution of the C352 for G results in a PTx-resistant  $Gi\alpha 2$  mutant that retains the ability to transmit signaling of GPCRs (9). PC3 cells were transiently transfected with wild type  $Gi\alpha 2$  and the  $Gi\alpha 2$ -C352G mutant. Expression of wild-type  $Gi\alpha 2$  and the  $Gi\alpha 2$ -C352G are shown in Fig. 2B (top). After pretreatment with PTx (200 ng/mL) overnight, OXT (100 nmol/L) treatment induced cell migration only in the PC3 cells that were transfected with the  $Gi\alpha 2$ -C352G

mutant (Fig. 2B). These results indicate that activated OXTR couples with  $Gi\alpha 2$  protein to exert OXT-induced PC3 cell migration.

#### The endogenous $Gi\alpha 2$ is essential for migration of prostate cancer cells

Control and  $Gi\alpha 2$ -targeting siRNAs were transfected into PC3 cells. RNA samples were harvested 72 hours after transfection. Total cell proteins were harvested at 72 and 96 hours after transfection. As shown in Fig. 3A,  $Gi\alpha 2$  mRNAs were the least abundant among the 3  $Gi\alpha$  isoforms in PC3 cell line. Control siRNA did not affect mRNA levels of  $Gi\alpha 1$ -3 in these cells. The  $Gi\alpha 2$ -targeting siRNA specifically reduced  $Gi\alpha 2$  mRNAs by 96% in this cell line (Fig. 3A). This  $Gi\alpha 2$ -targeting siRNA caused a significant reduction (~75%) in  $Gi\alpha 2$  protein levels in PC3 cells at 72 and 96 hours after transfection (Fig. 3B).

OXT and EGF induced cell migration in the PC3 cells that were pretreated with control siRNA (Fig. 3C). On the other hand, these effects of OXT and EGF were completely eliminated in the PC3 cells treated with the  $Gi\alpha 2$ -targeting siRNA (Fig. 3C). Inhibition of EGF-induced cell migration by the  $Gi\alpha 2$ -targeting siRNA was unexpected. To further examine whether  $Gi\alpha 2$  has a specific role in the EGF-induced cell migration, PC3 stable cell lines were generated to ectopically express wild-type  $Gi\alpha 2$  and the siRNA-resistant *Giα2* genes (Fig. 4A). The  $Gi\alpha 2$ -targeting siRNA completely eliminated EGF-induced cell migration in the PC3 cells that ectopically expresses wild-type  $Gi\alpha 2$  (Fig. 4A). However, the  $Gi\alpha 2$ -targeting siRNA failed to inhibit effects of EGF on migration of PC3 cells that expressed the



**Figure 3.** Gi $\alpha$ 2-targeting siRNA specifically silences expression of Gi $\alpha$ 2 in PC3 cells. A, RNA samples from PC3 cells were harvested 72 hours after transfection with control and Gi $\alpha$ 2-targeting siRNAs. Gi $\alpha$ 1-3 mRNA levels were detected with RT-PCR and real-time RT-PCR. No reverse transcriptase (RT) control samples were made by substitution of reverse transcriptase with RNase. Control siRNA did not affect mRNA levels of Gi $\alpha$ 1-3, the Gi $\alpha$ 2-targeting siRNA specifically reduced Gi $\alpha$ 2 mRNAs by 96%. *L-19* gene was amplified as a template control. B, total cell lysates from PC3 cells were harvested at 72 and 96 hours after siRNA transfection.  $\beta$ -actin was detected as a sample loading control. Band density was quantified and fold changes over 72 hours control siRNA treatment were calculated. Data were expressed as mean  $\pm$  SEM (A and B,  $n = 3$ ) and analyzed by ANOVA and Duncan's modified range tests. C, endogenous Gi $\alpha$ 2s are important for PC3 cell migration. OXT as well as EGF-induced cell migration in the PC3 cells that were treated with control siRNA. These effects of OXT and EGF were completely eliminated in the PC3 cells that were treated with the Gi $\alpha$ 2-targeting siRNA. Total cell lysates from PC3 cells were harvested after siRNA transfection followed by Western blot analysis with Gi $\alpha$ 2 antibody.  $\beta$ -actin was detected as a sample loading control. Data were summarized as mean  $\pm$  SEM ( $n = 5$ ) and analyzed by ANOVA and Duncan's modified range tests. Significant differences between groups in a given category ( $P < 0.05$ ) are designated with different lowercase letters.

siRNA-resistant *Gi $\alpha$ 2* gene (Fig. 4A). These data clearly support a critical role of Gi $\alpha$ 2 in EGF effects on cell migration. We have previously shown that EGF induces migration of DU145 prostate cancer cells whereas these cells are not responsive to OXT effects on cell migration (27). The Gi $\alpha$ 2-targeting siRNA, which caused a 93% reduction of Gi $\alpha$ 2 protein levels, blocked EGF-induced cell migration in DU145 cells (Fig. 4B).

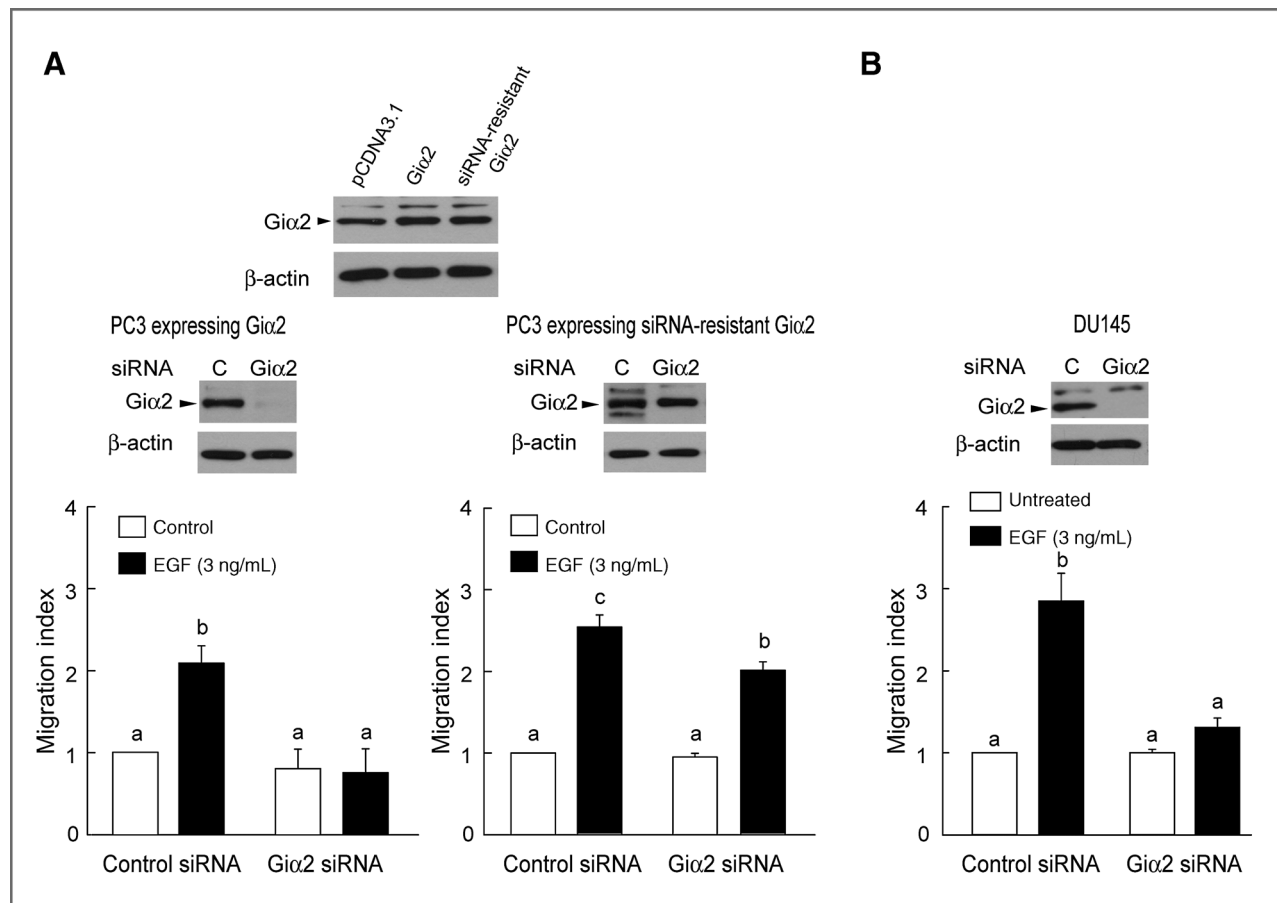
## Discussion

OXT is a peptide hormone that regulates normal prostate functions (22–24). However, the information is very limited on the effects of OXT on prostate cancer progression. In the present study, OXTR protein was detected only in prostate cancer cell lines, although OXTR mRNA is expressed in normal and prostate cancer cell lines (27). OXTR was also detected in the PHM1-41 cell lines that express endogenous OXTR (31). These data indicate that expression of OXTR is amplified in prostate cancer cells. Immunohistochemistry staining for OXTR has shown that expression of OXTR is negative in normal human prostate epithelium (26). However, OXTR has been shown to be expressed in hyperplastic as well as neoplastic prostate epithelium and to be increased in the neoplastic tissues when compared with the hyperplastic

tissues (25). The expression profile of OXTR in normal and prostate cancer cell lines reflects its expression in human prostate in normal and disease states. Bioinformatic mining of oncomine microarray database ([www.oncomine.org](http://www.oncomine.org)) has shown that expression of OXTR is significantly increased in metastatic prostate cancer specimens when compared with normal prostate specimens (32).

OXTR couples to multiple G proteins including Gq and Gi. In turn, these G proteins may convey OXT signaling to different intracellular pathways leading to distinct cellular functions (33). Activated OXTR has been shown to induce migration of PC3 cells (27). This effect of OXT is sensitive to PTx pretreatment, indicating the involvement of Gi/o-dependent signaling (27). Inhibition of the intracellular cAMP accumulation is the canonical function of activated Gi/o proteins (5). By using a luciferase reporter assay to monitor changes in intracellular cAMP levels, we show that OXT caused a dose-dependent inhibition of forskolin-induced luciferase activity. Hence, OXT activates Gi/o-dependent pathways in PC3 cells.

OXTR has been shown to couple to Gi $\alpha$ 3 in pregnant rat myometrium (29). Nevertheless, there is no evidence that excludes OXTR from coupling to other Gi/o $\alpha$  family members. In fact, OXTR has recently been shown to couple to all of the Gi/o $\alpha$  isoforms in transfected HEK293 cells



**Figure 4.** Endogenous *Giα2* plays a specific role in EGF-induced migration of prostate cancer cells. A, expression of *Giα2* was detected in total cell lysates from PC3 stable cell lines that ectopically express wild type *Giα2* or the siRNA-resistant *Giα2* genes. Total cell lysates from these cells were collected after *Giα2*-targeting siRNA followed by Western blot analysis with *Giα2* antibody.  $\beta$ -actin was detected as a sample loading control. The effect of EGF on cell migration was blocked by the *Giα2*-targeting siRNA in the PC3 cells that ectopically expresses wild-type *Giα2*. However, this effect of EGF became insensitive to the *Giα2*-targeting siRNA in the PC3 cell that expresses the siRNA-resistant *Giα2*. B, The *Giα2*-targeting siRNA blocked EGF-induced migration of DU145 cells. Total cell lysates from DU145 cells were harvested after siRNA transfection followed by Western blot analysis with *Giα2* antibody.  $\beta$ -actin was detected as a sample loading control. Data were expressed as mean  $\pm$  SEM (A,  $n = 3$ . B,  $n = 4$ ) and analyzed by ANOVA and Duncan's modified range tests. Significant differences between groups in a given category ( $P < 0.05$ ) are designated with different lowercase letters.

(34). Expression of *Gi/oα* isoforms has not been characterized in normal and prostate cancer cell lines. Although *Giα1-3* were the predominant *Gi/oα* family members that were expressed at the mRNA levels in PC3 cells, *Giα2* was most abundant isoform which was detected at the protein level in this cell line. Trace amounts of *Giα3* protein were detected in the membrane fraction whereas *Giα1* protein was completely absent in these cells. This finding cannot be explained by differential regulation of *Giα1-3* mRNAs because *Giα2* mRNA was expressed at the lowest level among the three *Giα* isoforms in PC3 cells. Another possibility is that there might be mutations to cause codon shift in the coding regions of *Giα1* and *Giα3* genes. However, this proved not to be the case because wild-type *Giα1* and *Giα3* genes were identified in PC3 cells. Therefore, both *Giα1* and *Giα3* could be diminished in PC3 cells by an unknown posttranscriptional mechanism, which will be studied in the future. In accordance with the present finding,

*Giα3* protein has been shown to be diminished in primary prostate cancer specimens (35). Ectopic expression of the *Giα2*-C352G mutant reversed the inhibition by PTx of OXT-induced cell migration in the transfected PC3 cells, suggesting that OXTR couples to *Giα2* that may mediate the OXT-induced migration of PC3 cells. To investigate whether the endogenous *Giα2* was involved in the OXTR-regulated PC3 cell migration, we used a specific siRNA to silence expression of *Giα2* gene. The *Giα2*-targeting siRNA eliminated the *Giα2* mRNA by 96%, but did not alter *Giα1* and *Giα3* mRNA levels in PC3 cells. A 93% reduction in *Giα2* mRNA was also observed in DU145 cells that were treated with the *Giα2*-targeting siRNA. These data indicate that the *Giα2*-targeting siRNA is very effective in knocking down expression of *Giα2* mRNAs in both PC3 and DU145 cell lines. The *Giα2*-targeting siRNA significantly blocked the expression of *Giα2* protein in PC3 cells, compared with the control siRNA-treated group. Under the validated

condition, the Gi $\alpha$ 2-targeting siRNA completely inhibited the OXT-induced cell migration in PC3 cells. These data indicate that the endogenous Gi $\alpha$ 2 is required for the OXT-induced migration of PC3 cells. The Gi $\alpha$ 2-targeting siRNA was also shown to inhibit EGF-induced cell migration in both PC3 and DU145 cell lines. Both Gi $\alpha$ 1 and Gi $\alpha$ 3 have been shown to play important roles in EGF-induced cell migration (36, 37). However, our study has provided the first line of evidence supporting a similar role of Gi $\alpha$ 2 in EGF function. Gi $\alpha$ 2 plays a specific role, because the Gi $\alpha$ 2-targeting siRNA was no longer effective to block the EGF-induced cell migration in the PC3 cell line that expresses a siRNA-resistant *Gi $\alpha$ 2* gene. Despite the fact that there is no comprehensive picture about how Gi $\alpha$ 2 is integrated in the EGF signaling, Gi $\alpha$ 2 appears to play an essential role in prostate cancer cell migration in response to EGF. Our knockdown studies also indicate that the trace amounts of Gi $\alpha$ 3 protein are unable to rescue OXT or EGF-induced cell migration in the absence of Gi $\alpha$ 2 protein.

In summary, the present study has provided direct evidence that support the expression of OXTR in prostate cancer cell lines. OXT treatment has been shown to activate the Gi/o $\alpha$ -dependent signaling. Gi $\alpha$ 2 is the most abundant member of the Gi/o $\alpha$  family that is detected at the protein

level in PC3 cells. Endogenous Gi $\alpha$ 2 is required for OXT- as well as EGF-induced cell migration in prostate cancer cells. These data suggest that Gi $\alpha$ 2 plays an essential role in prostate cancer cell migration.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** M. Zhong, B.T. Vo, S.A. Khan

**Development of methodology:** M. Zhong, B.T. Vo

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Zhong

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Zhong, S. Clarke, B.T. Vo

**Writing, review, and/or revision of the manuscript:** M. Zhong, B.T. Vo, S.A. Khan

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Zhong, S. Clarke

**Study supervision:** M. Zhong, S.A. Khan

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