GATA4 is a negative regulator of contractility in mouse testicular peritubular myoid cells

Yu-Qian Wang^{1,2}, Aalia Batool^{1,2}, Su-Ren Chen¹ and Yi-Xun Liu¹

¹State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China and ²University of Chinese Academy of Sciences, Beijing, China

Correspondence should be addressed to S-R Chen or Y-X Liu; Email: chensuren@ioz.ac.cn or liuyx@ioz.ac.cn

Abstract

Reduced contractility of the testicular peritubular myoid (PTM) cells may contribute to human male subfertility or infertility. Transcription factor GATA4 in Sertoli and Leydig cells is essential for murine spermatogenesis, but limited attention has been paid to the potential role of GATA4 in PTM cells. In primary cultures of mouse PTM cells, siRNA knockdown of GATA4 increased the contractile activity, while GATA4 overexpression significantly attenuated the contractility of PTM cells using a collagen gel contraction assay. Using RNA sequencing and qRT-PCR, we identified a set of genes that exhibited opposite expressional alternation between *Gata4* siRNA vs nontargeting siRNA-treated PTM cells and *Gata4* adenovirus vs control adenovirus-treated PTM cells. Notably, ion channels, smooth muscle function, cytokines and chemokines, cytoskeleton, adhesion and extracellular matrix were the top four enriched pathways, as revealed by cluster analysis. Natriuretic peptide type B (NPPB) content was significantly upregulated by GATA4 overexpression in both PTM cells and their culture supernatant. More importantly, the addition of 100 μ M NPPB could abolish the promoting effect of *Gata4* silencing on PTM cell contraction. Taken together, we suggest that the inhibitory action of GATA4 on PTM cell contraction is mediated at least partly by regulating genes belonging to smooth muscle contraction pathway (e.g. *Nppb*).

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Introduction

Spermatogenesis is fundamental to the establishment and maintenance of male fertility, while abnormal spermatogenesis will lead to male infertility/subfertility or various testicular tumors. It is generally considered that mammalian spermatogenesis is a complex sequential process of germ cell differentiation from primordial germ cells or spermatogonial stem cells (SSCs) to functional haploid sperm (Chen & Liu 2015, Chen *et al.* 2016c). In addition to germ cell development, spermatogenesis requires significant contributions of somatic cell populations, including testicular Sertoli cells, Leydig cells, peritubular myoid (PTM) cells, blood vessels and macrophages (Rebourcet *et al.* 2014, DeFalco *et al.* 2015). The importance of Sertoli cells, Leydig cells and macrophages in spermatogenesis has been fully revealed via cell ablation strategy and conditional knockout mouse models (Rebourcet *et al.* 2014, DeFalco *et al.* 2015). However, the regulatory roles and mechanism of PTM cells in testis development and spermatogenesis remain largely unknown.

PTM cells are the main cellular components of the wall of seminiferous tubules. They possess smooth muscle-like characteristics and are thought to be important for the intratesticular transport of immotile sperm. Loss of contractility (phenotypical switch) in human PTM cells

may contribute to sub- or infertility, because PTM cell markers, such as myosin heavy chain (MYH11) and alpha smooth muscle actin (α -SMA) are often lost or diminished in PTM cells of men with impaired spermatogenesis (Schell *et al.* 2010, Welter *et al.* 2013). Recent studies have shown that several PTM cell-expressed genes, such as colony-stimulating factor 1 (CSF1) (Oatley *et al.* 2009), androgen receptor (AR) (Welsh *et al.* 2009), leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) (Qian *et al.* 2013) and glial cell line-derived neurotrophic factor (GDNF) (Chen *et al.* 2014, 2016a), are essential for mouse spermatogenesis by knockout mouse models. Furthermore, contraction and relaxation of PTM cells are regulated by sympathetic innervation and paracrine and endocrine substances, including endothelin (EDN1, ET-1), angiotensin II (ANGII), platelet-derived growth factor (PDGF-BB), prostaglandin F2 α (PGF2 α), neurotransmitters and hormones (Mayerhofer 2013). However, the overall role of PTM cells and individual function of PTM cell-expressed genes remain poorly understood; especially, the intrinsic genes/transcription factors that regulate the PTM cell contractility have been largely neglected.

Transcription factor GATA4 is an important regulator of spermatogenesis. In murine Leydig cells, GATA4 regulates steroidogenesis and glycolysis (Bergeron *et al.* 2015, Schrade *et al.* 2015). Our previous study suggests that GATA4 in Sertoli cells is vital for establishment and

maintenance of the SSC pool by attenuating chemokine signaling, taking advantage of conditional knockout mice (Chen *et al.* 2015). Regulation of chemokines by GATA4 is further proved by a microarray-based gene expression study (Schrade *et al.* 2016). In addition to Leydig and Sertoli cells, GATA4 is expressed in the nuclei of PTM cells. However, the potential role of GATA4 (both *in vitro* and *in vivo*) in PTM cell population remains unknown.

Here, we assessed the impact of *Gata4* knockdown and overexpression on primary cultures of adult mouse PTM cells. Using a functional assay and complementary transcriptome analysis, we show that GATA4 negatively regulates PTM cell contraction, involving its target genes implicated in ion channels and smooth muscle function. Our *in vitro* study will provide the solid basic data for exploring the role of GATA4 in PTM cells and spermatogenesis using conditional knockout mice.

Materials and methods

Animals

C57BL/6J male mice were obtained from Vital River Laboratory (Beijing, China). All animal work was approved by the Institutional Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences.

Primary PTM cell isolation and culture

The PTM cell isolation protocol was mainly adopted from Chen *et al.* (2014). Briefly, PTM cells were isolated from 10-week-old C57BL/6J male mice. Enzyme digestion 1: tunica albuginea was removed and digested with 1 mg/mL Collagenase4 (Sigma-Aldrich) and 1 mg/mL DNase1 (Sigma-Aldrich) in PBS solution at 34°C in a water bath for 15 min and washed three times with PBS to remove interstitial cells. Enzyme digestion 2: the remaining seminiferous tubules were further digested with 1 mg/mL Collagenase 4 and 1 mg/mL DNase1 in PBS for 20 min at 34°C to release PTM and other cells. After precipitation, supernatant was collected and centrifuged for 10 min at 600g. Cells were resuspended in DMEM/F12, applied to the top of a Percoll (Sigma-Aldrich) step gradient (differing by 5% from 20 to 60% Percoll) and centrifuged at 4°C for 20 min at 800g. Most PTM cells were located at the 35–40% Percoll interface. The isolated PTM cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS) (BD Biosciences, CA, USA) at 37°C with 5% CO₂. The purity was determined by immunocytofluorescence with an antibody of PTM cell marker α -SMA (Abcam, ab5694).

siRNA transfection and adenovirus infection

For GATA4 knockdown, primary PTM cells were transiently transfected with a pool of four siRNAs targeting mouse *Gata4* (5'-AGAGAAUAGCUUCCGAACCA-3', 5'-GGAUAUGGGUGUCCGGGU-3', 5'-CUGAAUAAAUCUAAGACGC-3', 5'-GGACAUAAUACCCGCGUAA-3') or with nontargeting control siRNA (5'-UGGUUUACAUGUCGACUAA-3'; all from

GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen) at a final concentration of 0.1 μ M. The cells were harvested at 48 h after transfection for RNA sequencing and qRT-PCR analysis. To overexpress GATA4, the PDV4 adenovirus containing mouse *Gata4* cDNA (1329bp) was generated by GenePharma. Viral constructs were transduced into a 293T cell line, and a high titer (10^{10} IU/mL) of viral particles was obtained by four rounds of amplification. PTM cells were infected with *Gata4*-adenovirus (20 pfu/cell) and analyzed via qRT-PCR after 24 h. GenePharma green fluorescent protein (Ad-GFP) served as a negative control.

RNA sequencing

Total RNA (1 μ g for each samples) was extracted from *Gata4* siRNA-, *Gata4* adenovirus- and control oligonucleotide-treated PTM cells using TRIzol reagent (Tiangen, Beijing, China) as the manufacturer's instructions. Three duplicate samples for each treatment were included. Then, equal amounts of total RNA were analyzed using BGISEQ-500 platform (Fehlmann *et al.* 2016) and the sequencing procedure was performed by BGI (Shenzhen, China). The list of differentially expressed genes (with fold change cut-offs ≥ 2 or ≤ 0.5 and significance *P* value < 0.05) was provided as Supplementary Table 1 (see section on supplementary data given at the end of this article).

Quantitative RT-PCR (qRT-PCR)

Briefly, measurement of RNA integrity and synthesis of cDNA were performed as previously described (Chen *et al.* 2013). Each sample was measured in triple independent experiments. Samples CT values were normalized to *Gapdh* CT values and relative expression levels were calculated using the $\Delta\Delta$ CT method. Primer pairs were listed in Table 1.

Immunostaining

Mouse adult testes were harvested and fixed in 4% paraformaldehyde (PFA) overnight. Five-micron paraffin-embedded sections were cut for staining. Immunostaining methods were performed as described (Li *et al.* 2013). The sections were incubated with the primary antibody of MYH11 (Abcam, ab53219) or GATA4 (Santa Cruz, sc-1237, TX, USA) at 4°C overnight. For negative controls we incubated the sections with the corresponding IgG isotype instead of the antiserum. Fluorescent secondary antibodies were purchased from Cell Signaling Technology and nuclei were stained with DAPI (Beyotime, Shanghai, China). For immunohistochemistry, secondary and third antibodies were obtained from ZSGB-Bio (Beijing, China) and staining was visualized using a diaminobenzidine substrate kit (ZSGB-Bio).

Collagen gel contraction assay

Twenty-four hours after transfection with siRNA or adenovirus, PTM cells (without FBS) were used for a collagen contractility assay according to the manufacturer's instructions (Cell Biolabs, CA, USA). 2,3-Butanedione monoxime (Cell Biolabs), a cell contraction inhibitor, was used as a negative control. Pictures

Table 1 Primers used for qRT-PCR.

Gene	Primer (5'–3')	
<i>Gata4</i>	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
<i>Kcnk12</i>	TCATCGCCTACGGACTGTTC	TGAAGAGCGAGTAGATGCAGC
<i>Kcnq5</i>	GTCGGCGCAACGTCAGTA	AACCAACACAAGGAGAAAAACG
<i>Atp1a3</i>	TCTCAGATGTGTCCGTTCTTCT	TGGAAAGAGAGTGAAAGGCAAG
<i>Nalcn</i>	CAAAGATGCACATACGGGGAA	AAAGCCCGGATCATAATGAGTG
<i>Nppb</i>	GAGGTCACTCCTATCCTCTGG	GCCATTCCTCCGACTTTTCTC
<i>Gpr4</i>	GCTGGCGCTCTACCTGATG	AGGCGATGCTGATATAGATGTTG
<i>Ednrb</i>	GTGGCTTCTGGGGGTATGG	TCTTAGTGGGTGGCGTCATTA
<i>Agt</i>	TCTCCTTACCACAACAAGAGCA	CTTCTCATTACAGGGGAGGT
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTCA

of free-floating collagen gel lattices were taken 2 days after release and analyzed with ImageJ. The degree of contraction was evaluated by determining the area of the gel matrix before and after treatment.

Enzyme-linked immunosorbent assay

NPPB measurement from cell lysates and culture supernatant was performed using a mouse NPPB ELISA kit (JONLN, Shanghai, China), following the manufacturer's instructions. NPPB concentration (pg/mL) was determined by absorbance measurements at 450nm against a standard curve in a competitive assay using an ELISA reader.

Cell viability assay

Cell viability was determined by counting the ratio of live cells to total cells after transfection by an automated cell counter (Thermo Fisher Scientific).

Statistics

Data were compared for statistical significance using GraphPad Prism version 5.01 (GraphPad Software Inc.). Student's *t* tests

were used for the analyses. The data were presented as the mean $\pm$ S.E.M. of at least three independent experiments, and differences were considered statistically significant at **P* < 0.05 and ***P* < 0.01.

Results

Exploring the role of GATA4 in primary mouse PTM cells using gain- and loss-of-function approaches

To identify the expression pattern of GATA4 within mouse testes, we performed immunological staining of GATA4 and a PTM cell marker MYH11. In addition to Sertoli cells and Leydig cells, transcription factor GATA4 was specifically expressed in PTM cells (Fig. 1, arrowheads) at the periphery of seminiferous tubules within the testes. To investigate the role of GATA4 in this cell population, primary mouse PTM cells were isolated using Percoll discontinuous gradients and further identified by immunostaining of the PTM cell marker α -SMA (Fig. 2A). Approximately 94% of the isolated cells were α -SMA positive, indicating that we obtained primary PTM cells with high purity (Fig. 2B). Next, we used siRNA and adenovirus to inhibit or overexpress

Figure 1 GATA4 localization in PTM cells within mouse adult testes. (A) Immunofluorescence staining for a PTM cell marker MYH11 (green signals) on the tissue sections of 8-week-old testes. Nuclei were stained with DAPI. (B) GATA4 detection by immunohistochemical staining (brown signals) in 8-week-old testis sections. Scale bar, 50 μ m. Arrowheads indicate PTM cells at the periphery of seminiferous tubules.

Figure 2 PTM cell isolation and alternation of GATA4 expression. (A) Representative image of isolated cells staining with a PTM cell marker α -SMA (red signals). Nuclei were stained with DAPI. Scale bar, 10 μ m. (B) Histogram illustrating the purity of isolated PTM cells, indicated by the percentage of α -SMA-positive cells. (C) Immunocytochemistry staining of GATA4 (brown signals) in *Gata4* siRNA- and nontargeting siRNA-transfected PTM cells. Scale bar, 50 μ m. (D) Relative mRNA level of *Gata4* in *Gata4* siRNA- and nontargeting siRNA-transfected PTM cells. (E) Cell staining of GATA4 in *Gata4* adenovirus- and control adenovirus-infected PTM cells. Scale bar, 50 μ m. (F) Altered mRNA level of *Gata4* in PTM cells after *Gata4* adenovirus infection. In B, D, F, data are presented as the mean $\pm$ S.E.M. ** indicates $P < 0.01$ by Student's *t* test.

GATA4 expression in PTM cells, respectively. The morphology of PTM cells was normal and their viability was unaffected after transfection (Supplementary Fig. 1). Immunocytochemistry staining showed markedly reduced GATA4 expression in *Gata4* siRNA-treated cells (Fig. 2C) and increased expression after *Gata4* adenovirus infection (Fig. 2E). *Gata4* mRNA level was reduced by approximately 60% in cells treated with *Gata4* siRNA vs nontargeting siRNA-treated cells by qRT-PCR analysis (Fig. 2D). Conversely, *Gata4* adenovirus infection significantly increased the mRNA expression of *Gata4* (>8-fold) (Fig. 2F).

Negative regulation of PTM cell contraction by GATA4

Given that testicular PTM cells possess smooth muscle-like characteristics and GATA4 is well known to determine the contractile ability of cardiomyocytes (Ieda et al. 2010, Aries et al. 2014), we sought to assess the regulatory role of GATA4 on testicular PTM cell contraction. Accordingly, a collagen gel contraction assay was applied to PTM cells that were pretreated with *Gata4* siRNA, *Gata4* adenovirus or their nontargeting controls. As an evaluation index, the contraction area was calculated after the 2-day observation cycle. The morphology of PTM cells in collagen gel appeared normal and undistinguishable among groups (Supplementary Fig. 1). We found that siRNA knockdown of GATA4 in

PTM cells significantly increased the contractile activity of PTM cells (Fig. 3A and C). By contrast, adenovirus overexpression of GATA4 in PTM cells resulted in a 42% decrease in contractile activity of PTM cells, as the contraction area was significantly reduced (Fig. 3B and C). Furthermore, we found that *Gata4* siRNA-treated PTM cells showed obviously higher expression of α -SMA and more pronounced stress fibers than nontargeting controls and *Gata4* adenovirus-treated PTM cells (Fig. 3C). Collectively, these data suggest that GATA4 is a negative regulator of contractility in mouse PTM cells.

Changes of gene expression profiles in PTM cells influenced by GATA4

To gain mechanistic insight into the negative regulation of PTM cell contraction by transcription factor GATA4, we performed RNA sequencing of *Gata4* siRNA-treated, *Gata4* adenovirus-treated, and their nontargeting control PTM cells. The expression levels of 91 genes were significantly increased (fold change (G4_Adv/NT_Adv) ≥ 2 , P value < 0.05) in *Gata4* adenovirus-treated PTM cells; meanwhile, they were significantly downregulated (fold change (G4_siRNA/NT_siRNA) ≤ 0.5 , P value < 0.05) in *Gata4* siRNA-transfected PTM cells. A second class of 32 genes was negatively regulated by GATA4 in PTM cells (Fig. 4A). Cluster analysis revealed that the top four enriched pathways were ion channels (Fig. 4A and

Figure 3 Negative regulation of PTM cell contraction by GATA4. (A) Collagen contractility assay in PTM cells transfected with *Gata4* siRNA or *Gata4* adenovirus or their nontargeting controls for 2 days. (B) Quantitative data showing the contraction area (cm²) in *Gata4* siRNA-, *Gata4* adenovirus- and their control-treated PTM cells. Data are presented as the mean $\pm$ S.E.M. * indicates $P < 0.05$, ** indicates $P < 0.01$ by Student's t test. (C) Immunofluorescence images showing the distribution of α -SMA in treated PTM cells. α -SMA filaments organized in strongly labeled stress fibers after *Gata4* silencing. Scale bar, 10 μ m.

B), smooth muscle function (Fig. 4A and C), cytokines and chemokines (Fig. 4A and D), and cytoskeleton, adhesion and extracellular matrix (Fig. 4A and E).

GATA4 regulates genes implicated in ion channels and smooth muscle contraction

We hypothesize that genes implicated in ion channels ($n=11$) and smooth muscle function ($n=15$) are regulated by GATA4 and their dysregulation may contribute to the attenuated contractile ability of *Gata4* adenovirus-treated PTM cells. Consistent with RNA sequencing data, the mRNA levels of genes linked to ion channels, such as *Kcnk12*, *Kcnq5*, *Atpl3* were significantly upregulated in *Gata4* adenovirus-treated PTM cells (Fig. 5A, B and C), while *Nalc1* was negatively regulated by GATA4 as revealed by qRT-PCR (Fig. 5D). Furthermore, the mRNA levels of *Nppb* and *Gpr4*, two quiescence factors of contraction, were significantly elevated after *Gata4* adenovirus infection (Fig. 5E and F). Notably, GATA4 overexpression significantly attenuated mRNA levels of two well-known stimulators of PTM cell contraction, including *Ednrb* (encoding for endothelin receptor) and *Agt* (encoding for angiotensinogen) (Fig. 5G and H). In agreement with transcriptome and qRT-PCR data, we found that natriuretic peptide type B (NPPB) content was significantly upregulated after

Gata4 adenovirus treatment in both PTM cells and their culture supernatant (Fig. 5I). More importantly, the addition of 100 μ M NPPB could abolish the promoting effect of *Gata4* siRNA on testicular PTM cell contraction (Fig. 5J). Collectively, the inhibitory action of GATA4 on PTM cell contraction is mediated at least partly by regulating genes belonging to ion channels and smooth muscle contraction signaling (e.g. *Nppb*) (Fig. 5K).

Discussion

GATA4 is expressed in several testicular somatic cell populations (Sertoli cells, Leydig cells and PTM cells) that play distinct roles in spermatogenesis (Bielinska et al. 2007, Mazaud Guittot et al. 2007, Penny et al. 2017). We and peers have revealed that GATA4 in Sertoli cells is essential for establishment and maintenance of SSC pool (Chen et al. 2015), while GATA4 regulates steroidogenesis and glycolysis in Leydig cells (Schrade et al. 2015). Here, we aimed to investigate the role of GATA4 in PTM cells and found that PTM cell contraction was negatively controlled by GATA4 partly by regulating genes belonging to ion channels and smooth muscle contraction signaling (e.g. *Nppb*).

So far, four agonists (endothelin, angiotensin II, platelet-derived growth factor, prostaglandin F₂ α) have been reported to stimulate testicular PTM cell

Figure 4 Gene expression profile of *Gata4*-silenced and -overexpressed PTM cells. (A) RNA sequencing discovering 91 *GATA4*-positively regulated genes and 32 genes that are negatively regulated by *GATA4* in PTM cells. Table of biologic processes enriched in differentially expressed genes (KEGG analysis). (B, C, D and E) Expression levels (FPKM values) of selected gene sets, which are displayed with activities of ion channels (B), smooth muscle function (C), cytokines and chemokines (D), and cytoskeleton, adhesion and ECM (E) in *Gata4* siRNA-, *Gata4* adenovirus- and control-treated PTM cells. Red frames indicate genes that were further confirmed by qRT-PCR.

contraction (Tripiciano *et al.* 1998, Romano *et al.* 2006, 2007, Welter *et al.* 2014). Endothelin (also known as EDN1 or ET-1) has been shown to be produced by Sertoli cells and to bind EDNRB, a high-affinity receptor on

PTM cells, thus stimulating second messenger cascades and contractile response (Fantoni *et al.* 1993, Filippini *et al.* 1993, Romano *et al.* 2007). Our study suggests that *Ednrb* is repressed by *GATA4* in PTM cells; thus,

Ion channels:

Smooth muscle contraction:

Figure 5 Dysregulation of genes implicated in ion channels and smooth muscle contraction in *Gata4*-overexpressed PTM cells. (A, B, C and D) Differential expression of genes belonging to ion channels, such as *Kcnk12* (A), *Kcnq5* (B), *Atpla3* (C) and *Nalcn* (D) between *Gata4* adenovirus- and control-treated PTM cells. (E, F, G and H) The mRNA levels of genes implicated in smooth muscle contraction, such as *Nppb* (E), *Gpr4* (F), *Ednrb* (G) and *Agt* (H) in *Gata4*-overexpressed and control groups. (I) Analysis of the content of NPPB (pg/mL) in *Gata4* adenovirus- or nontargeting control-transfected PTM cells and their culture media by ELISA assay. M, culture media; C, cells. (J) Quantitative data showing the contraction area (cm²) in control-, *Gata4* siRNA alone-, *Gata4* siRNA plus 100 μM NPPB-treated PTM cells. Data are presented as the mean ± S.E.M. * indicates $P < 0.05$, ** indicates $P < 0.01$ by Student's *t* test. (K) Hypothetical and simplified model of critical factors (e.g. NPPB) involved in the regulation of PTM contraction by GATA4.

the EDN1 (ligand)-EDNRB (receptor) signaling may be attenuated after GATA4 overexpression. Based on the RNA sequencing and qRT-PCR data, we suggest that GATA4 negatively regulates PTM cell contraction likely via both depressing the expression of *Agt* (encoding for angiotensinogen) and *Ace* (encoding for angiotensin-converting enzyme) and upregulating the level of *Gpr4* that may be a reductor of angiotensin receptor affinity (Sanada et al. 2016, Sun et al. 2016). Angiotensin I (ANGI) is formed by the action of renin on AGT, and it is further converted to ANGII through removal of two C-terminal residues by the enzyme ACE (Welter et al. 2014). Notably, forced GATA4 expression upregulates both the mRNA and protein level of natriuretic peptide type B (NPPB), an agonist of smooth muscle cell contraction (Carvajal 2014, Orlandi et al. 2015). Addition of NPPB could abolish the promoting effect of *Gata4* silencing on PTM cell contraction, indicating NPPB is also an inhibitor for PTM cell contraction and involved in the regulation of PTM contractility by GATA4. Another GATA4 positively regulated gene that attracts our attention is pentraxin 3 (*Ptx3*). *Ptx3* has been identified as a major product of human PTM cells by secretome analysis (Flenkenthaler et al. 2014). A recent study further suggest that biglycan

(BGN) serves as a ligand of toll-like receptor 2 (TLR2) and increases the protein expression and secretion of pentraxin 3 (PTX3) in PTM cells (Mayer et al. 2016). However, the role of PTX3 in the testis and its relationship with transcription factor GATA4 and PTM cell contraction needs further investigation.

Studies on roles of ion channels in testicular PTM contraction do not exist. However, there is close relationship between smooth muscle cell contraction and ion channels, because mobilization of intracellular ions plays critical roles in initiating and modulating the intensity and duration of contraction response (Hakonarson & Grunstein 1998). Our high-throughput screening reveals that eleven genes belonging to ion channels are potentially regulated by GATA4, of which *Kcnq5*, *Rnd1*, *Nalcn* and *Kcnma1* are suggested to regulate smooth muscle cell contraction (Loirand et al. 1999, Jepps et al. 2009, Reini et al. 2015, Chen et al. 2016b, Mani et al. 2016). As testicular PTM cells are smooth muscle-like cells, further studies investigating the role of these ion channel-related genes on PTM cell contraction will be of great interest.

Interestingly, bioinformatics analysis and literature search of differentially expressed genes further revealed

that approximately 24% (17 of 72) of GATA4-regulated genes could be linked to EGF-EGF receptor (EGFR) pathway. The altered expression of these 17 genes was further confirmed by qRT-PCR (data not shown). EGF could be modified by a couple of GATA4-regulated genes, including *Nppb*, *Grem1*, *Bmp8b*, *Omd*, *Ptgs2* and *Atf3*. EGFR could be regulated by *Ubash3b*, *Ereg*, *Reps2*, *Fgfr4* and *Gprc5a*. Downstream signaling of EGF-EGFR pathway, such as RAS/RAF/MEK/ERK, STAT and PI3K/AKT/mTOR, could be targeted by *Elmod1*, *Olr1*, *Cish*, *Pvrl4*, *Atp1a3* and *Igf2b1*. Collectively, our preliminary results suggest that the EGF-EGFR pathway may be involved in downstream signaling of GATA4 in PTM cells and is potential critical for PTM cell contraction.

Two previous studies investigated the roles of AR and GDNF in PTM cells and spermatogenesis using flox mice and *Myh11*-Cre strain (Welsh *et al.* 2009, Chen *et al.* 2016a). It is essential to further observe the *in vivo* role of *Gata4* in PTM cells by generating *Gata4*-conditional knockout mouse models. However, we found embryonic lethality in *Gata4*^{flox/flox}, *Myh11*-Cre mice. Accordingly, screening of PTM cell-specific markers should be of great concern, because *Myh11*-Cre is expressed in all kinds of smooth muscle cells, including, but not limited to PTM cells (Chen & Liu 2016).

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0148>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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