Biochemical and Biophysical Research Communications 426 (2012) 65–70

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The local microenvironment instigates the regulation of mammary tetratricopeptide repeat domain 9A during lactation and involution through local regulation of the activity of estrogen receptor α

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ARTICLE INFO

Article history:
Received 2 August 2012
Available online 14 August 2012

Keywords:
TTC9A regulation
ERalpha reactivation
Mammary gland

ABSTRACT

Tetratricopeptide repeat domain 9A (TTC9A) belongs to a family of TTC9 proteins. Its induction by progesterone in breast cancer cells was associated with marked growth inhibition and induction of focal adhesion. TTC9A interacts specifically with actin-binding protein tropomyosin Tm5NM-1 which stabilizes actin filament and focal adhesion. However, the function of TTC9A is still obscure. This study exploited mice model to characterize the regulation of TTC9A gene expression during mammary development and explored possible mechanisms of TTC9A gene regulation. It was demonstrated that mammary TTC9A expression is distinctively down-regulated in gland undergoing functional differentiation (lactation) and up-regulated during involution. Furthermore, TTC9A expression during lactation and involution is regulated by the factors in the local microenvironment. This is illustrated with teat sealing model in which the teat sealed glands (undergoing involution) expressed significantly higher levels of TTC9A protein and mRNA than the contralateral non-sealed lactating glands. Importantly, this local induction of TTC9A expression upon involution coincided with the re-activation of estrogen receptor α (ERα). Together with the observation that TTC9A is a direct ERα target gene, we propose that the fall and rise of TTC9A levels during lactation and involution is caused by the changes of ERα activity that is in turn regulated by the factors in the microenvironment.

1. Introduction

Tetratricopeptide repeat domain 9A (TTC9A) is a member of TTC9 family proteins that also include TTC9B and TTC9C. Human TTC9A gene was first identified by sequence analysis of a cDNA clone from a brain cDNA library [1]. It was confirmed to code for a 222 amino acid TTC9A protein in human using mouse polyclonal antibody [2]. Human TTC9B and TTC9C share 46% and 35% homology with TTC9A in amino acids sequence, respectively [3]. However, TTC9B and TTC9C identities have yet to be confirmed at the protein level.

TTC9A protein contains three tetratricopeptide repeat (TPR) domains. The TPR domain is a 34 amino acid (aa) consensus motif found in tandem repeats of varying numbers in a large family of TPR-containing proteins [4–6]. The TPR motifs generally form an antiparallel α-helical hairpin [7–9]. Clustering of these hairpins in tandem generates a domain with a grooved surface that can conveniently grasp another polypeptide. The flexible TPR motif therefore presents an elegant evolutionary solution for coordinating protein interactions [10]. The functions of TPR-containing proteins are diverse and include cell cycle control [11], RNA splicing [12], protein transport especially protein import [13], regulatory phosphate turnover [14], and protein folding [15]. TPR domain-containing proteins also play an integral part in steroid hormone receptor signaling. Hip, Hop, FKBP51, FKBP52 and cyclophllin 40 are essential for steroid receptor assembly and maturation [6,10,16].

Studies have suggested that TTC9A may play a role in the regulation of cell growth but its precise mode of action is cellular context-dependent. TTC9A was induced by mitogens, estrogen and growth factors in breast cancer cells MCF-7 [2]. TTC9A expression is also significantly higher in breast cancer tissues than the adjacent normal tissues [17]. These indicate that TTC9A expression is positively associated with cell proliferation and the presence of growth-stimulating signals. On the other hand, induction of TTC9A by progesterone in PR-transfected breast cancer cells MDA-MB-231 was associated with marked growth inhibition [18].

The induction of TTC9A by progesterone was also associated with induction of stress fibers and focal adhesion [2,19]. The involvement of TTC9A in cell adhesion may be linked to its specific interaction with tropomyosin Tm5NM-1 (3). Tm5NM-1 is an actin

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.036

binding protein that was reported to promote the stability of stress fibers and inhibits disassembly of focal adhesions in a number of cell lines [20,21]. It is plausible that Tm5NM-1 partners with TTC9A to regulate the stability of actin filament and cell-matrix interaction.

To understand potential functions TTC9A in the regulation of cell proliferation and adhesion, we exploited mice model to study the regulation of TTC9A gene expression during various stages of mammary development. The mammary gland undergoes cycles of growth, differentiation and involution during the reproductive cycle [22–24]. There is rapid mammary growth during the first two weeks of pregnancy, followed by morphological and functional differentiation, leading to lactation. The program of functional differentiation in the lactating mammary gland is switched off upon weaning, when the mammary gland undergo involution characterized by programmed cell death and tissue remodeling [25]. Here we studied TTC9A expression during various stages of mammary development and explored mechanisms that regulate TTC9A expression. The study demonstrated that TTC9A expression is positively regulated as a function of mammary growth and negatively associated with functional differentiation. More interestingly, the regulation of TTC9A expression during lactation and involution is mediated through the local microenvironment. The study also provided evidence that this local regulation of TTC9A expression is mediated by de-activation and re-activation of estrogen receptor alpha.

2. Material and methods

2.1. Chemical

17-β-Estradiol Benzoate, Phosphate Buffered saline, Dimethylsufoxide (DMSO), Aprotinin, Sodium Vanadate and Ponceau Red were from Sigma Aldrich (St. Lious, MO). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was from Promega (Fitchburg, WI), Triton X-100, Pepstatin A, Phenylmethylsulfonyl fluoride, Leupeptin were from Roche (Switzerland, Basel) and Sodium fluoride (Merck, NJ, USA). Nexaband Skin Sealer was obtained from Abbott laboratories (Illinois, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Antibody for GAPDH was obtained from Amersham Biosciences (Buckinghamshire, UK). TTC9A polyclonal antibody was generated in mice using full-length recombinant TTC9A protein as was reported previously [2].

2.2. Animal care and experimental procedures

Balb/c mice were obtained from the Sembawang Laboratory Animals Centre, National university of Singapore (NUS). All in vivo procedures and animal care followed the guidelines on the Care and Use of Animals for Scientific Purposes (2004) set by National Advisory Committee for Laboratory Animal Research (NACLAR) in Singapore. They were housed at the School of Biological Sciences (SBS), Nanyang Technological University under the standard specific pathogen free (SPF) conditions and fed ad libitum.

For timed pregnancy studies, three 8-week-old female mice were housed with one male mouse and the morning on which a cervical plug was found was designated as day 0 of pregnancy. The day of parturition was designated day 0 of lactation. Pups were removed 21 days after parturition unless otherwise indicated. The day when pups were removed is designated as day 0 of involution.

To evaluate if TTC9A expression is modulated by the circulating hormones or the local environment, mammary teats on the left sides were sealed using the surgical adhesive agent, Nexaband to induce involution whereas the mammary glands on the right side continued lactating. Mice were monitored after sealing to ensure complete teat closure and uninterrupted nursing on open teats. Paired open and closed glands were harvested at 48 h and 7 day time points after teat closure for analysis of TTC9A gene expression.

To determine if estrogen regulates TTC9A expression in vivo, immature female mice of 16 days old were injected subcutaneously with 20 μg/kg of 17β-estradiol benzoate in sesame oil. The control mice were injected sesame oil only. Mice were injected twice and sacrificed after 48 h. Organs were harvested and snap-frozen in liquid nitrogen.

To collect tissues from mice for protein and RNA analysis, mice were anesthetized with a combination of ketamine and xylazine and sacrificed by cervical dislocation. Tissues were dissected and snap-frozen in liquid nitrogen before stored at −80 °C. Prior to Protein and RNA extraction, the frozen tissues were grounded to power with mortar and pestle cool with liquid nitrogen.

2.3. Protein extraction and Western blotting analysis

Approximately 20 mg of grounded tissue powder was homogenised in cold lysis buffer containing 100 mM NaF, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF and the cocktail of proteinase inhibitors (5 μg/ml pepstatin A, 5 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM Na3VO4). Protein lysates were cleared by centrifuging at 14,000 rpm for 20 min at 4 °C. Protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay protocol from Pierce (Rockford, Illinois).

For Western blotting analysis of TTC9A expression, 20 μg of total protein from each tissue was analyzed and membranes were probed with TTC9A antibody, followed with GAPDH antibody to verify the amount of protein loading. The TTC9A and GAPDH band intensities were analyzed with GS800 densitometer. The relative TTC9A protein expression was then determined after normalizing the TTC9A expression with GAPDH.

2.4. RNA extraction and real-time RT-PCR

Approximately 50 mg of grounded tissue power was used for total RNA extraction using Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). 5 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed with SYBR Green master mix on an ABI Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA). Primers for mouse TTC9A, PRB, Greb1, Ampreg, Sprr2A, FOXA1 and ER alpha were used to analyze their gene expression in tissue samples (Supplement Table 1). Mouse acidic ribosomal phosphoprotein (36B4) primers were included in each experiment to normalize the quantity of cDNA used. The PCR for each gene fragment was performed in triplicate, and each primer set was repeated twice.

2.5. Statistical analysis

The Mann Whitney non parametric statistical analysis was carried out for most of the data in the experiment. The observation was considered significant if the p value determined after the statistical analysis was <0.05.

3. Results and discussion

Mouse TTC9A codes for a 219 aa protein. It can be conveniently detected in mouse embryonic stem cells and day 11.5 embryos (Supplementary Fig. 1A). All the organs from mouse embryos at 18.5 days express TTC9A (Supplementary Fig. 1B) as all the organs in adult mice do (Supplementary Fig. 1C). It is also notable that

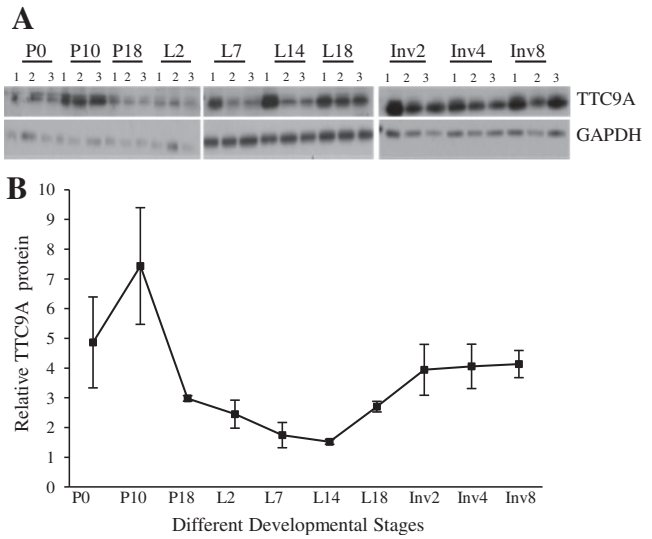

Fig. 1. TTC9A is differentially regulated during Mammary gland development. Mammary gland of Balb/c mice was harvested at different stages of development, total proteins extracted and probed with TTC9A mice polyclonal antibody. (A) Western blot showed that TTC9A was regulated at pregnancy, lactation and involution. P0, P10, P18 stands for pregnant day 0, day10 and day 18 respectively. L2, L7, L14, L18 stands for lactation day 2, day 7, day 14 and day 18 respectively. Inv2, Inv4 and Inv8 stands for involution day 2, day 4 and day 8, respectively. (B) Densitometry data of relative TTC9A protein expression from Fig. 1A with GAPDH levels as loading controls (Mean ± SEM, $n = 3$). *Note:* Most mice in the experiment had 6–8 pups. But mouse 1 at L7 and L14 had only two pups due to cannibalism and expressed much higher levels of TTC9A than other mice of the same stage. Hence mouse 1 at L7 and L14 were considered as outliers and not included in the densitometry calculation since they do not represent lactation state.

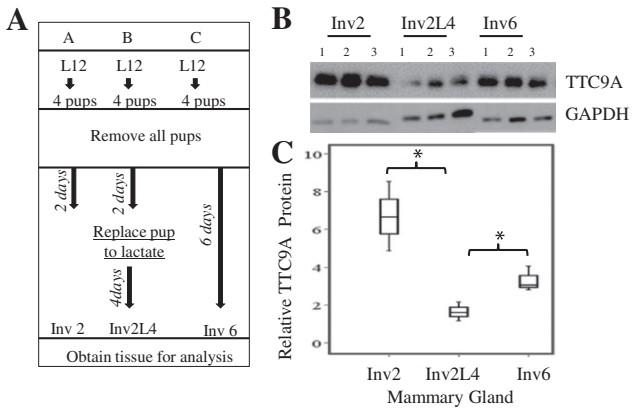

Fig. 2. Restoration of lactation after involution down-regulates TTC9A protein. (A) Experimental design for studying the effect of restoration of lactation on mammary TTC9A expression. Pups were removed from the mothers at day 12 of lactation to induce involution. Group Inv2 was at 48 h involution, group Inv2L4 was made to re-lactate with pups for 4 days after involution for 48 h (Inv2) and group Inv6 continued involution for 6 days. (B) Restoration of lactation at Inv2L4 reduced TTC9A protein levels significantly compared with that at Inv2 and Inv6. (C) Box plot illustrating densitometry data of relative TTC9A protein expression with GAPDH as loading controls (Mean ± SEM, $n = 3$). Mann–Whitney non-parametric analysis was performed on the data to determine statistical significance. $^{*}p < 0.05$.

TTC9A expression level is the lowest in liver. The relative low level of TTC9A in liver were also observed in human tissue blot [2]. Although TTC9A is ubiquitously expressed in all organs from the embryonic cell stage, its function in organ or animal development is unclear.

3.1. TTC9A expression is regulated during the cycle of mammary development

Work in mice and some other animal models showed that early to mid-pregnancy is associated with rapid mammary growth followed by structural and functional differentiation during late pregnancy [22,23]. Once lactation ceases through either natural weaning or pups removal, the mammary glands undergo involution characterized by apoptosis and tissue remodeling. Fig. 1 shows that the highest levels of TTC9A protein was observed at day 10 of pregnancy when mammary cell proliferation index is the highest [26]. Significant decrease of mammary TTC9A protein was observed from day 10 to day 18 of pregnancy when the mammary gland has initiated structural and functional differentiation [27]. This was followed by progressive decline of TTC9A protein levels at day 2, 7 and 14 of lactation. These data implies that TTC9A expression is suppressed in functionally differentiated mammary gland. This notion is strengthened by two additional observations. First, TTC9A protein level goes up when milk yield declined. Milk production in laboratory mice generally peaks at day 14 to 15 of lactation and declines markedly by day 18 [28]. Accordingly, mammary TTC9A protein level went up at day 18 of lactation and this relatively high level of TTC9A persisted in involuting mammary gland. Second, a mouse with fewer pups, which is associated with lower milk yield, is also associated with higher TTC9A expression. As is shown in Fig. 1A, mice 1 at lactation day 7 (L7) and 14 (L14)

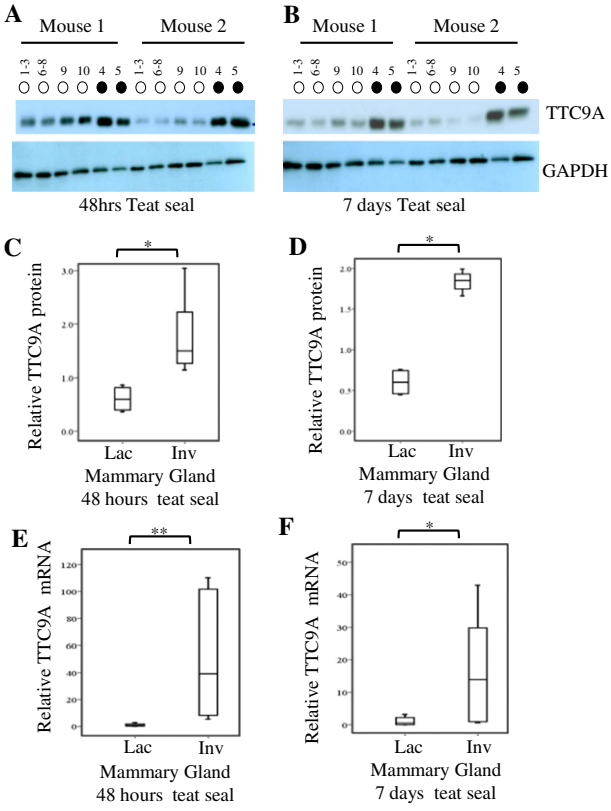

Fig. 3. Involution-associated up-regulation of mammary TTC9A expression is mediated by the local microenvironment. The 4th and 5th mammary glands (●) were sealed at day 7 of lactation to induce involution while the 9th, 10th and three pairs of cervical-thoracic glands (1–3 and 6–8) (○) were left open for lactation. Mammary glands were harvested at 48 h (A, C and E) or 7 days (B, D and F) and analysed for TTC9A protein and mRNA expression. Figure A and B show Western blotting analysis of TTC9A expression; Figure C and D are densitometry data of figure A and B, respectively, after normalizing against GAPDH. Relative TTC9A mRNA expression at 48 h (E) and 7 days (F) are presented as box plot after normalizing against the 36B4 mRNA levels by RT-PCR analysis. The TTC9A protein and mRNA was significantly higher in the sealed glands than the open glands. $^{*}p < 0.05$, $^{**}p < 0.01$. Lac- Lactating, Inv- Involution.

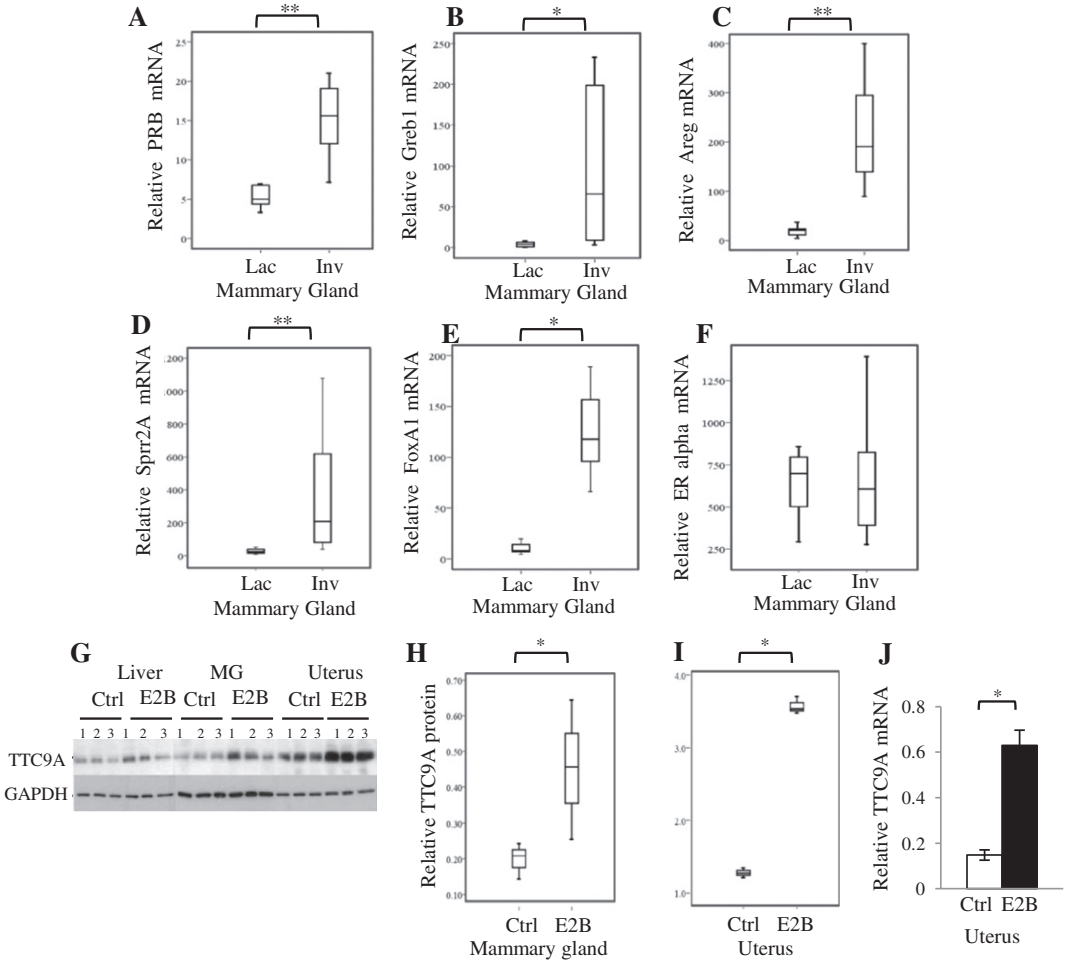

Fig. 4. Up-regulation of mammary TTC9A in involution is associated with ERα reactivation (A–F). Significant increase in mRNA of estrogen regulated genes PR (A), Greb1 (B), Areg (C), Sprr2A (D) and FOXA1 (E) in the sealed versus the open gland but there was no significant change in the ERα mRNA (F). Data obtained from RNA samples of four pairs of mammary gland of two mice. Estrogen induces mammary and uterine TTC9A expression in mice (G–J). Two weeks Balb/c mice were injected with estradiol benzoate (E2B) for 48 h. (G) Western blot shows TTC9A expression in control and E2B treated liver, mammary gland and uterus. GAPDH was used as loading control. Box plot ($n = 3$) shows significant increase in relative TTC9A protein expression in the mammary gland (H) and uterus (I) on E2B treatment. Relative TTC9A mRNA increased by threefold in the uterus (J) on E2B treatment. 36B4 was used as the cDNA input control. Data analyzed by Mann–Whitney non-parametric statistics. $^{*}p < 0.05$, $^{**}p < 0.01$.

with only 2 pups (due to cannibalism) had much higher TTC9A protein than other mice with 6–8 pups. These observations suggest a negative association between TTC9A expression and functional differentiation of the mammary gland.

The data described so far indicate that mammary TTC9A protein expression is up-regulated during the phase of rapid mammary growth, decreases with lactation and bounces up when the mammary gland undergoes involution. We further tested if restoration of lactation after weaning is able to emulate the decrease of TTC9A expression. In this experiment, the litter size is normalized to 4 per mother mouse (Fig. 2A). Lactating mice at post-partum day 12 were weaned by pup removal and divided into 3 groups as is illustrated in Fig. 2A. The mammary gland of group Inv2 were collected after 2 days of involution (Inv2); group Inv2L4 mice were given back the pups for restoration of lactation at Inv2 and the mammary glands were collected after 4 days of lactation (Inv2L4); the mammary gland from group Inv6 were collected after 6 days of involution. It is clear that the mammary glands with re-established lactation (Inv2L4) expressed significantly less TTC9A protein compared to mammary glands from day 2 involution and day 6 involution (Inv6) (Fig. 2B and C). Therefore, TTC9A expression is specifically regulated according to the differentiation states of the mammary gland.

3.2. TTC9A expression during lactation and involution is regulated by the local microenvironment

We next looked into possible mechanisms of lactation- and involution-associated TTC9A expression changes. It has long been believed that mammary involution and the associated mammary cell death is initiated by the withdrawal of lactogenic hormones such as prolactin and glucocorticoids [25]. To determine if involution-induced increase of TTC9A expression level is the result of the systemic change of hormonal activity or is regulated by the local environment, we used a mice model in which the 4th and 5th mammary teats were sealed with surgical adhesive agent Nexaband to induce involution while the other mammary glands continued lactating. The open (unsealed) and closed (sealed) mammary glands were harvested at 48 h and 7 days after the procedure. Note that 4 samples of lactating (open) mammary tissue (from 1–3, 6–8, 9th to 10th mammary gland) and 2 involuting (sealed) mammary tissue were analyzed for TTC9A protein and mRNA expression. Although the glands were under the influence of the same systemic hormonal milieu, the involuting (sealed) glands express significantly higher levels of TTC9A compared to the contra lateral lactating (open) gland at both 48 h (Fig. 3A and C) and 7 days' time points (Fig. 3B and D). TTC9A mRNA in

involuting glands was increased by an average of 50-fold after 48 h compared to the contralateral lactating controls (Fig. 3E), and by 20-fold after 7 days (Fig. 3F). It is plausible that the increase of TTC9A gene expression in involuting mammary gland is part of the genetic program initiated under the influence of local environment in association with apoptosis and tissue remodeling.

3.3. TTC9A up-regulation during involution is associated with ERα re-activation

We then explored possible regulator of TTC9A gene expression. Progesterone receptor (PR) is an ERα-dependent target gene. It was reported over three decades ago that PR was absent from the lactating mammary glands as a result of ERα inactivation. In contrast, PR was detectable in the contra lateral thelectomized, non-lactating mammary glands of the same mouse [29,30]. We asked if the up-regulation of TTC9A in the contralateral sealed glands at 48 h post-sealing was associated with up-regulation of ERα target genes in general. The mRNA levels of 4 bona fide ERα target genes (PR, GREB1, amphiregulin (Areg) and Sprr2A) were compared between 4 pairs of lactating glands and the contra lateral involuting glands by real-time RT-PCR (Fig. 4). Expectedly, there were significantly higher levels of expression of all four genes in the contralateral sealed, involuting mammary glands than the lactating glands. The average fold of increase for PR (Fig. 4A), GREB1 (Fig. 4B), amphiregulin (Fig. 4C) and Sprr2A (Fig. 4D) was approximately 3, 18, 14 and 20, respectively. The data thus confirmed that TTC9A up-regulation in involuting mammary gland is associated with up-regulation of ERα target genes at 48 h post-involution. More interestingly, FOXA1, an ERα–DNA interaction licensing factor [31], was also significantly increased in the involuting mammary glands compared to the contralateral lactating glands (Fig. 4E). ERα inactivation in lactating glands has been associated with the inability of ERα to bind to chromatin in vitro [33,34]. FOXA1 is essential for ERα regulation of gene transcription through facilitating ERα-chromatin interaction [31,35,36]. It is likely in this context that FOXA1 up-regulation is critical for the re-activation of ERα in the involuting mammary gland through chromatin remodeling which in turn result in the up-regulation of TTC9A gene expression.

We next investigated if TTC9A is a target gene of ERα in the mammary gland. Balb/c mice at 2-week's old were injected with estradiol benzoate (E2B) in sesame oil or with sesame oil only. Uteri, mammary gland and liver were harvested 48 h later for analysis of TTC9A expression. The mice responded to E2B treatment with expected increase of uterine weight (data not shown). E2B stimulated significant increases of both TTC9A protein in the uterus and mammary glands (Fig. 4G–J). TTC9A mRNA is also induced by estrogen shown with uterine samples, confirming that TTC9A is induced by estrogen at the transcriptional levels. Indeed, analysis of TTC9A gene sequence using Dragon ERE Finder version 2 [32] revealed two putative ERE sequences (−346 to −363 and −1010 to −1027 from transcription start site) with a sensitivity of 97% and 87%, respectively. Hence TTC9A is likely a direct ERα target gene.

In summary, this study revealed that TTC9A expression is up-regulated during rapid mammary growth in the mid-pregnancy, down-regulated during lactation and elevated during mammary involution. Forced involution and restoration of lactation also emulated the aforementioned changes of TTC9A expression. Furthermore, unilateral teal sealing experiments suggest that the fall and rise of TTC9A during lactation and involution are independent of systemic hormones and was therefore regulated by paracrine or autocrine factors in the local microenvironment. Importantly, this local regulation of TTC9A expression was positively associated with local activity of ERα and the expression of ERα licensing factor FOXA1. Since TTC9A gene contains two canonical ERE motifs and is induced by estrogen, we propose that the changes of TTC9A expression is caused by the local regulation of ERα activity and the concurrent regulation of FOXA1 is at least partly responsible for the re-activation of ERα in the involuting mammary gland. Based on the knowledge that TTC9A binds specifically to tropomyosin Tm5NM1 [17] and Tm5NM1 functions to stabilize actin filaments and inhibits the disassembly of focal adhesions [20,21], we speculate that the up-regulation of TTC9A during involution may function together with Tm5NM1 to stabilize ECM structure to minimize tissue disintegration during mammary cell apoptosis and tissue remodeling. This research was funded by the Biomedical Research Council of Singapore.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.036.

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