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Estrogen, tamoxifen, and Akt modulate expression of putative housekeeping genes in breast cancer cells

Khyati N. Shah, Jesika S. Faridi*

Department of Physiology and Pharmacology, Thomas J. Long School of Pharmacy and Health Sciences, University of the Pacific, 3601 Pacific Ave., Stockton, CA 95211, United States

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ABSTRACT

Clinically, Akt overexpression has been associated with tamoxifen resistance, and multiple *in vitro* breast cancer models of tamoxifen resistance have been developed. In order to study the mechanism of this tamoxifen resistance, differential gene expression studies have been performed utilizing quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Since accurate data normalization requires the use of a stable reference gene, the goal of this study was to identify the most stable reference gene for RT-qPCR (from a panel of putative housekeeping genes) that remains unaltered despite estrogen or tamoxifen treatment or stable overexpression of active Akt. Gene expression of nine candidate genes was determined in parental and Akt overexpressing MCF-7 breast cancer cells treated with estrogen, tamoxifen, or vehicle, and gene stability was analyzed using two different statistical models. Based on our results, we suggest *RPL13A* as suitable internal reference gene that is both stable and remains unaltered in MCF-7 cells regardless of estrogen or tamoxifen treatment or Akt overexpression. We also validated that expression levels for *RPL13A,* as well as *RPLP0* (another member of the RPL protein family), remain unaltered after estrogen and tamoxifen treatment in the ER positive ZR-75-1 cell line and ER negative MDA-MB-468 breast cancer cell line. Both *RPL13A* and *RPLP0* levels were also stable in normal and tumor mammary tissue from Her2 overexpressing mice. In addition, our work emphasizes the importance of a preliminary study to validate each reference gene that will be used for RT-qPCR.

1. Introduction

Breast cancer is the most common cancer in American women with an estimated 207,090 newly diagnosed cases in 2010. In the United States, about 39,840 women are expected to die from breast cancer, and about 1 in 8 women (between 12 and 13%) will develop breast cancer over the course of her lifetime [1]. Selective estrogen receptor modulators (SERMs) such as tamoxifen are an important initial regimen to treat breast cancer with 30% of treated patients developing systemic recurrence due to resistance. Overexpression of the receptor tyrosine kinases, HER2, EGFR, IGFR, and the signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways, following their activation are frequently involved in SERM resistant breast cancers [2,3].

Akt, also known as protein kinase B (PKB), exists as three closely related isoforms Akt1, Akt2, and Akt3. The enzymatic activity of Akt1 is increased by phosphorylation on its two critical amino acid residues namely threonine at 308 and serine at 473. The phosphorylated form of Akt, also known as p-Akt or active Akt, induces many downstream signaling cascades and has implicated in drug resistance [4]. In one study, the overexpression of p-Akt was detected in 58% of breast cancer samples and p-Akt was confirmed as a negative prognostic marker [5]. Several studies have reported that activation of Akt in breast cancer predicts a worse outcome in tamoxifen treated patients. Survival analyses have revealed that patients that were p-Akt positive were more prone to relapse with distant metastatic cancer. High cytoplasmic expression of Akt1 and Akt3 were correlated with high active Akt (p-Akt) which is implicated in endocrine resistance and an increased incidence of metastatic tumors [6].

Estrogen exerts its biological action via estrogen receptors (ERs), which act as ligand-activated transcription factors to regulate various physiological processes including gene expression [7]. ERs regulate gene expression via binding to estrogen responsive elements (EREs) or binding to Sp or Ap-1 complexes [8]. In addition, half ERE sites that are in close proximity to Sp and Ap-1 sites may govern estrogen responses in estrogen responsive gene promoters [9]. The SERM, tamoxifen can activate ER mediated induction of promoters regulated by Ap-1 and Sp [8]. In addition, growth factor activation of the PI3K/Akt pathway has been shown to activate the estrogen receptor and mediate tamoxifen resistance in breast cancer. Cross-talk between Akt and estrogen signaling involves activation of ER transcriptional pathways as well as activation of the PI3K/Akt and other signaling cascades [3]. Moreover, the PI3K/Akt

* Corresponding author. Tel.: +1 209 946 2964; fax: +1 209 946 2857.
E-mail address: jfaridi@pacific.edu (J.S. Faridi).

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pathway promotes a potent survival signal, and many researchers have demonstrated that activation of Akt confers chemotherapeutic drug resistance. We have previously developed active Akt overexpressing MCF-7 breast cancer cells that are able to form xenografts even in the presence of 4-hydroxy-tamoxifen [2,10].

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) has become the method of choice for quantifying gene expression of biological samples [11–13]. Accurate data normalization requires the use of a stable reference gene [6,7]. Many researchers have commonly used putative housekeeping genes (HKGs) as control reference genes without validating HKG expression for each tissue and experimental condition [12,14]. Specifically in the estrogen-dependent MCF-7 breast cancer cell line, there has been no such extensive study reported examining the expression of commonly used HKGs with respect to experimental treatments like as estrogen and tamoxifen, which are known to alter the expression of many genes and perhaps even HKGs. In this current study, we used our previously developed active Akt overexpressing breast cancer cell lines along with parental MCF-7 breast cancer cells, in order to identify stable reference genes that remain unaltered after treatment with estrogen and tamoxifen [2]. To study the effect of treatment on HKGs, we investigated the utility of a panel of nine housekeeping genes representing different functional classes and gene families as internal reference genes for normalizing real-time PCR data after experimental treatments with estrogen or tamoxifen treatment and Akt overexpression.

2. Materials and methods

2.1. Cell culture and treatment

Late-passage MCF-7 cells were originally received from Dr. Adeline Hackett at the Peralta Cancer Institute (Oakland, CA) [16]. MCF-7 cells were previously transfected with myristoylated Akt1 or Akt3 [10] and were maintained in Advanced DMEM-F12 containing phenol-red and supplemented with 5% FBS, streptomycin, and penicillin (Invitrogen, Carlsbad, CA) [10]. The ZR-75-1 cells were a kind gift from ChemGenex Pharmaceuticals (Menlo Park, CA) and were originally purchased from ATCC. The ZR-75-1 cells were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FBS, streptomycin, and penicillin (Invitrogen, Carlsbad, CA). The MDA-MB-468 cells were a kind gift from Dr. Wade Russu (University of the Pacific, Stockton, CA) and were originally purchased from ATCC. The MDA-MB-468 cells were maintained in Advanced DMEM supplemented with 5% FBS, streptomycin, and penicillin (Invitrogen, Carlsbad, CA). 17β-estradiol (E2) and 4-hydroxy-tamoxifen (Tam) were purchased from Sigma (St. Louis, MO). Cells were washed with HBSS (Invitrogen, Carlsbad, CA) and kept in phenol-red-free DMEM-F12 media (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal-stripped-FBS (Invitrogen, Carlsbad, CA) for 48 h. Cells were then washed with HBSS and treated with 10^{-8} M ethanol (V), E2, and Tam in phenol-red-free, serum-free DMEM-F12 for 24 h.

2.2. Western blot analyses

Cells were disrupted in Akt lysis buffer (all components from Sigma, St. Louis, MO), and Western blot analyses were performed as previously published [2]. Briefly, lysates were clarified by centrifugation for 15 min at 15,000 × *g*. The protein concentration was measured, and an equal amount of protein from each cell type was used for Western blotting. Akt phosphorylation was determined by probing Western blots with p-Akt–Ser^{473} antibody (Cell Signaling, Danvers, MA). Detection of bound antibody was carried out after a subsequent incubation with secondary antibody and using West Pico Chemiluminescence Reagent (Pierce, Rockford, IL).

2.3. Animal tissue

FVB-Tg(MMTV-Erbb2)NK1Mul/J mice were obtained commercially from the Jackson Laboratory (Bar Harbor, ME). During all studies, mice were maintained on ordinary Purina mouse chow and water *ad lib*. All experiments involving the use of mice were performed in accordance with protocols approved by the Institutional University Animal Care and Use Committee of The University of the Pacific (Stockton, CA). Tissues were collected from mouse mammary tumors and corresponding normal mammary tissue. Tissues were snap frozen until RNA extraction.

2.4. RNA extraction and quantification

Total RNA was isolated using RNeasy according to manufacturer's protocol (Qiagen, Valencia, CA). The concentration and A_{260}/A_{280} ratio were measured by UV absorbance. Quality was also determined on an ethidium bromide (Sigma, St. Louis, MO) stained 1.5% agarose gel (Invitrogen, Carlsbad, CA), and Kodak 1D Image Analysis Software (CareStream Health, Rochester, NY) was used to determine the intensity and ratio of the 28S to 18S ribosomal RNA bands.

2.5. RT-qPCR

RT-qPCR reactions were performed using SYBR green technology with primers against human *GAPDH*, *ACTB*, *RPL13A*, *PGK1*, *TFRC*, *PPIA*, *HPRT1*, *B2M*, *GUSB* and *RPLP0* (Real Time Primers, Elkins Park, PA) and *pS2* (Invitrogen, Carlsbad, CA) (Table 1). The qScript one-step RT-qPCR kit (Quanta Biosciences, Gaithersburg, MD) was used according to the manufacturer's instructions. Reverse transcription was carried out at 95 °C for 3 min, followed by amplification for 50 cycles of 30 s at 95 °C and 45 s at 60 °C with a final extension of 1 min at 95 °C. Primer efficiencies (*E*) were determined for all

Table 1
Putative HKG and primer descriptions.

Gene	Name	Accession no.	Size	Region	Forward primer	Reverse primer	Efficiency
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.2	238	95–332	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG	93.8%
ACTB	Actin, beta, cytoplasmic	NM_001101.2	234	736–969	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	98.4%
RPL13A	Ribosomal protein L13a	NM_012423.2	126	487–612	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	99.9%
RPLP0	Ribosomal protein, large, P0	NM_001002	191	402–592	GGAGAAACTGCTGCCTCATA	GGAAAAAGGAGGTCTTCTCG	95.7%
PGK1	Phosphoglycerate kinase 1	NM_000291.2	198	1085–1282	CTGTGGGGGTATTTGAATGG	CTTCCAGGAGCTCCAAACTG	101.1%
TFRC	Transferrin receptor	NM_003234.1	210	2042–2251	CGCTGGTCAGTTCGTGATTA	GCATTCCCGAAATCTGTTGT	101.0%
PPIA	Peptidylprolyl isomerase A	NM_021130.2	158	409–566	TTCATCTGCACTGCCAAGAC	TCGAGTTGTCCACAGTCAGC	97.0%
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1	NM_000194.1	94	496–589	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	97.5%
B2M	Beta-2 microglobulin	NM_004048.2	86	589–674	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	96.1%
GUSB	Glucuronidase, beta	NM_000181.1	171	1657–1827	AAACGATTGCAGGGTTTCAC	CTCTCGTCGGTGACTGTTCA	97.4%
pS2	Trefoil factor 1	NM_003225	173	116–288	GCCCAGACAGAGACGTGTACA	TCACACTCCTCTTCTGGAGGG	103.8%

primers using the 10-fold serial dilution method with the equation, $E = 10^{[-1/\text{slope}]}$. Each sample was analyzed in triplicate along with a negative control (noRT) for each gene using RNA samples from three independent experiments. At each cycle, the amount of fluorescence was quantified using a My iQ™ single color real time PCR detection system iCycler (Bio-Rad, Hercules, CA), and the cycle at which the signal rose above threshold (Ct) was determined. Fold change was determined using the comparative $2^{-\Delta\Delta Ct}$ method [11].

2.6. Determination of gene stability

For stability comparison of candidate reference genes, we applied the software geNorm, version 3.4 [12] and NormFinder [15]. Ct values were converted into relative quantities for analysis by logarithmic transformation of raw Ct values with geNorm to generate stability value *M* for the most stable genes. geNorm calculates the gene expression stability as measure *M* for a reference gene and generates the ranking of the tested genes according to their expression stability. NormFinder calculates the stability values for candidate reference genes for normalization. A low stability value indicates a low combined intra and intergroup variation proves high expression stability.

2.7. Statistical analysis

Statistical analyses were performed with Minitab student 12 (Minitab Inc., State college, PA) and p values < 0.05 were considered statistically significant. Two-sample t tests were used to compare vehicle treated cells with estrogen or tamoxifen treated parental, Akt1, or Akt3 overexpressing MCF-7 cells, ZR-75-1, MDA-MB-468 cells respectively.

3. Results

3.1. Confirmation of RNA integrity and primer specificity

To identify an appropriate reference gene in MCF-7 cells that remains unaltered regardless of treatment or Akt overexpression, ten putative HKG primer sets were investigated (Table 1). All RNA samples were examined for their concentration, purity, and integrity. The absorbance ratio at 260/280 of the RNA samples was in the range from 1.82 to 2.07 (1.95 ± 0.03) and indicated pure and protein-free RNA. The ribosomal RNA ratio was generated based on the ratio of ribosomal bands (28S/18S ratio > 1.75) on 1% agarose gels. Each primer set was verified to be specific by *in silico* analysis via stringent parameters using the FastPCR software [17]. The amplification specificity for each RT-qPCR analysis was confirmed by a single peak in melt curve analysis. The PCR products were confirmed by a single band of the expected size in 2.5% agarose gel electrophoresis. Primer efficiencies were determined using the slope of the standard curve obtained by serial dilution and ranged as 93.8–103.98% (Table 1). Of the ten putative HKGs, all were expressed in MCF-7 cells. *RPL13A* was the highest expressed putative HKG, and *HPRT1* was the lowest expressed putative HKG with average Ct values of 14 and 28 in vehicle treated MCF-7 cells, respectively.

3.2. Estrogen and tamoxifen alter HKG expression

RT-qPCR was performed to determine the fold change in gene expression between the vehicle, estrogen, and tamoxifen treated MCF-7 cells for a panel of nine putative HKGs. Only the expression of *RPL13A*, *TFRC*, and *GUSB* were not significantly altered in MCF-7 cells after estrogen and tamoxifen treatment in MCF-7 cells (Fig. 1A). In Akt1 overexpressing MCF-7 cells, the expression of *GAPDH*, *RPL13A*, *TFRC*, *B2M*, and *GUSB* were not significantly altered after estrogen and tamoxifen treatment (Fig. 1B). In Akt3 overexpressing MCF-7 cells, only the expression of *RPL13A*, was not significantly altered after estrogen and tamoxifen treatment (Fig. 1C).

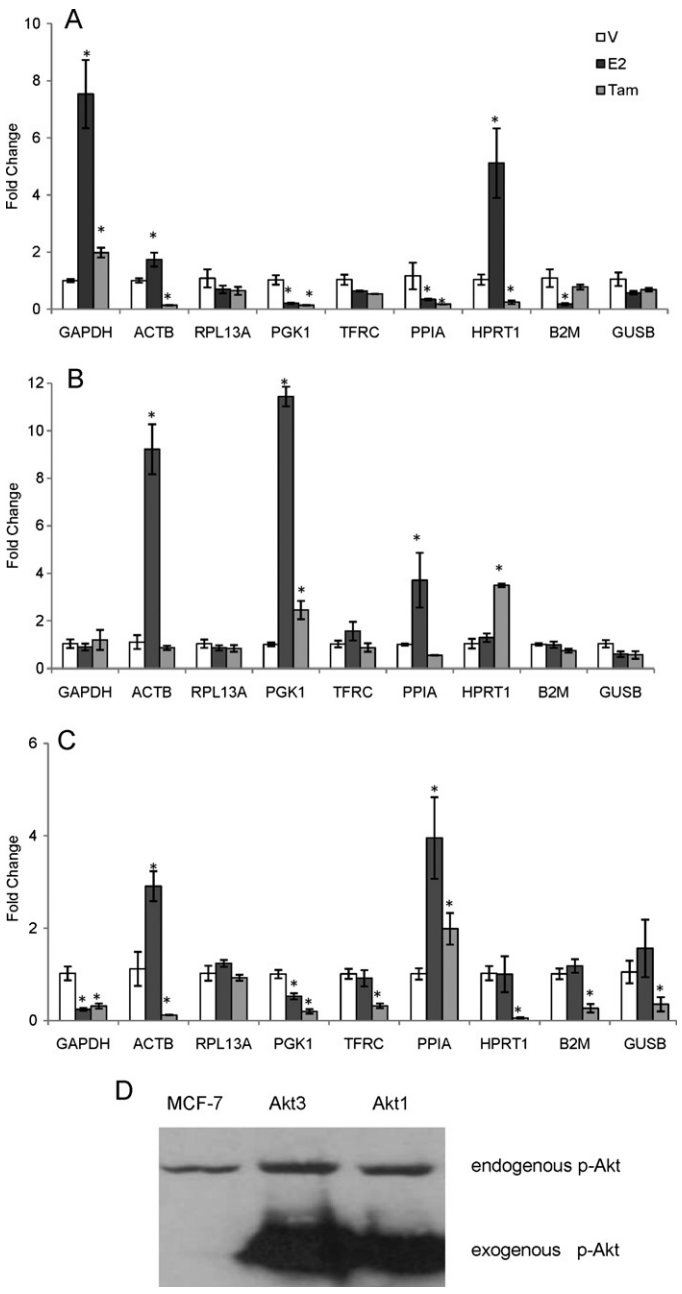

Fig. 1. Estrogen and tamoxifen modulate the expression of putative HKGs in MCF-7 cells. The fold change relative to vehicle treated parental MCF-7 was determined for (A) parental, (B) Akt1 overexpressing, and (C) Akt3 overexpressing MCF-7 cells after treatment with vehicle (V), E2, or Tam for 24 h relative to vehicle control. Error bars represent mean standard error of three individual experiments done in triplicate. Comparisons between groups were made two-sample t-tests (*$p < 0.05$). (D) The levels of active, phosphorylated Akt in the parental MCF-7 cells, the stable transfected clone of Akt3, and the stable transfected clone of Akt1 expressing MCF-7 cells are shown as assessed by Western blotting (WB) with an antibody to the pSer^{473} site. Both endogenous and transfected p-Akt levels are shown for cells grown in complete media.

3.3. Overexpression of active Akt alters HKG expression

In order to isolate the effect of active Akt overexpression on the expression of putative HKGs, the fold change of each HKG in Akt1

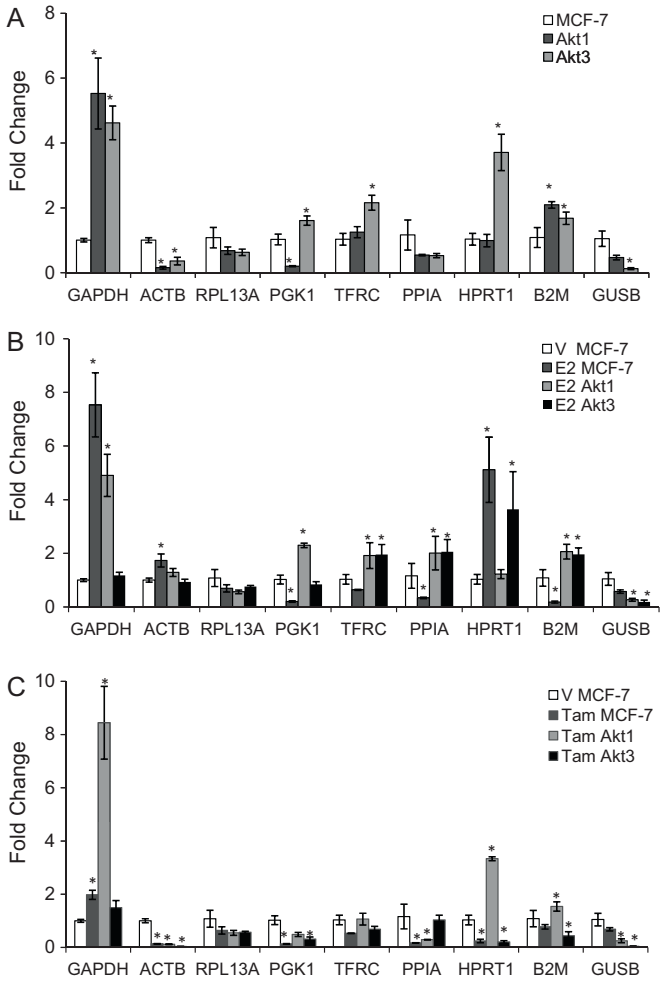

Fig. 2. Akt modulates the expression of putative HKGs in MCF-7 cells. Fold change relative to vehicle treated parental MCF-7 cells was determined for (A) vehicle, (B) E2, or (C) Tam treated parental, Akt1, and Akt3 overexpressing MCF-7 cells. Error bars represent mean standard error of three individual experiments done in triplicate. Comparisons between groups were made with two-sample *t*-tests (*$p < 0.05$).

or Akt3 overexpressing cells relative to parental MCF-7 cells was determined. Only the expression of *RPL13A* and *PPIA* were not significantly altered with both isoforms of Akt after vehicle treatment (Fig. 2A). In estrogen treated parental, Akt1, and Akt3 overexpressing MCF-7 cells as compared to vehicle treated parental MCF-7 cells, only the expression of *RPL13A* was not significantly altered in any estrogen treated cell line (Fig. 2B). *GAPDH* was unaltered in Akt3 cells treated with estrogen, while *HPRT1* was unaltered in Akt1 cells treated with estrogen as compared to vehicle treated MCF-7 cells. In tamoxifen treated parental, Akt1, and Akt3 overexpressing MCF-7 cells as compared to vehicle treated parental MCF-7 cells, the expression of *RPL13A* and *TFRC* were not significantly altered in any of the tamoxifen treated cells as compared to vehicle treated MCF-7 cells (Fig. 2C).

3.4. *RPL13A is an unaltered and highly stable gene*

The fold change of each putative HKG in estrogen or tamoxifen treated parental, Akt1, and Akt3 overexpressing MCF-7 cells as compared to vehicle treated parental MCF-7 cell was determined. Only one putative HKG, *RPL13A*, whose expression was not significantly ($p > 0.05$) altered by treatment or Akt overexpression in MCF-7 cells was identified. To analyze the expression stability of the nine

Table 2
Expression stability values of reference genes calculated by Normfinder and geNorm programs.

Rank	Gene	NormFinder	geNorm
		Stability[a]	Stability[a]
1	*TFRC*	0.229	1.25
2	*RPL13A*	0.475	1.38
3	*PGK1*	0.764	1.59
4	*PPIA*	0.822	1.63
5	*B2M*	0.841	1.65
6	*GAPDH*	0.875	1.71
7	*HPRT1*	0.927	1.77
8	*ACTB*	1.047	1.89
9	*GUSB*	1.106	1.94

[a] High expression stability is indicated by low stability value.

candidate reference genes, gene stability in parental, Akt1 overexpressing, and Akt3 overexpressing MCF-7 cells was determined using geNorm [12] and NormFinder [15] (Table 2 and Fig. 3). Using geNorm, the top two ranked genes had *M* values below 1.5, suggesting high expression stability and low combined variation, also consistent with NormFinder. Although *TFRC* was the overall most stable reference gene, this gene was significantly altered by treatment as well as Akt overexpression. The second ranked gene for expression stability, *RPL13A*, was the only significantly unaltered putative HKG after treatment or Akt overexpression in MCF-7 cells.

Since *RPL13A* is both highly stable and unaltered by treatment or Akt overexpression, the remaining eight HKGs were normalized to *RPL13A* using $2^{-\Delta\Delta Ct}$ method [11]. Fold change relative to vehicle treated MCF-7 cells is shown in Table 3 with values that were significantly altered ($p < 0.05$) indicated in bold text. Expression of *GAPDH* and *HPRT1* are significantly increased and *B2M* is significantly decreased after estrogen treatment in parental MCF-7 cells. Yet, expression of *ACTB, PGK1*, and *PPIA* are significantly decreased after tamoxifen treatment in parental MCF-7 cells. Only *GAPDH* expression is significantly increased in both vehicle treated Akt overexpressing cells suggesting that this gene is also Akt regulated.

To validate the possible utility of *RPL13A* as unaltered reference gene in other breast cancer cell lines, we investigated the effect of estrogen and tamoxifen treatment on *RPL13A* in the estrogen positive breast cancer cell line ZR-75-1(ER+/PR−) and as well as the estrogen negative MDA-MB-468 (ER-/PR−) cell line [19]. Our analysis shows that *RPL13A* is relatively unaltered regardless of estrogen and tamoxifen treatment in all three breast cancer cell lines (Fig. 4A). Since *RPL13A* is in the ribosomal protein L (RPL) family of proteins, we examined the effect of estrogen and tamoxifen

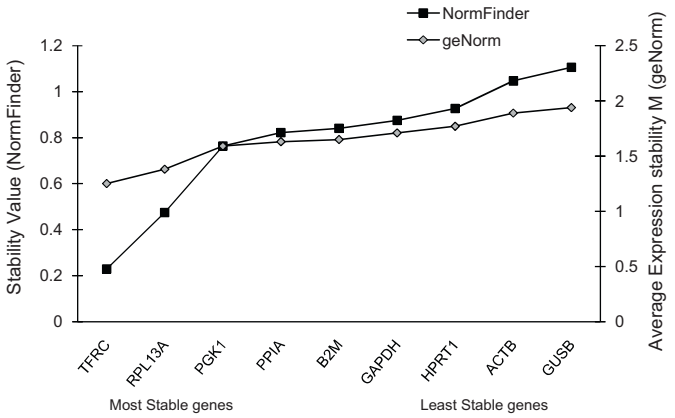

Fig. 3. GeNorm and NormFinder analysis of putative HKGs. High expression stability is indicated by a low stability value. The top two ranked genes had *M* values below 1.5 using geNorm suggesting high expression stability and low combined variation.

Table 3
Putative housekeeping genes normalized to *RPL13A*.

	MCF-7			Akt1			Akt3		
	Vehicle	Estrogen	Tamoxifen	Vehicle	Estrogen	Tamoxifen	Vehicle	Estrogen	Tamoxifen
GAPDH	1.05 ± 0.21	**11.10 ± 1.47**	3.28 ± 0.47	**8.58 ± 1.32**	**8.61 ± 0.31**	**11.25 ± 2.17**	**7.95 ± 1.28**	1.54 ± 0.10	**4.75 ± 1.09**
ACTB	1.04 ± 0.20	2.65 ± 0.57	**0.25 ± 0.47**	**0.30 ± 0.09**	2.33 ± 0.34	**0.24 ± 0.06**	0.54 ± 0.10	1.22 ± 0.07	**0.07 ± 0.01**
PGK1	**1.17 ± 0.42**	0.31 ± 0.05	**0.24 ± 0.05**	0.39 ± 0.04	**4.16 ± 0.34**	0.99 ± 0.32	2.76 ± 0.69	**1.13 ± 0.21**	0.56 ± 0.07
TFRC	**1.02 ± 0.15**	0.98 ± 0.14	0.92 ± 0.21	2.46 ± 0.58	**3.29 ± 0.39**	2.05 ± 0.58	**3.69 ± 0.83**	2.59 ± 0.49	**1.19 ± 0.01**
PPIA	1.04 ± 0.18	0.52 ± 0.09	**0.30 ± 0.06**	1.06 ± 0.15	**3.40 ± 0.65**	0.57 ± 0.11	0.86 ± 0.08	2.74 ± 0.63	2.74 ± 0.63
HPRT1	1.05 ± 0.24	**7.30 ± 0.96**	0.40 ± 0.42	2.07 ± 0.68	2.20 ± 0.32	**6.47 ± 1.25**	**5.90 ± 0.16**	**4.64 ± 1.61**	0.38 ± 0.16
B2M	**1.04 ± 0.21**	**0.29 ± 0.09**	1.39 ± 0.42	**4.06 ± 0.53**	**3.64 ± 0.05**	**2.85 ± 0.17**	2.83 ± 0.56	2.63 ± 0.47	0.77 ± 0.26
GUSB	**1.02 ± 0.15**	0.86 ± 0.11	1.13 ± 0.15	0.97 ± 0.27	0.97 ± 0.2	0.46 ± 0.12	**0.20 ± 0.03**	**0.24 ± 0.09**	**0.08 ± 0.05**

Fold change in gene expression is shown relative to control (vehicle treated parental MCF-7 cells) for three independent experiments done in triplicate. Values shown in bold text are significantly different than control ($p < 0.05$).

treatment on another RPL family member, *RPLP0*, in the MCF-7, ZR-75-1, and MDA-MB-468 breast cancer cell lines. Both *RPL13A* and *RPLP0* levels were fairly stable in all cell lines and treatments (Fig. 4A and B). Since MCF-7, ZR-75-1, and MDA-MB-468 breast cancer cell lines express normal levels of Her2, we also examined *RPL13A* and *RPLP0* expression levels in normal and tumor mammary tissue from Her2 overexpressing mice (FVB-Tg(MMTV-Erbb2)NK1Mul/J). Both *RPL13A* and *RPLP0* levels were fairly stable in both normal and tumor mammary tissue from Her2 overexpressing mice (Fig. 4C).

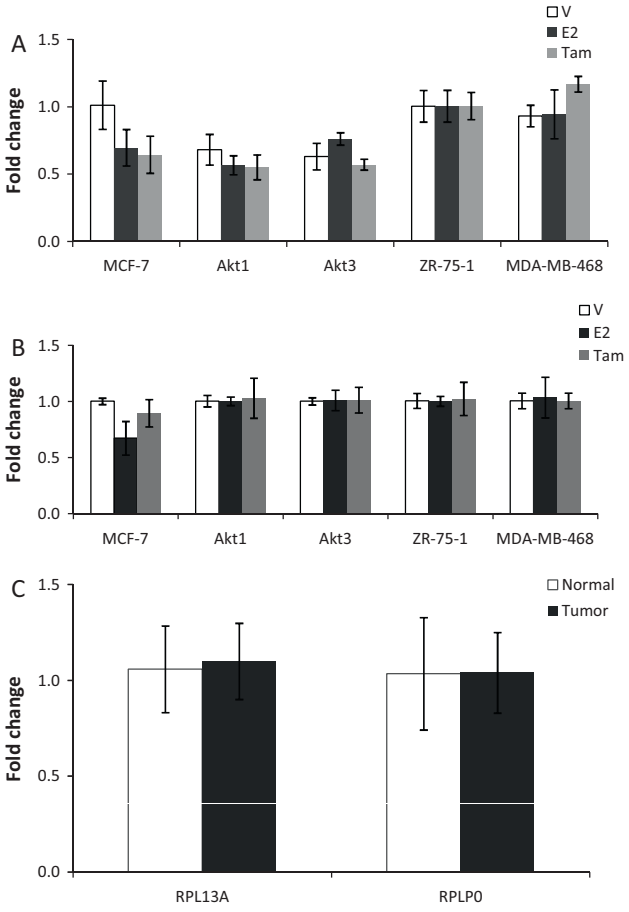

Fig. 4. *RPL13A* and *RPLP0* are unaltered by estrogen or tamoxifen treatment in breast cancer cells and tumors. The fold change of *RPL13A* (A) and *RPLP0* (B) in estrogen (E2) or tamoxifen (Tam) treated parental, Akt1, and Akt3 overexpressing MCF-7 as well as ZR-75-1 and MDA-MB-468 breast cancer cells as compared to vehicle (V) treated parental cells was determined. (C) The fold change of *RPL13A* and *RPLP0* in Her2 overexpressing normal as compared to tumor mammary tissue was determined. Error bars represent mean standard error of three individual experiments done in triplicate. Comparisons between groups were made with two-sample *t*-tests (*$p < 0.05$).

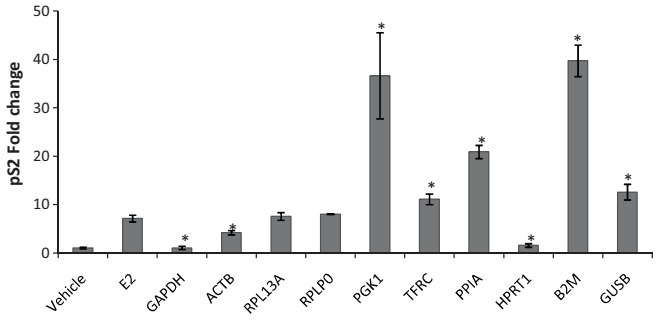

Fig. 5. Estrogen alters HKG expression and normalization of *pS2*. After 24 h treatment, the induction of *pS2* gene expression with estrogen (E2) was 7-fold higher than with vehicle in MCF-7 cells. To determine the effect of normalizing this estrogen-regulated gene to HKGs, the Ct values of *pS2* were normalized to Ct values from each of the ten putative HKGs for estrogen treatment as compared to vehicle treatment in MCF-7 cells. pS2 fold change was determined using the comparative $2^{-\Delta\Delta Ct}$ method. Error bars represent mean standard error of three experiments done in triplicate. Comparisons between groups were made with two-sample *t*-tests (*$p < 0.05$), control versus E2 for each HKG normalization.

3.5. Estrogen alters HKG expression and normalization of pS2

We observed a 7.1-fold increase in the expression of a known estrogen-regulated gene *pS2* in estrogen-treated as compared to vehicle-treated MCF-7 cells (Fig. 5) [18]. This E2 induction in *pS2* expression levels with estrogen was similar to our previously published data in MCF-7 cells [2]. To determine the effect of normalizing this estrogen-regulated gene to HKGs, the Ct values of *pS2* were normalized to Ct values from each of the ten putative HKGs. Only *RPL13A* and *RPLP0* yielded fold inductions which were not significantly different ($p = 0.72$, $p = 0.272$) than the "E2" *pS2* fold change. After *pS2* expression was normalized to *PGK1*, *TFRC*, *PPIA*, *B2M*, and *GUSB*, the normalized fold changes were significantly greater and after normalization to *GAPDH*, *ACTB*, and *HPRT1*, were significantly lower than the "E2" fold change. Case in point, normalizing *pS2* expression to *GAPDH* showed a loss of any induction with estrogen treatment of this well established estrogen-responsive gene.

4. Discussion

The ideal internal reference gene for quantitative gene expression studies should remain unaltered regardless of experimental conditions. To the best of our knowledge, this study is the first comparison of a panel of putative HKGs with regard to their utility as normalizers or internal reference genes after treatment with estrogen or tamoxifen in MCF-7 cells. Additionally, our study is the first to examine the effect of overexpressing active Akt on the expression of putative HKGs in any cell or tissue type. There are only a

few articles about reference gene evaluation in breast tissue and one report that shows GAPDH is regulated by estrogen, showing a dose-dependent increase in expression, in MCF-7 cells [20,21].

Therefore, we first evaluated the effect of estrogen or tamoxifen treatment on internal reference gene expression. From the panel of commonly used putative HKGs, only the expression of *RPL13A*, *TFRC*, and *GUSB*, remained significantly unaltered after treatment with either estrogen or tamoxifen treatment in parental MCF-7 cells (Fig. 1A). Our studies show that *GAPDH* and *HPRT1* are induced; whereas, *PGK1* and *B2M* are repressed in the presence of estrogen in parental MCF-7 cells, suggesting the possibility that these four genes are estrogen-regulated genes. Although these four genes do not contain classical EREs in their sequences, we have identified that all four genes contain half EREs as well as Sp and Ap-1 binding sites. It is known that half ERE sites that are in close proximity to Sp and Ap-1 sites and ER/Sp and ER/Ap-1 pathways may govern estrogen responses in estrogen responsive gene promoters. Using the transcription factor binding predictive tool, SABiosciences' Text Mining Application/UCSC Genome Browser, we were able to predict that the *GAPDH* promoter has two ER-alpha (−7988 and +352), three Ap-1 (−4919, −3200, and +1074), and one Sp1 binding site (+43) relative to the transcription start site.

Ap-1 dependent gene expression has also been shown to be regulated by Akt [22]. Since we have predicted the presence of Ap-1 sites in the promoter region of *GAPDH*, this explains the significant increase in *GAPDH* expression in Akt overexpressing cells as compared to parental MCF-7 cells. Therefore, our results indicate that *GAPDH* is regulated by both estrogen and Akt, suggesting significant dual regulation of this gene and possibly other genes with Ap-1 binding sites as well. Other researchers have also suggested a point of convergence as well as cross-talk of these two pathways [23]. Lastly, we observed Akt isoform differences in specific putative HKG genes. However, the mechanism for altered gene regulation by different Akt isoforms is still unclear and may be a topic for future studies.

Since treatment induced changes in HKG expression can greatly alter the interpretation of gene expression data, we examined the effect of normalizing *pS2* gene expression to our panel of HKGs. In doing so, we observed *pS2* fold changes that ranged from 1.06 ± 0.34 (*GAPDH*) to 39.68 ± 3.25 (*B2M*) upon normalizing this gene of interest to our panel of HKGs. Normalization to HKGs that were induced by estrogen treatment resulted in low *pS2* gene expression. For example, *GAPDH* expression was induced 7.53 ± 1.20 fold upon estrogen treatment (Fig. 1A) and normalizing *pS2* expression to *GAPDH* resulted in a loss of any induction with estrogen treatment of this well established estrogen responsive gene. Conversely, very high *pS2* normalized gene expression was observed for HKGs that were repressed by estrogen treatment. For example, *B2M* expression was repressed 0.18 ± 0.04 upon estrogen treatment (Fig. 1A) and normalizing *pS2* expression to *B2M* resulted in an inflated induction with estrogen treatment. After normalization of *pS2* expression to either *RPL13A* or *RPLP0*, we have confirmed that both of these two *RPL* family members are good candidate internal reference genes in breast cancer cells. This finding is consistent with several reports that have suggested the use of RPL family members as internal reference genes in ovarian tissue [24], human prostate cancer (LNCaP) cells and human breast cancer (T-47D and ZR-75-1) cells [25], and in renal carcinoma [26].

In conclusion, our present study has identified *RPL13A* from a panel of nine putative HKGs as the most suitable internal reference gene that is both stable and unaltered in MCF-7 cells after treatment with estrogen, tamoxifen, or Akt overexpression. We also validated that *RPL13A* and *RPLP0* (another member of the RPL family protein) expression levels remain unaltered after estrogen and tamoxifen treatment in ER positive ZR-75-1 and ER negative MDA-MB-468 breast cancer cell lines. Both *RPL13A* and *RPLP0* levels were fairly stable in both normal and tumor mammary tissue from Her2 overexpressing mice. Since Akt overexpression has been associated with tamoxifen resistance, our work may also be applicable to gene expression studies in tamoxifen resistant breast tumor samples or *in vivo* models of tamoxifen resistance. In addition, our work emphasizes the importance of a preliminary study to validate each reference gene that will be used for RT-qPCR. Future studies should focus on identifying the precise mechanism for the estrogen and Akt regulation of these putative HKGs.

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