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Regulation of TIM-3 expression in a human T cell line by tumor-conditioned media and cyclic AMP-dependent signaling

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ABSTRACT

T cell immunoglobulin and mucin domain-3 (TIM-3) expression increases in exhausted T cells, which inhibits T cell function. TIM-3 expression is supposedly up-regulated in tumor-bearing individuals via chronic antigenic stimulation of T cells. Considering the immunosuppressive nature of the tumor microenvironment, we investigated whether tumor-secreted molecules might enhance TIM-3 expression in Jurkat T cells. We observed that TIM-3 expression was increased by the activation of prostaglandin (PG) E_2 and cyclic AMP (cAMP) signaling pathways. Adenylate cyclase activation led to protein kinase A (PKA)-dependent upregulation of the TIM-3 minimal promoter region and of upstream conserved non-coding sequences. TIM-3 expression in Jurkat T cells was increased by the exposure to breast tumor cell-conditioned media partially through the interaction between PGE_2 and its receptor, EP4. Our results propose that tumor-secreted molecules such as PGE_2, which activates PKA and EPAC, may regulate TIM-3 expression in T cells.

1. Introduction

TIM-3 is a transmembrane surface protein regulating T cell function (Monney et al., 2002; Sabatos et al., 2003; Sanchez-Fueyo et al., 2003), which was originally identified as a Th1 cell marker, as it was not expressed in Th2 cells (Sabatos et al., 2003). TIM-3 attenuates T cell proliferation and cytokine production (Hastings et al., 2009; Jones et al., 2008). In addition, its interaction with galectin-9, one of its ligands, decreases $CD4^+$ T cell cytokine production and survival (Zhu et al., 2005). Despite a report that TIM-3 plays a stimulatory role in T cell activation (Gorman et al., 2014), it is generally regarded as an inhibitory molecule and a marker of exhausted T cells, as its expression has been detected in chronically activated T cells that exhibit functional insufficiency (Jones et al., 2008).

The tumor microenvironment modulates the T cell response to evade anti-tumor immunity (Joyce and Fearon, 2015). Owing to the action of cancer-associated fibroblasts and myeloid-derived suppressor cells (MDSCs), T cells are excluded from the vicinity of cancer cells and are trapped in the extracellular matrix surrounding the tumor cells (Molon et al., 2011; Zhang et al., 2003). The tumor microenvironment is hypoxic because the abundant extracellular matrix exerts pressure on the tumor blood vessels and increases the metabolic rate of the tumor cells (Forster et al., 2017; Joyce and Fearon, 2015). Hypoxia triggers MDSC accumulation and expression of the inhibitory immune checkpoint molecule PDL-1 (Forster et al., 2017; Joyce and Fearon, 2015). Furthermore, tumor cells produce immunoregulatory exosomes and/or soluble molecules such as PGE_2, cytokines, and adenosine, which may play immunosuppressive roles (Clayton et al., 2011; Guo et al., 2017; Olesch et al., 2015; Ristimaki et al., 2002). PGE_2, ATP, and adenosine may increase intracellular cAMP concentration by interacting with their specific receptors (Clayton et al., 2011; Di Virgilio and Adinolfi, 2017; Sugimoto and Narumiya, 2007). In addition, tumor cells transfer cAMP into T cells through gap junctions and convert naïve/effector T cells into senescent T cells (Ye et al., 2014). Furthermore, $TIM\text{-}3^+$ T cell number increases in the tumor bed and is associated with poor prognosis (Li et al., 2012); however, regulation of TIM-3 expression in the tumor microenvironment has not been well-studied.

Modulation of inhibitory immune checkpoint molecules is

Abbreviations: CM, conditioned media; cAMP, cyclic adenosine monophosphate; CNS, conserved non-coding sequence; CLTA-4, cytotoxic T-lymphocyte-associated protein 4; DMEM, Dulbecco's modified Eagle's medium; EPAC, exchange factor directly activated by cAMP; FBS, fetal bovine serum; HIF-1α, hypoxia-inducible factor 1-α; IBMX, 3-isobutyl-1-methylxanthine; MDSCs, myeloid-derived suppressor cells; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PGE_2, prostaglandin E_2; PKA, protein kinase A; TIM-3, T cell immunoglobulin and mucin domain-3

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considered a promising approach for cancer treatment. Currently approved targets for cancer therapy include CTLA-4 and PD-1 (Das et al., 2017), and blocking antibodies against these molecules have improved the survival of some patients with melanoma, renal cancer, Hodgkin's disease, or lung cancer (Das et al., 2017). TIM-3 is a candidate for cancer therapy, as its blockade had an anti-tumor effect in mouse tumor models (Lee et al., 2010; Ngiow et al., 2011). Furthermore, TIM-3 and serum levels of IL-15 correlate with the clinical outcome of anti-CTLA-4 Ab treatment (Tallerico et al., 2017). Therefore, understanding the mechanism via which TIM-3 expression is regulated in T cells may lead to the identification of methods for enhancing anti-tumor immunity. TIM-3 expression is possibly induced by persistent antigen stimulation of T cells in tumor-bearing individuals. However, as IL-15, IL-2, or IL-7 upregulate TIM-3 expression in the absence of antigenic stimulation (Mujib et al., 2012), tumor-secreted molecules may affect TIM-3 expression in T cells. In this study, we tested this possibility by either treating Jurkat T cells with regulators of cAMP-dependent signaling or exposing them to breast tumor cell-conditioned media.

2. Materials and methods

2.1. Chemical reagents

PGE_2, IBMX, forskolin, PKA inhibitor H-89, EPAC inhibitor ESI09 and EP4 inhibitor ONO-AE3-208 were purchased from Sigma-Aldrich, St. Louis, MO, USA. The EPAC activator 8-CPT-2′-O-ME-cAMP and the PKA activator 6-Bnz-cAMP were purchased from Merck Millipore, Darmstadt, Germany. The P2Y11 agonist NF546 was purchased from Tocris Bioscience (Bristol, UK).

2.2. Cell lines and cultures

We cultured the human Jurkat T cell line and breast cancer cell lines (HCC1937, MDA-MB-231, and T-47D, BT20, HCC1954, HCC70, HCC2218, HCC1419, HCC1428) in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco), at 37 °C in 5% CO_2. The breast tumor cell line MCF7 and the Chang liver cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the co-culture, HCC1937, MDA-MB-231, T-47D, MCF7, or Chang cells ($1 \sim 2 \times 10^5$) were seeded in the wells, and 16 h later, Jurkat T cells (5×10^5) were added to the wells. Tumor cell-conditioned media (CM) were collected from 16-h cultures of tumor cell lines. Jurkat T cells were exposed to tumor cell-CM or stimulated with agonists for 6 h. When inhibitors were used, each inhibitor was added 30 min prior to tumor-CM or forskolin treatment, except for the EP4 inhibitor, ONO-AE3-208, which was added 60 min prior to treatment.

2.3. Western blotting

Jurkat T cells (1×10^6) were lysed in 100 μL lysis buffer containing protease inhibitors (Calbiochem, Billerica, MA, USA). The lysate was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The blots were first probed with anti-TIM-3 (R & D Systems, Minneapolis, MN, USA) or anti-β-actin antibodies (Bethyl Laboratories, Montgomery, TX, USA) and then with either anti-goat IgG (Invitrogen, Carlsbad, CA, USA) or anti-rabbit IgG conjugated with horseradish peroxidase (Life Technologies, Thermo Fisher, Waltham, MA, USA). The blots were developed using a chemiluminescence kit (GE Healthcare, Little Chalfont, UK).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from Jurkat T cells using RNAiso (Takara Bio, Kusatsu, Japan), from which cDNA was synthesized using oligo-dT primers and PrimeScript RTase (200 U/μL) (Takara Bio). qRT-PCR was then performed using the cDNA, SYBR gene Ex Taq Premix (Takara), and the following primer sets: human TIM-3 forward and reverse primers 5′-TCCAAGGAGCTTACCACCAG-3′ and 5′-GCCAATGTGGATATT TGTGTTAGATT-3′, and human actin forward and reverse primers 5′-TGGCACCCAGCACAATGAA-3′, 5′-CTAAGTCATAGTCCGCCTAG-3′. The PCR was run on an ABI PRISM 7500 sequence detection system. TIM-3 mRNA level was normalized to actin mRNA level.

2.5. Assessment of PGE_2 concentration

PGE_2 level in the culture supernatant was estimated using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lansing, MI, USA) according to the manufacturer's instruction. Briefly, 25 μL sample or standard solution was mixed with 50 μL diluted enzyme conjugate and then incubated at room temperature for 1 h. The substrate was then added to each well, and the absorbance was measured at 450 nm.

2.6. Plasmid construction

Luciferase reporter vectors containing TIM-3 promoter alone were previously constructed (Kim et al., 2012; Yun et al., 2016). In brief, the TIM-3 promoter DNA fragment, which was amplified from genomic DNA of HMC-1 cells, was inserted into the pGL3-basic vector (Promega, Madison, WI, USA), yielding the T3U-luc reporter vector. Additionally, four conserved noncoding sequences (CNSs) of the 5′ upstream regulatory region of *TIM-3* were amplified using PCR with appropriate primer sets and the genomic DNA of Jurkat T cells. Each CNS was inserted upstream of the *TIM-3* proximal promoter region in the T3U (290)-luc vector.

2.7. Luciferase reporter assay

In total, 1×10^6 Jurkat T cells were transfected with a mixture of 3.6 μg luciferase reporter plasmid and 0.4 μg pEGFP-N1 vector (Clontech, Mountain View, CA, USA) using a microporator (Digital Bio, Seoul, Republic of Korea). The cells were incubated in 2 mL complete medium for 24 h and then stimulated with forskolin (20 μM) or tumor-CM for 6 h. The frequency of cells expressing GFP was assessed using flow cytometry on the FACSCanto II system (BD Biosciences, Franklin Lakes, NJ, USA). The luciferase activity in the cell lysate was measured using the luciferase assay system kit (Promega). Luciferase activity was normalized to the frequency of GFP-expressing cells, which represented transfection efficiency.

2.8. Fractionation of conditioned media

We fractionated the 38 mL of 16 h-cultured HCC1937-CM using sequential centrifugation (300 × *g* for 10 min, 2000 × *g* for 20 min, 9600 × *g* for 20 min, and then 150,000 × *g* for 70 min) at 4 °C. The supernatant was subjected to the next round of centrifugation. A portion of the supernatant was collected after each centrifugation step and the pellet was collected after the final centrifugation step. The final pellet containing exosomes was resuspended in 4 mL of RPMI 1640 containing 10% FBS.

2.9. Flow cytometry

Jurkat T cells were labeled with monoclonal anti-human TIM-3 Ab conjugated with phycoerythrin (R&D systems, Minneapolis, MN) and

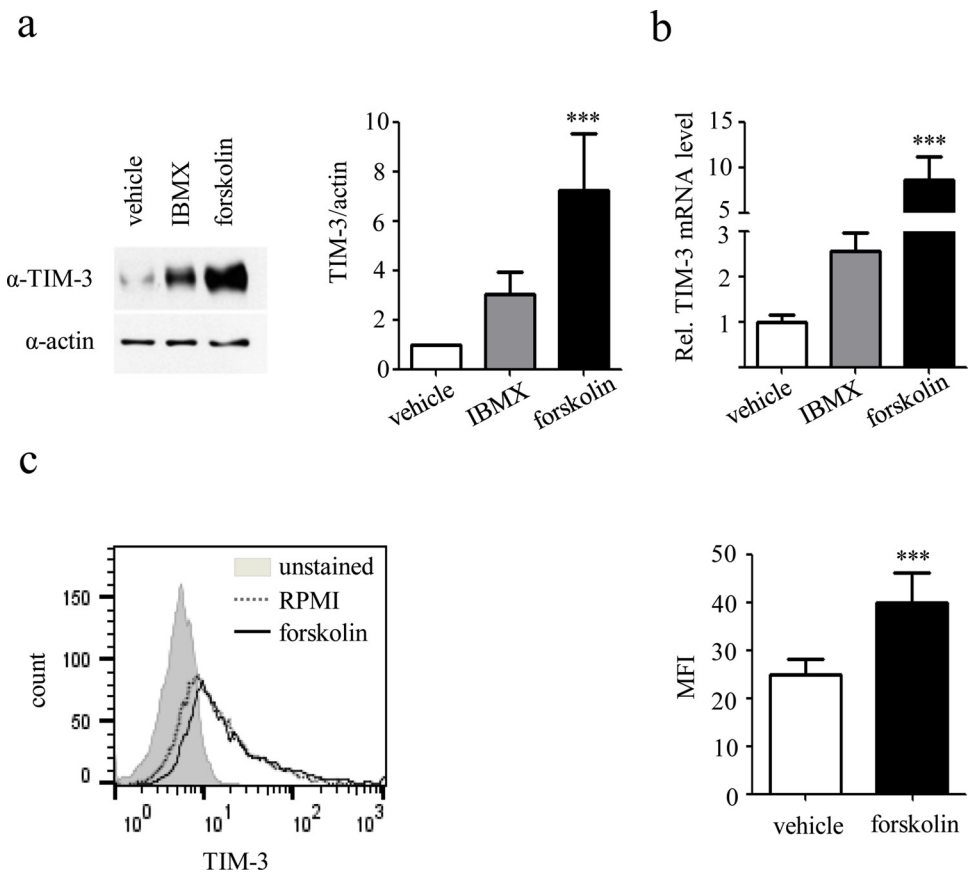


Fig. 1. Forskolin increases TIM-3 expression. TIM-3 protein (a, c) and mRNA (b) levels were analyzed in Jurkat T cells stimulated with IBMX (100 μM) or forskolin (20 μM) by western blotting (a), qRT-PCR (b), and flow cytometry (c). MFI: mean fluorescence intensity. Data are expressed as mean ± SD (n ≥ 5). *P < 0.05, **P < 0.005, ***P < 0.0005 vs vehicle.

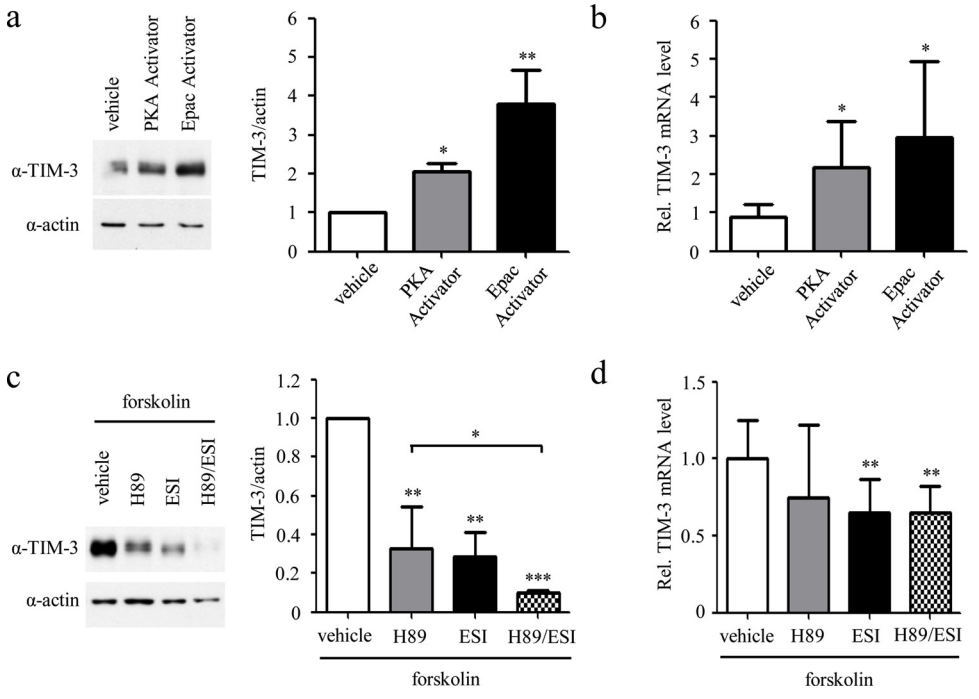


Fig. 2. PKA and EPAC activation increases TIM-3 expression. Jurkat T cells were stimulated with PKA activator (100 μM), EPAC activator (100 μM), or forskolin (20 μM) for 6 h. H89 (20 μM) and/or ESI09 (10 μM) were added to Jurkat T cell culture 30 min prior to forskolin addition. TIM-3 protein (a, c) and mRNA (b,d) levels were analyzed using qRT-PCR and western blotting, respectively. Data are expressed as mean ± SD (n ≥ 5). *P < 0.05, **P < 0.005, ***P < 0.005 vs vehicle.

then analyzed using FACS CantoII.

2.10. Statistical analysis

The statistical significance of differences between three or more groups was assessed by using the Kruskal-Wallis test with Dunn's multiple comparison test. Student's *t*-test was performed to analyze the differences between two groups. Differences were considered significant at $p < 0.05$.

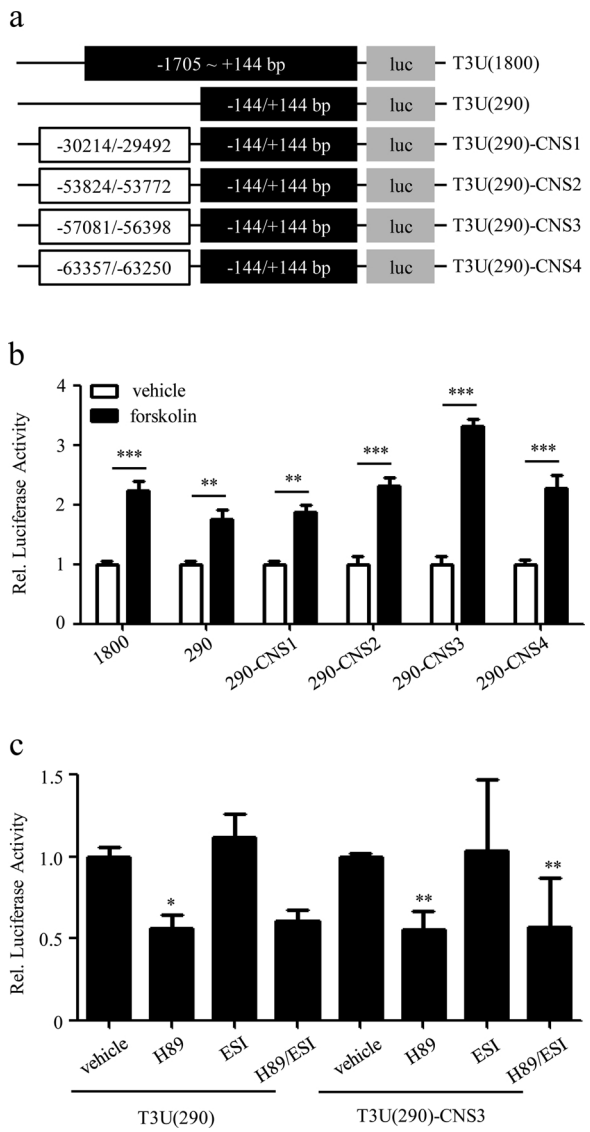


Fig. 3. TIM-3 gene promoter and upstream conserved non-coding sequences are responsive to forskolin. (a) Diagram of luciferase reporter vectors containing minimal or long *TIM-3* promoter (filled square) alone or with additional CNS (open square). (b, c) Jurkat T cells were transfected with the indicated luciferase reporter vector together with a GFP expression vector. At 24 h post-transfection, these cells were stimulated with forskolin (20 μM) for 6 h (b) or pretreated with H89 (20 μM) and/or ESI09 (10 μM) 30 min prior to forskolin addition (c). Luciferase activity was normalized to the GFP expression level and relative luciferase activity against vehicle-treated luciferase activity was calculated. Data are expressed as mean ± SD (n ≥ 6). Student's *t*-test (b). Kruskal-Wallis test with Dunn's multiple comparison test (c). **P < 0.01, ***P < 0.001 vs vehicle.

3. Results

3.1. Forskolin increases TIM-3 expression in Jurkat T cells

Given that tumor cells may upregulate the intracellular level of cAMP in T cells through the release of cAMP-elevating molecules such as PGE_2 and ATP or cAMP-containing exosomes (Berglund et al., 2013; Clayton et al., 2011; Sreeramkumar et al., 2012; Sugimoto and Narumiya, 2007), we first investigated the effect of intracellular cAMP upregulation on TIM-3 expression in Jurkat T cells (Fig. 1). Jurkat T cells were treated with chemicals increasing intracellular cAMP levels such as IBMX, a cAMP degrading enzyme phosphodiesterase inhibitor, and forskolin, a cAMP-producing adenylyl cyclase activator. Forskolin significantly increased TIM-3 mRNA and protein levels (P < 0.005). Although there was no statistical significance, IBMX also increased TIM-3 mRNA and protein levels.

Generally, intracellular cAMP elevation activates PKA and EPAC (Mosenden and Tasken, 2011). Hence, we examined the effect of PKA and EPAC activation on TIM-3 expression in Jurkat T cells (Fig. 2). The PKA activator increased TIM-3 mRNA and protein levels by approximately 2-fold, whereas the EPAC activator increased it by 3 to 4-fold (P < 0.05). Furthermore, the PKA and EPAC inhibitors, H89 and ESI09, respectively, suppressed forskolin-induced TIM-3 expression (mRNA levels were reduced to 65% ~ 74.7% of control level and protein levels to 9.8% ~ 32.7%) (P < 0.005). These results indicate that the cAMP signaling pathway regulates TIM-3 expression in Jurkat T cells through PKA and EPAC activation.

3.2. TIM-3 promoter and upstream CNS are responsive to forskolin treatment

To investigate whether the TIM-3 promoter or 5′ upstream CNS is involved in forskolin-induced TIM-3 expression, we performed luciferase reporter assays using the indicated luciferase reporter vectors (Fig. 3). Forskolin treatment significantly enhanced luciferase activity driven by all reporter vectors containing the *TIM-3* minimal promoter (-144 to +144 bp from the start of transcription initiation site of *TIM-3*) with respect to that in untreated controls. The T3U(290)-CNS3 luc vector further increased forskolin-induced luciferase activity with respect to that induced by the T3U(290)-luc vector, implying that the forskolin-responsive regions might be within the *TIM-3* minimal promoter and CNS3 (P < 0.01). The forskolin-stimulated luciferase activity was suppressed by PKA inhibition but not by EPAC inhibition, indicating that TIM-3 minimal promoter and CNS3 might respond to PKA activation.

3.3. PGE_2 induces TIM-3 expression in Jurkat T cells

Since the intracellular cAMP level may increase in cells treated with PGE_2 or extracellular ATP through interaction with their respective receptors, EP4 or P2Y11 (Dreisig and Kornum, 2016; Sugimoto and Narumiya, 2007), we evaluated TIM-3 expression levels in Jurkat T cells treated with PGE_2 or the P2Y11 agonist NF546 (Fig. 4). PGE_2 treatment increased TIM-3 protein and mRNA levels in a concentration dependent manner (P < 0.05). However, NF546 did not affect TIM-3 expression. Of note, the intracellular cAMP level in NF546-treated cells was not assessed. To further examine the role of PGE_2 in regulating TIM-3 expression, we used the EP4 receptor inhibitor prior to treatment with PGE_2. The level of surface TIM-3 was increased by PGE_2 treatment but not in the presence of the EP4 inhibitor. These results indicate that PGE_2 upregulates TIM-3 expression via its interaction with EP4.

3.4. Several breast tumor cell lines increase TIM-3 expression in Jurkat T cells in a contact-independent manner

We next assessed whether tumor cells modulated TIM-3 expression in Jurkat T cells (Fig. 5). Co-culture of Jurkat T cells with the breast tumor HCC1937 cell line led to significantly higher TIM-3 protein levels at both 6 and 24 h (P < 0.01) than in the control (Jurkat T cells alone); however, co-culture with MDA231, T-47D, MCF7, or Chang Liver cell line did not show this effect (Fig. 5a). We next evaluated TIM-3 expression in Jurkat T cells incubated for 6 h in tumor cell-conditioned media (CM) that was collected from a 16 h culture of four breast tumor cell lines (HCC1937, MCF7, MDA231, or T-47D) (Fig. 5b). HCC1937CM significantly elevated TIM-3 protein level compared to control medium (P < 0.05); however, MCF7CM, MDA231CM, or T-47DCM did not. The features of HCC1937 cells differ from those of the three other breast tumor cells. HCC1937 cells originated from a primary tumor of ductal carcinoma, whereas MCF7 and MDA231 were established from pleural

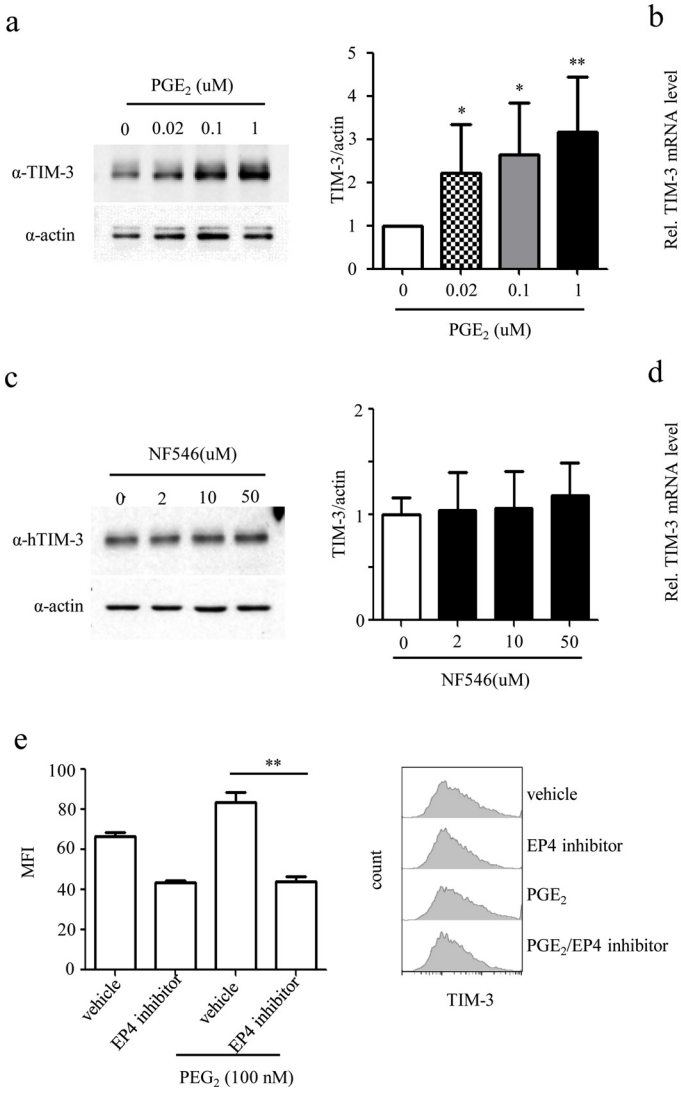


Fig. 4. PGE_2 induces TIM-3 expression. TIM-3 protein (a, c) and mRNA (b, d) expression in Jurkat T cells treated with the indicated concentration of PGE_2 (a, b) or P2Y11 agonist NF546 (c, d) for 6 h. The TIM-3 mRNA and protein levels were analyzed using qRT-PCR and western blotting, respectively. Data are expressed as mean ± SD ($n \geq 5$). (e) PGE2 (100 nM) was added to the culture of Jurkat T cells that had been pretreated with EP4 inhibitor (1 μM) for 1 h. Six hours later, TIM-3 expression was analyzed by flow cytometry. Representative histogram and a plot showing the mean fluorescence intensity. Data are expressed as mean ± SD ($n = 4$). *$P < 0.05$, **$P < 0.005$.

effusion of metastatic adenocarcinoma, and T47D from pleural effusion of invasive ductal carcinoma (Kao et al., 2009). To verify whether the ability to upregulate TIM-3 expression was an exclusive property of HCC1937CM, we examined the effect of another tumor cell-CM on TIM-3 expression. According to the subtypes identified by Kao et al. (2009) based on genomic and transcriptional profiling, HCC1937 is the basal A subtype, which is characterized by the BRCA1 signature, whereas MDA231 is the basal B subtype with a epithelial-mesenchymal transition signature, and MCF7 as well as T47D are luminal subtypes with good prognosis signatures. Thus we selected three cell lines (BT20, HCC1954, and HCC70) belonging to the basal A subtype, and three cell lines (HCC2218, HCC1419, and HCC1428) of the luminal subtype. Compared to control media, HCC1954CM and HCC70CM significantly enhanced TIM-3 protein expression ($P < 0.05$) (Fig. 5c), showing that this ability was not limited to HCC1937 cells. We then analyzed TIM-3 mRNA levels under these conditions (Fig. 6). HCC1937CM significantly elevated TIM-3 mRNA level at 1 h and further increased it at 6 h, compared to control medium ($P < 0.05$). Taken together, these results clearly indicate that certain breast tumor cells may promote TIM-3 expression in Jurkat T cells in a contact-independent manner.

Considering the immunomodulatory nature of tumor cell-derived exosomes, we determined whether the exosome-containing fraction of HCC1937CM might be responsible for TIM-3 induction in Jurkat T cells (Supplementary Fig. 1). The supernatants obtained after the serial centrifugation of HCC1937CM still increased TIM-3 protein levels, but the exosome-containing fraction did not, implying that soluble molecules may play a role in regulating TIM-3 expression.

3.5. PGE_2 is produced by TIM-3-inducing breast tumor cell lines and is involved in TIM-3 upregulation in Jurkat T cells

We assessed PGE_2 levels in HCC1937CM, HCC1954CM, HCC70CM and MCF7CM (Fig. 7a and b). PGE_2 concentration in the 16-h HCC1937CM was approximately 4-fold and 2-fold higher than that of Jurkat T cell-CM and MCF7CM, respectively ($P < 0.05$). PGE_2 concentrations were also elevated in HCC1954CM and HCC70CM. We next explored whether PGE_2 released in tumor cell-CM was critical for the modulation of TIM-3 expression. We pretreated Jurkat T cells with an EP4 inhibitor and then incubated them in the indicated tumor cell-CM in the presence or absence of the EP4 inhibitor (Fig. 7c and d). As expected, the EP4 inhibitor reduced the TIM-3 expression on the surface of Jurkat T cells. Concordantly, the EP4 inhibitor decreased the TIM-3 protein level in the cell lysate when Jurkat T cells were incubated in HCC70CM. However, the EP4 inhibitor did not decrease the TIM-3 protein level when incubated in HCC1937CM and HCC1954CM. These results indicate that PGE_2 might control the tumor cell-CM induced TIM-3 expression on the surface of Jurkat T cells. Further, PGE_2 in HCC70CM might play a major role in controlling TIM-3 protein expression but in HCC1937CM and HCC1954CM, other molecules might play a larger role.

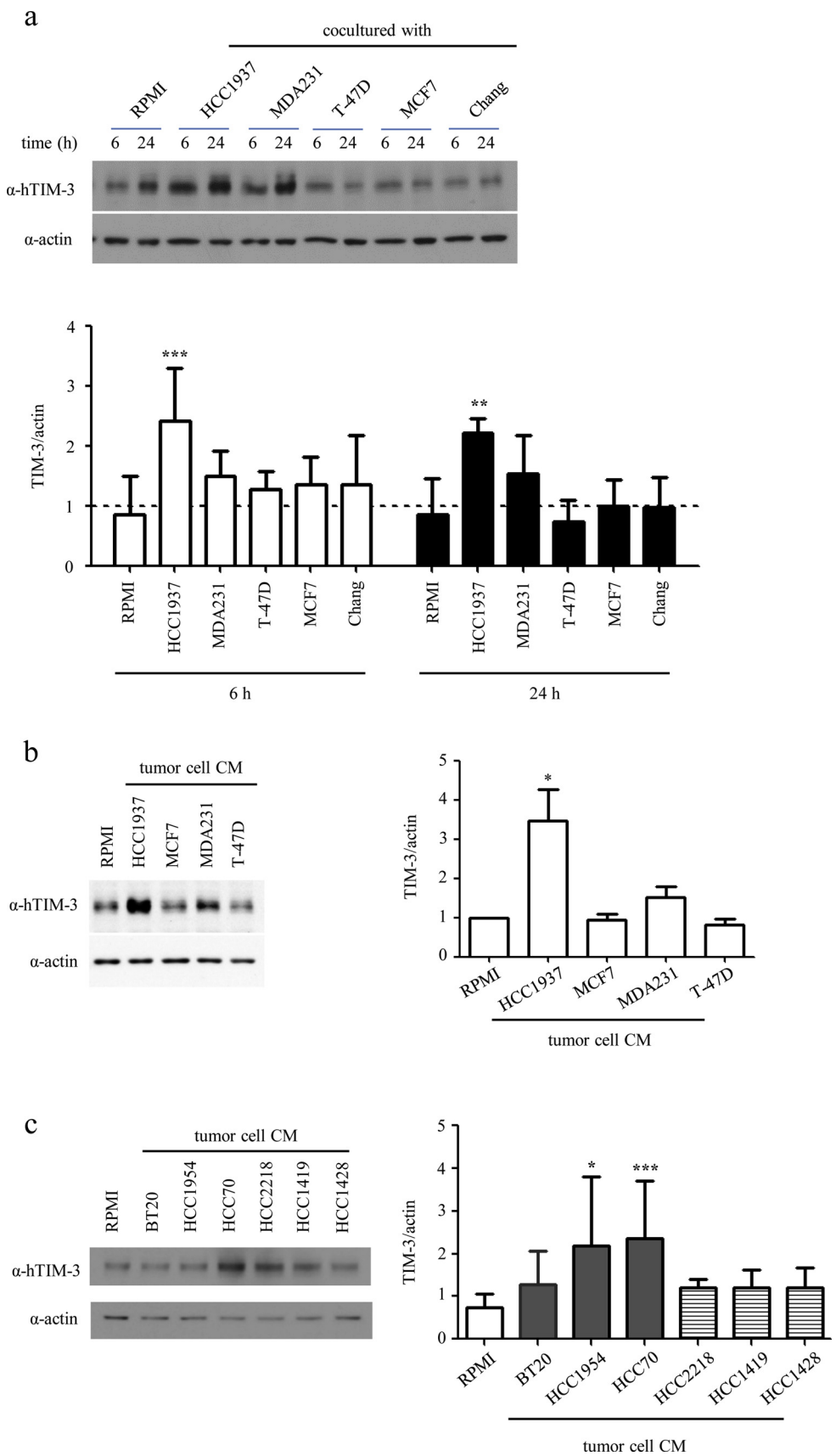


Fig. 5. Several breast tumor cell lines increase the TIM-3 protein level in Jurkat T cells. TIM-3 protein levels in Jurkat T cells co-cultured with the indicated tumor cell line for the indicated time (a) or incubated with the 16 h-conditioned media (CM) of the indicated tumor cell line for 6 h (b, c). Representative western blots and plots of normalized data from four or more independent experiments. Chang: Chang liver cell. Data are expressed as mean ± SD. *P < 0.05, **P < 0.005, ***P < 0.0005.

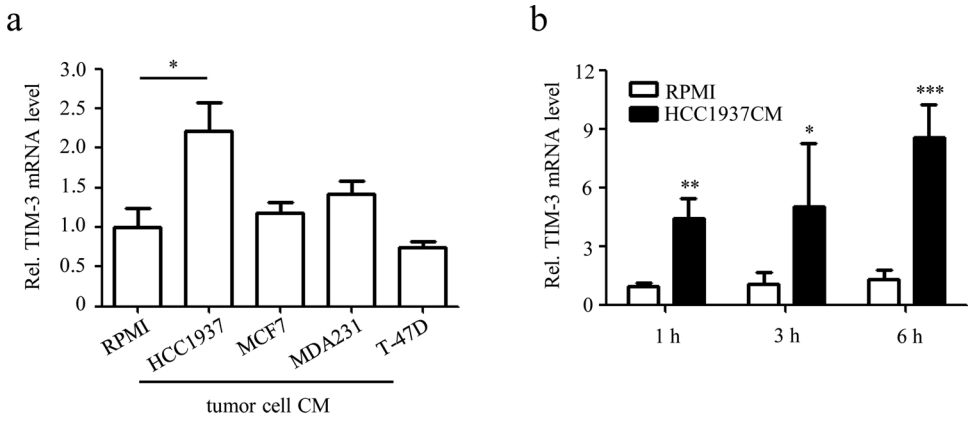


Fig. 6. HCC1937-conditioned medium increases *TIM-3* mRNA expression in Jurkat T cells. TIM-3 expression of Jurkat T cells incubated for 6 h in the 16 h-conditioned media (CM) of the indicated tumor cell line (a). HCC1937-CM induced TIM-3 mRNA expression kinetics in Jurkat T cells (b). TIM-3 mRNA levels assessed by RT-qPCR. Data are expressed as mean ± SD ($n \geq 5$). *P < 0.05, **P < 0.005, ***P < 0.0005 vs. RPMI.

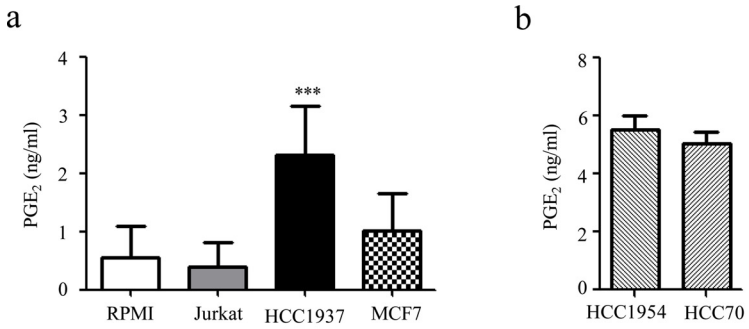
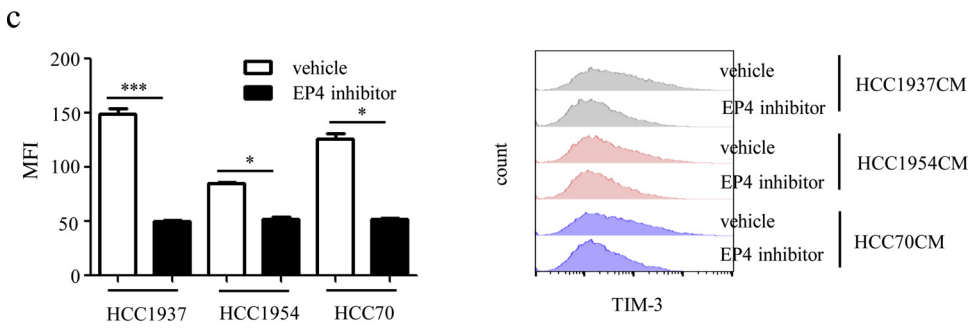
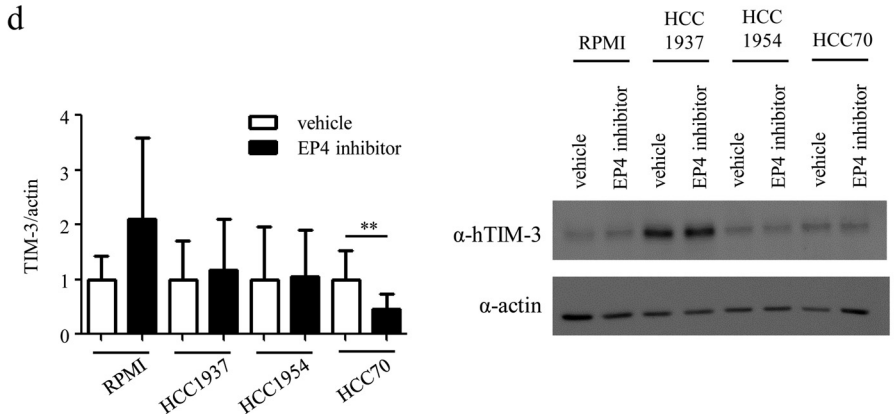


Fig. 7. PGE_2 contained in breast tumor cell-conditioned media increases TIM-3 protein expression in Jurkat T cells. PGE_2 concentration in the 16 h-conditioned medium of the indicated tumor cells (a, b). Data are expressed as mean ± SD ($n \geq 5$). ***P < 0.001 vs RPMI. Jurkat T cells were pretreated with EP4 inhibitor (1 μM) for 1 h and then incubated with the indicated tumor cell-CM in the presence or absence of EP4 inhibitor for 6 h (c, d). TIM-3 expression was analyzed by flow cytometry (c) and Western blotting (d). Representative histogram and a plot showing mean fluorescence intensity (c). Data are expressed as mean ± SD ($n = 4$ for c and $n \geq 5$ for d).





3.6. PKA and EPAC inhibitors decrease HCC1937CM-induced TIM-3 protein levels

Next, we investigated the relationship between PKA and EPAC activation and HCC1937CM induced TIM-3 expression. We analyzed TIM-3 expression in HCC1937CM-incubated Jurkat T cells in the presence or absence of PKA and/or EPAC inhibitor (H89 and/or ESI09) (Fig. 8). TIM-3 mRNA and protein expression was differentially affected

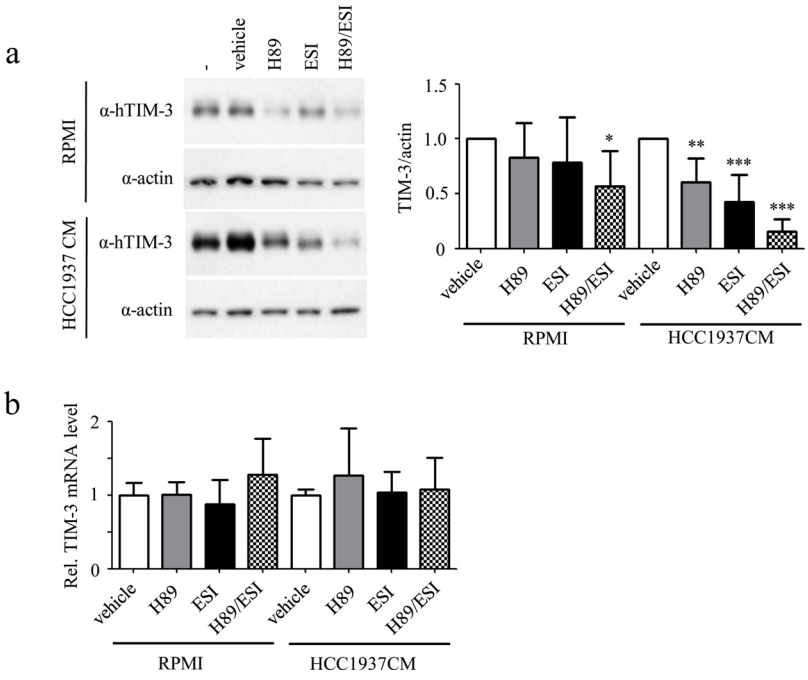


Fig. 8. Inhibitors of PKA and EPAC suppress HCC1937-conditioned media-induced TIM-3 protein expression in Jurkat T cells. Jurkat T cells were pretreated with H89 (20 μM) and/or ESI09 (10μM) for 30min and then incubated in the complete RPMI medium, or HCC1937CM for 6h. TIM-3 protein (a) and mRNA (b) levels were analyzed using western blotting and qRT-PCR, respectively. -: no pretreatment; vehicle: solvent of H89 and ESI09. Data are expressed as mean ± SD (n ≥ 6). *P < 0.05, **P < 0.005, ***P < 0.0005 vs. vehicle.

by H89 and/or ESI09 treatment. H89 and/or ESI09 treatment did not reduce HCC1937-CM-induced TIM-3 mRNA level but clearly reduced the protein level ($P < 0.001$) with respect to that in the vehicle control. Unexpectedly, the H89 and ESI09 combination slightly lowered the basal TIM-3 protein level in control RPMI media- incubated Jurkat T cells ($P < 0.05$). These results suggest that PKA and EPAC are involved in HCC1937CM-induced TIM-3 protein expression in Jurkat T cells.

4. Discussion

In this study, we demonstrated that intracellular cAMP elevation induces TIM-3 expression and promoter/enhancer activity in Jurkat T cells, through activation of its downstream effectors, PKA and EPAC. Further, PGE_2 and the tumor-CM containing PGE_2 increased TIM-3 expression by interacting with EP4.

This is the first report of TIM-3 regulation by the cAMP signaling pathway. Currently known signal molecules or transcription factors affecting TIM-3 expression are T-bet, MEK, c-Jun, and nuclear factor interleukin 3 regulated (NFIL3) in T cells, T-bet in HCV-infected monocyte/macrophages, and Hif1-α in ischemic microglial cells (Anderson et al., 2010; Koh et al., 2015; Kossatz et al., 2016; Yi et al., 2017; Yoon et al., 2011; Yun et al., 2016; Zhu et al., 2015). Previously, intracellular cAMP elevation or PKA activator has been reported to promote CTLA-4 transcription and protein expression in EL4 cells and primary CD4 + T cells (Li et al., 2013; Vendetti et al., 2002). However, the relation between cAMP signaling and TIM-3 expression has not been reported. We demonstrated that forskolin, which elevates intracellular cAMP through direct activation of adenylate cyclase, increased TIM-3 expression at both mRNA and protein levels. Forskolin-induced PKA activation was required for the increase in TIM-3 minimal promoter and 5′ upstream CNS activity, resulting in TIM-3 transcription. Forskolin-induced EPAC activation might be involved in TIM-3 transcription based on the results obtained using the EAPC activator and inhibitor. However, the TIM-3 promoter and the 5′ upstream CNS region did not respond to EPAC activation. Further study is needed to verify this possibility, as well as the question as to whether activation of PKA and EPAC regulates TIM-3 expression at the post-transcriptional level.

We show that PGE_2 modulates TIM-3 expression in Jurkat T cells by interacting with EP4. Of the four PGE_2 receptors, Jurkat T cells express EP3 and EP4 (Blaschke et al., 1996; Gerlo et al., 2004), which increase intracellular calcium and cAMP levels, respectively (Gerlo et al., 2004). An EP3- and EP4-dependent PGE_2 modulation of prolactin expression was reported in human T cells (Gerlo et al., 2004). However, as we focused on the cAMP signaling pathway, we did not examine the role of EP3 in PGE_2-mediated TIM-3 induction.

We demonstrated that PGE_2 in breast tumor cell-CM was involved in controlling TIM-3 expression in Jurkat T cells. Notably, over 50% of invasive and over 80% of metastatic breast tumors were reported to produce high amount of PGE_2-synthesizing cyclooxygenase 2 (Harris et al., 2014). Breast tumor cells produce more PGE_2 in the presence of interleukin-1β released from macrophages (Hou et al., 2011). Further studies are warranted to investigate the role of PGE_2 in the modulation of TIM-3 expression in tumor-infiltrated T cells.

We should mention one of unresolved findings of our study. Forskolin increased TIM-3 protein expression by seven-fold in the cell lysate but by approximately two-fold on the cell surface. This may be due to the difference in detection methods: western blotting and flow cytometry use polyclonal and monoclonal anti-TIM-3 Ab, respectively. On the other hand, the apparent discrepancy might be due to the lack of a required signal for Tim-3 transport to the plasma membrane. Consistently, forskolin did not increase CEACAM1 expression, which facilitates TIM-3 protein surface localization (data not shown, Huang et al., 2015).

In conclusion, we showed the ability of several breast tumor cell lines to increase TIM-3 expression in Jurkat T cells in a contact-independent but PGE_2-EP4/PKA/EAPC signaling-dependent manner. We propose that TIM-3 expression in effector T cells may increase in tumor microenvironment in an Ag-independent mode. The activated tumor-Ag specific T cells and bystander T cells recruited to the tumor tissue may be exposed to PGE_2 released from tumor cells, independently of the encounter with a cognate Ag, after which TIM-3 expression may be increased. Further studies are required to test this model.

Author contribution

S. Yun performed most of the experiments and drafted some of the figures; BK Lee performed flow cytometric and statistical analysis, and drafted some of the figures. K. Komori constructed some of the reporter vectors; BG Lee, and MJ Lee performed experiments; K. Kim contributed to data interpretation; S. Park designed the research, interpreted the

data, and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.12.006.

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