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Pig endothelial protein C receptor is functionally compatible with the human protein C pathway

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Running head: Pig EPCR is compatible with human TBM

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Abstract

Background: Endothelial protein C receptor (EPCR) plays an anticoagulant and anti-inflammatory role by promoting the activation of protein C by thrombin bound to thrombomodulin (TBM). Incompatibility between pig TBM and human/primate thrombin is thought to contribute to dysregulated coagulation in pig-to-primate organ xenografts, and expression of human TBM (hTBM) in pigs has shown benefit in preclinical models. However, it is not known whether there are incompatibilities – or molecular barriers -- between endogenous pig EPCR (pEPCR) and transgenically expressed human TBM.

Aim: To clone and express pEPCR, and determine its function in the human protein C pathway *in vitro*.

Methods: pEPCR cDNA was generated from pig lung RNA by RT-PCR. Primate COS-7 transfectants expressing various combinations of human and pig TBM and EPCR were incubated with human thrombin and human protein C, and tested for TBM co-factor activity.

Results: The predicted protein sequence of pEPCR shared 72.3% amino acid sequence identity with hEPCR, and residues critical for protein C binding were conserved. COS-7 cells transfected with hEPCR, pEPCR or vector showed minimal TBM cofactor activity (0.13 ± 0.04, 0.13 ± 0.02 and 0.14 ± 0.06 U, respectively). The cofactor activity of hTBM-transfected cells (1.18 ± 0.29 U) was 8-fold higher than vector-transfected cells ($p = 0.004$) and further increased 4-fold and 3-fold by co-transfection with hEPCR (5.01 ± 1.12 U, $p = 0.004$) or pEPCR (3.73 ± 0.65 U, $p= 0.003$), respectively.

Conclusions: Our data show that pEPCR is largely compatible with the human TBM/thrombin complex, when expressed on COS-7 cells *in vitro*, promoting the activation of human protein C. These findings suggest that endogenous pEPCR will enhance the activity of transgenic hTBM in the xenograft setting.

Keywords

Xenotransplantation, thrombomodulin, endothelial protein C receptor, protein C pathway, coagulation

Abbreviations used

APC	activated protein C
EPCR	endothelial protein C receptor
FITC	fluorescein isothiocyanate
GTKO	α1,3-galactosyltransferase gene-knockout
h	human
MFI	mean fluorescence intensity
p	pig
PAEC	porcine aortic endothelial cells
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcription - polymerase chain reaction
TBM	thrombomodulin

Authorship statement

E.J.S. participated in the research design, performance of the research, data analysis, writing and critical revision of the manuscript.

C.J.M. and J.C.R participated in the research design, performance of the research, data analysis, and critical revision of the manuscript.

N.F. participated in the performance of the research, data analysis, and critical revision of the manuscript.

S.C.R. participated in the research concept, design, and critical revision of the manuscript.

P.J.C. secured funding and participated in the research concept and design, data analysis, and writing of the manuscript, and carried the main responsibility for the study.

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Introduction

Xenotransplantation is a possible solution to the limited supply of human donor organs available for transplantation. The pig is a suitable donor due to its physiological and morphological similarity with humans (1), and significant progress has been made by genetically modifying donor pigs to prevent rejection of porcine xenografts (2). Dysregulated coagulation has frequently been a barrier to long-term survival in pig-to-nonhuman primate preclinical models (3). Therefore, one of the goals of the genetic approach is to overcome cross-species molecular incompatibilities affecting the regulation of coagulation, notably the failure of pig thrombomodulin (pTBM) to interact efficiently with human thrombin (4-6).

The protein C pathway is a key regulator of coagulation and inflammation in the microvasculature. The integral endothelial protein TBM binds circulating thrombin, blocking its procoagulant activity and converting it to an anticoagulant by changing its substrate specificity from prothrombin to protein C. Activated protein C (APC) inhibits coagulation by irreversibly inactivating factors Va and VIIIa (7). TBM thus acts as a cofactor for thrombin's activation of protein C. A second transmembrane-anchored molecule, endothelial protein C receptor (EPCR), significantly enhances TBM cofactor activity by binding protein C and presenting it to the TBM/thrombin complex (8). EPCR also has TBM-independent anti-inflammatory, anti-apoptotic and cytoprotective effects via binding of APC and cleavage of PAR-1 (9, 10).

Our *in vitro* studies have shown that pTBM binds human thrombin but is a poor cofactor for the subsequent activation of human protein C (6). We have also demonstrated that overexpression of hTBM protects against transplant-related injury

in a transgenic mouse model (11). Several groups have generated hTBM-expressing transgenic pigs, and although the results from preclinical models are not yet conclusive, they suggest that hTBM protects against the development of dysregulated coagulation (12-15). However, in the context of a porcine xenograft, it is not known whether endogenous pig EPCR (pEPCR) will be functionally compatible with hTBM expressed by transgenic pig endothelial cells.

To address this question, we cloned pEPCR and expressed it in transfected cells to examine its ability to enhance hTBM cofactor activity for human thrombin-mediated activation of human protein C. We report here that pEPCR shares a high degree of homology with human EPCR (hEPCR) at both the gene and protein level, and demonstrates functional compatibility with the human protein C pathway *in vitro*.

Materials and methods

cDNA preparation, PCR and RACE

EAhy926 (human endothelial) cells and SV40-transformed porcine aortic endothelial cells (SVAP) cells were grown as described (16). Total RNA was isolated from the cells using TRIzol (Life Technologies, Scoresby, Australia) according to the manufacturer's protocol. WT pig lung total RNA was provided by James Godwin (16). cDNA was prepared from total RNA using Superscript III reverse transcriptase, random hexamers and oligo (dT) (Life Technologies) according to the manufacturer's protocol. PCR was performed using *Taq* DNA polymerase (Invitrogen, Mount Waverley, Australia). Rapid amplification of cDNA ends (RACE) was performed using the SMART RACE kit (BD Biosciences, North Ryde, Australia).

Transfection of COS cells

The mammalian expression plasmid pBOS-FLAG was used to construct vectors for the expression of FLAG-tagged hTBM, pTBM, hEPCR or pEPCR. 5 x 10^6 COS-7 cells were transiently transfected with a total of 5 µg of DNA by electroporation using a Gene Pulser (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) at 0.2V and 960µFd. 48 hrs after transfection, cells were analyzed by flow cytometry using FITC-labelled antibodies to detect expression of FLAG (Clone K, WEHI, Parkville, Victoria,

Australia), hTBM (Clone IA4, WEHI), or hEPCR (rat anti-mouse hEPCR Clone RCR-252, BD Biosciences). The anti-hTBM and anti-hEPCR mAbs did not cross-react with pig (data not shown).

Thrombomodulin cofactor activity assay

TBM cofactor activity was determined essentially as previously described (6). 48 hrs after transfection, $5x10^4$ cells were incubated for 30 min with 0.1 U/ml human thrombin and 150 nM human protein C (Merck-Millipore, Bayswater, Victoria, Australia) in a 100 µl reaction at 37°C. The reaction was terminated by addition of 4 µl 2U/ml hirudin (Merck), and the concentration of APC generated (nM) was measured using the Spectrozyme PCa chromogenic substrate (Vital Diagnostica, Castle Hill, New South Wales, Australia), by measuring absorbance at 405 nm using a FLUOStar Omega kinetic plate reader (BMG Labtech, Offenburg, Germany). Cofactor activity was calculated as Units, where 1 U = 1 nM APC/5×10^5 cells/30 min. Data were corrected for transfection efficiency using the mean fluorescence intensity (MFI) of an aliquot of cells stained for hTBM and analyzed by flow cytometry.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM) of 5-7 independent experiments, performed in triplicate. Data were analyzed using unpaired Student t Test (2-tailed).

Results

pEPCR and hEPCR are highly similar at the protein and gene levels

Pig lung was used as a source of pEPCR RNA because the lung is an organ which is rich in endothelial cells. A 331-bp product was amplified by PCR of pig lung cDNA using a degenerate upstream primer (5´-TCTTCGAWGTGGCTGGTGAATGG-3´) based on homology between human, bovine and mouse EPCR, and a downstream primer (5´-TTGCGGCCGCGGAGAGTAATTAACATCGC-3´) specific for hEPCR. A product of the same size was generated when cDNA from the human endothelial cell

line EAhy926 cDNA, but not from the immortalized pig aortic endothelial cell line SVAP, was used. The failure to generate an EPCR product from SVAP cells was unlikely to be due to the quality of the SVAP cDNA, because pTBM could be readily amplified from the same preparation (data not shown).

The sequence of the pig lung PCR product was 87% identical to that of bovine EPCR, and was used to design specific primers for rapid amplification of DNA ends (RACE). This generated a 0.6 kb 3′ RACE product extending the pEPCR cDNA sequence to a polyA tail. Attempts to generate a 5′ RACE product were unsuccessful. Instead, the 5′ sequence of pEPCR cDNA was extended by PCR using a primer for the 5′-untranslated region of hEPCR (5′-CGAGCCAGGAACCCAGGT) and the hEPCR downstream primer listed above. The resulting 1.4 kb putative pEPCR cDNA sequence contained an open reading frame encoding a predicted 242-residue protein, including an 18-residue signal sequence (17), a 195-residue extracellular domain, a 23-residue transmembrane domain (identified using the program at www.cbs.dtu.dk/services/TMHMM), and a 6-residue intracellular domain (Fig. 1). The extracellular domain contained a 167-residue MHC class I/CD1 domain characteristic of hEPCR. The mature pEPCR protein was 72.3% identical to mature hEPCR (Fig. 2). In the critical protein C-binding MHC I/CD1 domain, identity was 73.7%, increasing to 83.8% when conserved residues were included. Of 10 residues in hEPCR known to be essential for binding of human APC (18), marked by red asterisks in Fig. 2, 7 were identical and 3 were conserved in pEPCR. In addition, the 4 *N*-linked glycosylation sites in hEPCR, which are not critical for protein C binding but may contribute to stability (18), were conserved in pEPCR.

Comparison of the pEPCR cDNA sequence with the porcine genome revealed 99.5% identity with a region of chromosome 17 (sequence ID: NC_010459.5). The comparison indicated that the pEPCR gene spans 4.6 kb and contains 4 exons (>120 bp and approximately 250, 280 and 700 bp) separated by 3 introns of approximately 2.6 kb, 370 bp and 240 bp. This structure is similar to that of the hEPCR gene on chromosome 20 (19, 20), with the exception that the second intron of hEPCR is larger (1217 bp). The pEPCR amino acid sequences predicted from the cDNA (Fig. 1) and the available genomic sequence were identical.

pEPCR interacts with human TBM/thrombin to promote activation of human protein C in vitro

COS-7 cells, which have negligible TBM cofactor activity (6), were transiently transfected with expression vectors for FLAG-tagged hTBM, hEPCR and pEPCR; alone or in combination. Flow cytometric analysis of hTBM-transfected cells using an hTBM-specific antibody showed strong expression of hTBM that was not significantly affected by co-transfection with hEPCR or pEPCR (Fig. 3). Expression of hEPCR was also comparable between experiments: the mean fluorescence intensity (MFI) for hTBM/hEPCR was 221.5 ± 36.9 versus 47.0 ± 3.6 for vector-transfected cells (both n = 6). Expression of pEPCR was not measured due to the lack of a specific detection antibody.

48 hrs after transfection, cells were analyzed for TBM cofactor activity in the presence of human thrombin and human protein C using the Spectrozyme PCa chromogenic assay. As expected, cells transfected with vector, hEPCR or pEPCR alone, in the absence of hTBM, showed minimal cofactor activity (0.14 ± 0.06, 0.13 ± 0.04 and 0.13 ± 0.02 U, respectively). Cofactor activity of cells transfected with pTBM was also minimal (0.18 ± 0.05 U, p = 0.578 vs vector-transfected), as previously reported (6). Cofactor activity of hTBM-transfected cells (1.18 ± 0.29 U) was 8-fold higher than that of vector-transfected cells (p = 0.004), and was increased a further 4-fold or 3-fold by co-transfection with hEPCR (5.01 ± 1.12 U, p = 0.004) or pEPCR (3.73 ± 0.65 U, p = 0.003), respectively (Fig. 4). We did not attempt to measure the anti-inflammatory and cytoprotective/anti-apoptotic activity of EPCR because these functions are dependent on PAR-1, which is not expressed in COS-7 cells (21).

Discussion

Transgenic expression of hTBM in donor pigs is aimed at preventing dysregulated coagulation by bypassing the molecular incompatibility between pTBM and human thrombin/protein C (3). Recent evidence suggests that hTBM expression improves vascular thromboregulation and thereby prolongs solid organ xenograft survival (13, 22). However, the cross-species compatibility of another important component of the

protein C pathway, EPCR, has not previously been examined. We therefore asked whether endogenous pEPCR would enhance the activity of hTBM *in vitro* as representative of the setting of a transgenic organ xenograft.

To address this question, we cloned pEPCR and compared its sequence and activity to that of hEPCR. Interestingly, we were able to generate pEPCR cDNA from pig lung but not from a pig aortic endothelial cell line. This was somewhat surprising since EPCR appears to be preferentially expressed in large vessels, at least in humans (23), but may be explained by altered gene expression in cell lines compared to primary cells. The comparison of pig and human EPCR protein sequences revealed a high degree of homology. Notably, 83.8% of residues were identical or conserved in the MHC I/CD1 domain, which binds the Gla domain of protein C/APC (24). It was therefore unsurprising that co-expression of pEPCR with hTBM in transfected cells significantly increased the latter's cofactor activity, demonstrating that pEPCR facilitates activation of human protein C by the human TBM/thrombin complex *in vitro*. While the 3-fold increase was relatively modest, it was not significantly different to that achieved with co-expression of hEPCR (**4**-fold), which in turn was similar to that previously reported for hEPCR *in vitro* (approximately 5-fold) (8). Furthermore, a study in primates suggests that these *in vitro* measures considerably underestimate the *in vivo* impact of EPCR on TBM cofactor activity (25).

Nevertheless, while our data and those of others (13) suggest that there is no absolute need to co-express hEPCR with hTBM, it may still be beneficial. Analysis of sera from baboons which had rejected porcine cardiac xenografts revealed the presence of elicited antibodies directed against several porcine cell surface proteins including CD46, CD59 and EPCR (26). Failure to completely prevent the generation of these antibodies may contribute to a vicious cycle of inflammation and coagulation, but conceptually this could be nullified by transgenic expression of the human homologs of the porcine complement and coagulation regulators, if these were appropriately modified in a post-translational manner. Furthermore, although not specifically addressed here, supraphysiological expression of human EPCR may amplify its own TBM-independent cytoprotective effects.

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Figure Legends

Figure 1. cDNA and predicted amino acid sequence of pEPCR.
The thin underlining designation denotes the predicted signal peptide; amino acids, which are numbered relative to the mature protein after signal peptide processing. Extracellular cysteines are circled, and putative *N*-glycosylation sites are boxed. The thick and dashed underlines indicate the MCH class I/CD1 and transmembrane domains, respectively.

Figure 2. Homology between the protein sequences of pig and human EPCR.
Vertical bars and colons indicate identity and conservation of amino acid residues, respectively. Red asterisks denote hEPCR residues that have been shown to be essential for human APC binding (18). Other annotations are as described for Fig. 1.

Figure 3. hTBM expression in transfected COS-7 cells.
A) Flow cytometric profiles of COS-7 cells transiently transfected with hTBM ± hEPCR or pEPCR, showing similar hTBM expression. B) Quantitation of hTBM expression averaged over 5-7 independent experiments, showing reproducible transfection efficiency. Vector and hTBM, n = 7; hTBM/pEPCR, n = 5; hTBM/hEPCR, n = 6. MFI: mean fluorescence intensity.

Figure 4. TBM cofactor activity of transfected COS-7 cells.

A) Cofactor activity for human thrombin-mediated activation of human protein C, assayed by Spectrozyme PCa. Cofactor activity was significantly increased by hTBM expression, and further significantly increased by co-expression of hEPCR or pEPCR. Vector, pTBM and hTBM: n = 7; hTBM/pEPCR: n = 5; hTBM/hEPCR: n = 6. B) Typical Spectrozyme PCa plots.

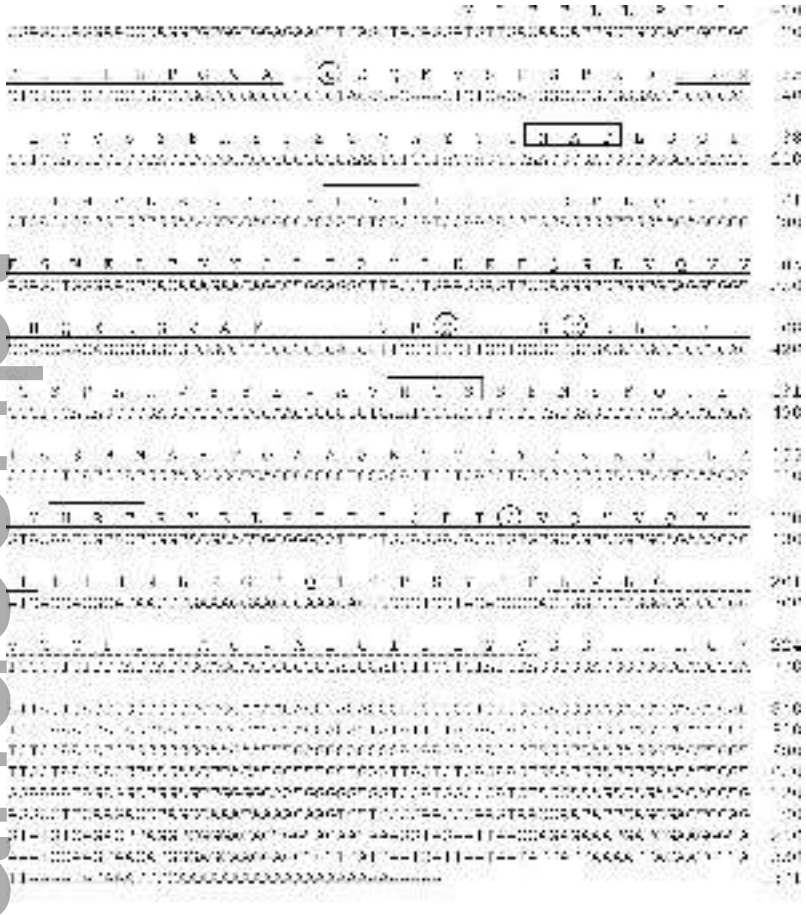

Figure 1: cDNA and predicted amino acid sequence of pEPOR. The thin underlining designates the putative signal peptide; amino acids, which are numbered relative to the mature protein after signal peptide processing. Extracellular cysteines are circled, and putative N-glycosylation sites are boxed. The thick and dashed underlines indicate the WSXWS and transmembrane domains, respectively.

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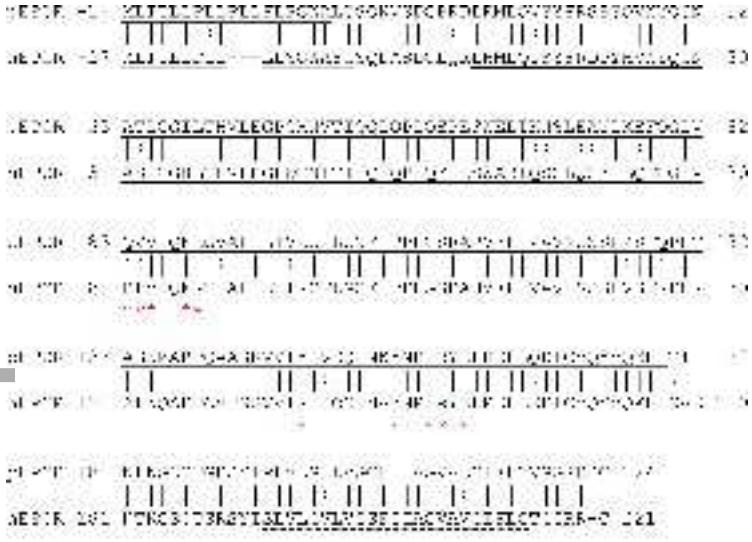

Figure 2. Homology between the protein sequences of pig and human EPOR. Vertical bars and colons indicate identity and conservation of amino acid residues, respectively. Red asterisks denote EPOR residues that have been shown to be essential for human EPO binding [15]. Other structural features are described in [9].

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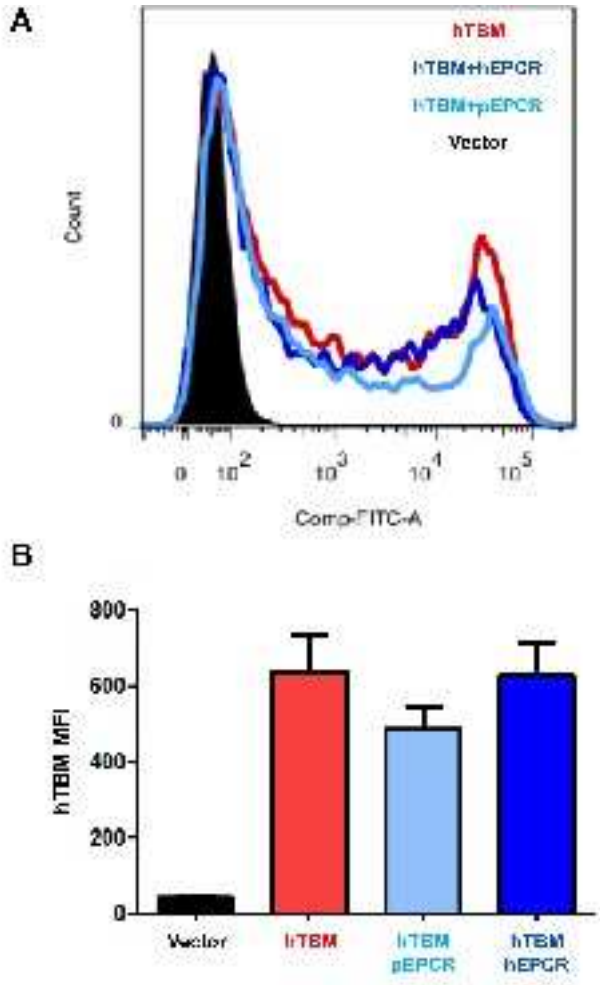

Figure 3. hTBM expression in transfected COS-7 cells. A) Flow cytometric profiles of COS-7 cells transiently transfected with hTBM, hEPCR or pEPCR, showing similar hTBM expression. B) Quantification of hTBM expression averaged over five independent experiments, showing reproducible transfection efficiency. Vector and hTBM, n=7, hTBM/pEPCR, n=5, hTBM/hEPCR, n=5. MFI, mean fluorescence intensity.

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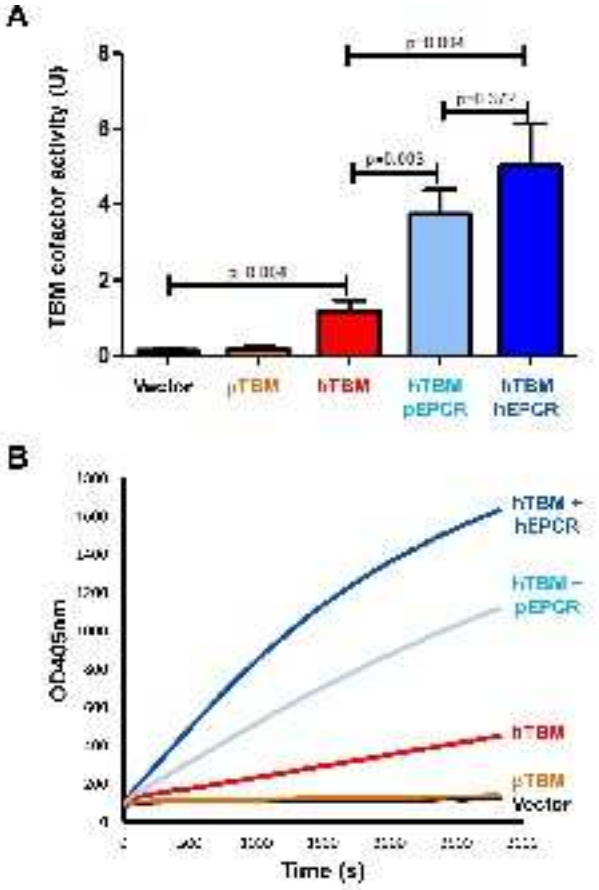

Figure 4. TBM cofactor activity of transfected COS 7 cells. A) Cofactor activity for human [illegible] thrombin-mediated activation of human protein C, assayed by spectrophotometric PCa. Cofactor activities were significantly increased by TBM expression alone, but were significantly increased by co-expression of hEPCR or pEPCR. Vector, µTBM and hTBM, n=7. hTBM/pEPCR [illegible] hTBM/hEPCR [illegible]. B) Typical spectrophotometric PCa plots.

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