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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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5 **Abstract**

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

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

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

20 **Results:** The predicted protein sequence of pEPCR shared 72.3% amino acid
21 sequence identity with hEPCR, and residues critical for protein C binding were
22 conserved. COS-7 cells transfected with hEPCR, pEPCR or vector showed minimal
23 TBM cofactor activity (0.13 ± 0.04 , 0.13 ± 0.02 and 0.14 ± 0.06 U, respectively). The
24 cofactor activity of hTBM-transfected cells (1.18 ± 0.29 U) was 8-fold higher than
25 vector-transfected cells ($p = 0.004$) and further increased 4-fold and 3-fold by co-
26 transfection with hEPCR (5.01 ± 1.12 U, $p = 0.004$) or pEPCR (3.73 ± 0.65 U, $p =$
27 0.003), respectively.

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

1

2 **Keywords**

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

5 **Abbreviations used**

6 APC activated protein C

7 EPCR endothelial protein C receptor

8 FITC fluorescein isothiocyanate

9 GTKO α 1,3-galactosyltransferase gene-knockout

10 h human

11 MFI mean fluorescence intensity

12 p pig

13 PAEC porcine aortic endothelial cells

14 RACE rapid amplification of cDNA ends

15 RT-PCR reverse transcription - polymerase chain reaction

16 TBM thrombomodulin

17 **Authorship statement**

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

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

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

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

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

4

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7 Council of Australia (558408).

8 **Introduction**

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

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

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

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

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

12 **Materials and methods**

13 ***cDNA preparation, PCR and RACE***

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

23

24 ***Transfection of COS cells***

25 The mammalian expression plasmid pBOS-FLAG was used to construct vectors for
26 the expression of FLAG-tagged hTBM, pTBM, hEPCR or pEPCR. 5×10^6 COS-7
27 cells were transiently transfected with a total of 5 μ g of DNA by electroporation using
28 a Gene Pulser (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) at 0.2V and
29 960 μ Fd. 48 hrs after transfection, cells were analyzed by flow cytometry using FITC-
30 labelled antibodies to detect expression of FLAG (Clone K, WEHI, Parkville, Victoria,

1 Australia), hTBM (Clone IA4, WEHI), or hEPCR (rat anti-mouse hEPCR Clone RCR-
2 252, BD Biosciences). The anti-hTBM and anti-hEPCR mAbs did not cross-react
3 with pig (data not shown).

4

5 ***Thrombomodulin cofactor activity assay***

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

17

18 ***Statistical analysis***

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

22 **Results**

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

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

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

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

25 Comparison of the pEPCR cDNA sequence with the porcine genome revealed
26 99.5% identity with a region of chromosome 17 (sequence ID: NC_010459.5). The
27 comparison indicated that the pEPCR gene spans 4.6 kb and contains 4 exons
28 (>120 bp and approximately 250, 280 and 700 bp) separated by 3 introns of
29 approximately 2.6 kb, 370 bp and 240 bp. This structure is similar to that of the
30 hEPCR gene on chromosome 20 (19, 20), with the exception that the second intron
31 of hEPCR is larger (1217 bp). The pEPCR amino acid sequences predicted from the
32 cDNA (Fig. 1) and the available genomic sequence were identical.

1

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

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

13 48 hrs after transfection, cells were analyzed for TBM cofactor activity in the
14 presence of human thrombin and human protein C using the Spectrozyme PCa
15 chromogenic assay. As expected, cells transfected with vector, hEPCR or pEPCR
16 alone, in the absence of hTBM, showed minimal cofactor activity (0.14 ± 0.06 , $0.13 \pm$
17 0.04 and 0.13 ± 0.02 U, respectively). Cofactor activity of cells transfected with
18 pTBM was also minimal (0.18 ± 0.05 U, $p = 0.578$ vs vector-transfected), as
19 previously reported (6). Cofactor activity of hTBM-transfected cells (1.18 ± 0.29 U)
20 was 8-fold higher than that of vector-transfected cells ($p = 0.004$), and was increased
21 a further 4-fold or 3-fold by co-transfection with hEPCR (5.01 ± 1.12 U, $p = 0.004$) or
22 pEPCR (3.73 ± 0.65 U, $p = 0.003$), respectively (Fig. 4). We did not attempt to
23 measure the anti-inflammatory and cytoprotective/anti-apoptotic activity of EPCR
24 because these functions are dependent on PAR-1, which is not expressed in COS-7
25 cells (21).

26 **Discussion**

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

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

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

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

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13

14 **Figure Legends**

15 **Figure 1. cDNA and predicted amino acid sequence of pEPCR.**

16 The thin underlining designation denotes the predicted signal peptide; amino acids,
17 which are numbered relative to the mature protein after signal peptide processing.
18 Extracellular cysteines are circled, and putative *N*-glycosylation sites are boxed. The
19 thick and dashed underlines indicate the MCH class I/CD1 and transmembrane
20 domains, respectively.

21

22 **Figure 2. Homology between the protein sequences of pig and human EPCR.**

23 Vertical bars and colons indicate identity and conservation of amino acid residues,
24 respectively. Red asterisks denote hEPCR residues that have been shown to be
25 essential for human APC binding (18). Other annotations are as described for Fig. 1.

26

27 **Figure 3. hTBM expression in transfected COS-7 cells.**

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

1

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

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

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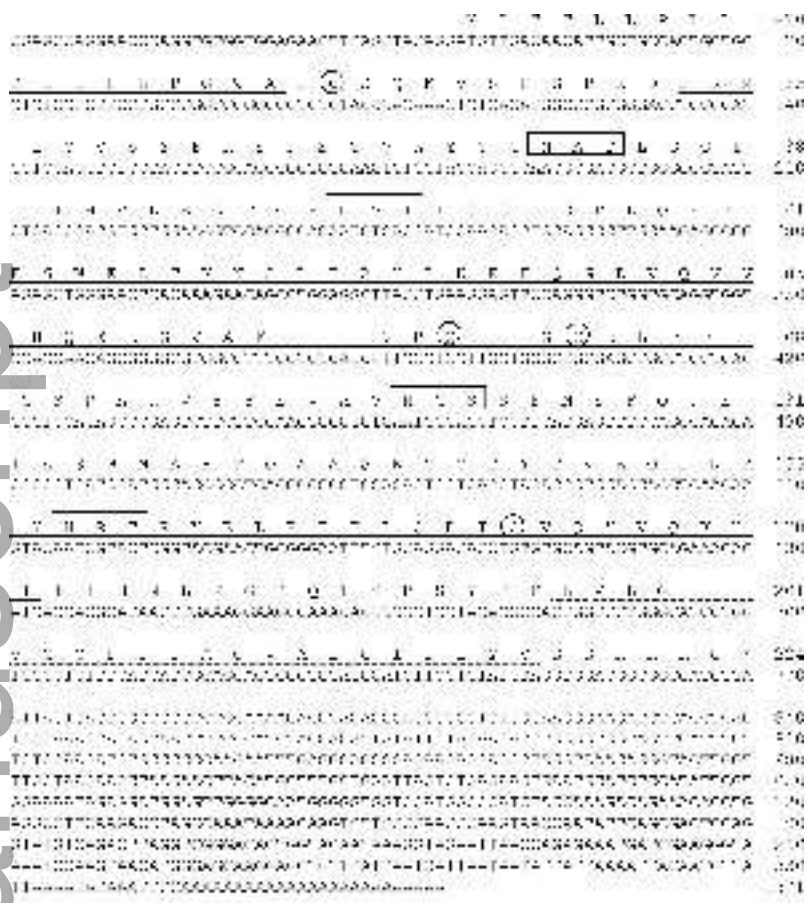


Figure 1. cDNA and predicted amino acid sequence of pPCR. The thin underlining designation identifies the predicted signal sequence amino acids, which are numbered relative to the mature protein after signal peptide processing. Secondary structures are predicted, and putative N-glycosylation sites are shown. The thin and dashed underlines indicate the M1E class p1D and II motifs, respectively.

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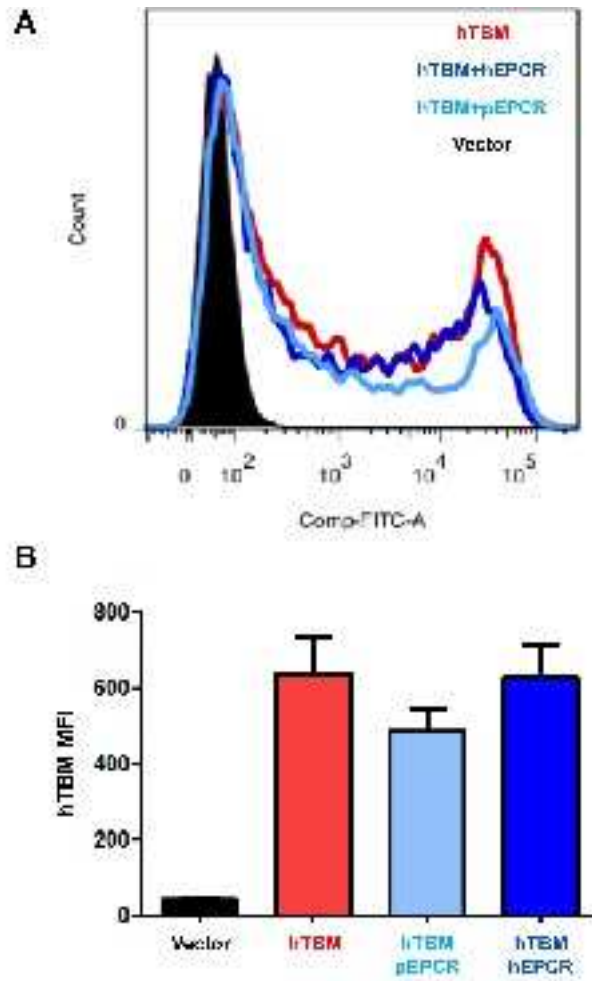


Figure 3. hTBM expression in transfected COS-7 cells. A) Flow cytometry analysis of COS-7 cells transiently transfected with hTBM, hEPCR or pEPCR, showing similar hTBM expression. B) Quantification of hTBM expression averaged over five independent experiments, showing reproducible transfection efficiency.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

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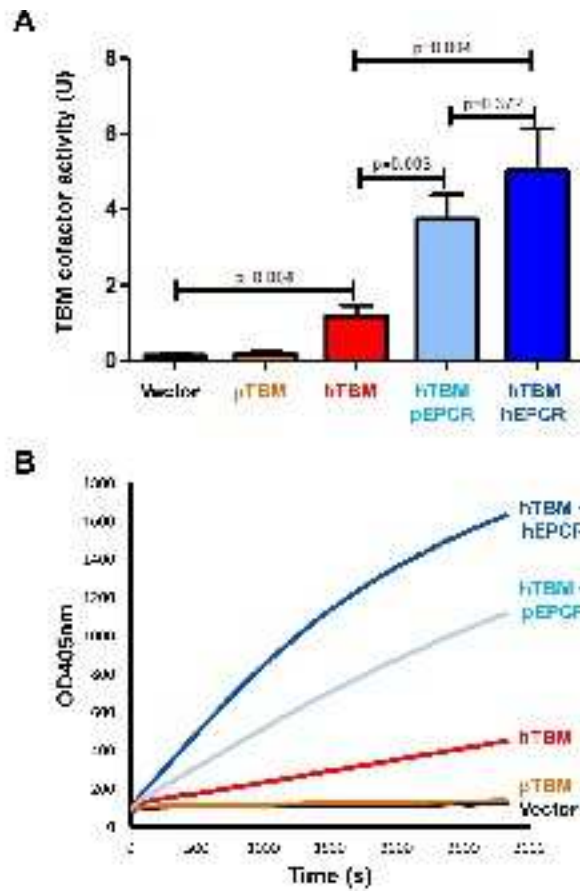


Figure 4. TBM cofactor activity of transfected COS 7 cells. **A)** Cofactor activity for human thrombin-mediated activation of human protein C, assayed by spectrometric PCa. Cofactor activity was significantly increased by hTBM expression, and further significantly increased by co-expression of hEPCR or pEPCR. Vector, pTBM and hTBM (n=7, hTBM/pEPCR = 5, hTBM/hEPCR = 6). **B)** Typical spectrometric PCa plots.

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