# Regulatory Sequences of the Porcine *THBD* Gene Facilitate Endothelial-Specific Expression of Bioactive Human Thrombomodulin in Single- and Multitransgenic Pigs

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**Background.** Among other mismatches between human and pig, incompatibilities in the blood coagulation systems hamper the xenotransplantation of vascularized organs. The provision of the porcine endothelium with human thrombomodulin (hTM) is hypothesized to overcome the impaired activation of protein C by a heterodimer consisting of human thrombin and porcine TM.

**Methods.** We evaluated regulatory regions of the *THBD* gene, optimized vectors for transgene expression, and generated hTM expressing pigs by somatic cell nuclear transfer. Genetically modified pigs were characterized at the molecular, cellular, histological, and physiological levels.

**Results.** A 7.6-kb fragment containing the entire upstream region of the porcine *THBD* gene was found to drive a high expression in a porcine endothelial cell line and was therefore used to control hTM expression in transgenic pigs. The abundance of hTM was restricted to the endothelium, according to the predicted pattern, and the transgene expression of hTM was stably inherited to the offspring. When endothelial cells from pigs carrying the hTM transgene—either alone or in combination with an aGalTKO and a transgene encoding the human CD46—were tested in a coagulation assay with human whole blood, the clotting time was increased three- to four-fold ($P<0.001$) compared to wild-type and aGalTKO/CD46 transgenic endothelial cells. This, for the first time, demonstrated the anticoagulant properties of hTM on porcine endothelial cells in a human whole blood assay.

**Conclusions.** The biological efficacy of hTM suggests that the (multi-)transgenic donor pigs described here have the potential to overcome coagulation incompatibilities in pig-to-primate xenotransplantation.

**Keywords:** Xenotransplantation, Coagulation incompatibility, Thrombomodulin, Transgenic donors, Human thrombomodulin, Transgenic donor pigs.

(*Transplantation* 2014;97: 138–147)

This study was supported by the Deutsche Forschungsgemeinschaft (DFG Transregio Research Unit 535 and Transregio Collaborative Research Center 127) and the Swiss National Science Foundation (32003B_135272).

The authors declare no conflicts of interest.

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A.W. participated in the design of the study, performed characterization of endothelial cells and participated in data interpretation and writing of the article. A.Ba. performed genomic analysis of the pigs and managed pig housing. A.K.B. performed analysis of biological function. E.K., A.Bl., and R.W. performed expression analysis of the pigs. W.B. performed evaluation studies of vectors. S.H. performed flow cytometric analysis. M.K., V.Z., and B.K. performed nuclear transfer and embryo transfer. C.F. participated in the analysis of founder pigs. J.M.A. and B.R. participated in the design of the study and data interpretation. R.S. designed evaluation studies of the vectors. H.N. provided scientific support in nuclear transfer. R.R. designed analysis of biological function. D.A. provided multitransgenic pigs. E.W. participated in the design of the study and in data interpretation and writing of the article. N.K. participated in the design of the study, performed bioinformatics analysis and vector construction, and participated in data interpretation and writing of the article.

E.W. and N.K. equally contributed to this study.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Received 4 April 2013. Revision requested 25 August 2013.

Accepted 15 August 2013.



ISSN: 0041-1337/14/9702-138

DOI: 10.1097/TP.0b013e3182a95cbc

The use of multitransgenic donor pigs, as well as the advanced preoperative and postoperative care of nonhuman primates, has led to impressive progress in the xenotransplantation of vascularized organs over the last decade, tempting one to think about its clinical implementation in the near future (*1*). In particular, the survival of pig-to-baboon heart transplants for up to 8 months inspires these ambitions (*2*). Yet, such feats are still exceptional, and the immunosuppressive regimens that have led to these promising data are far from clinical application owing to the severe side effects that result from systemic and high-dose administration of drugs. An appealing approach to circumvent these complications would be the genetic tailoring of donors because this allows the local but sustained application of an active agent. In a recent article, we demonstrated the applicability of such a strategy by using islet transplants from transgenic pigs that sufficiently suppressed T-cell–mediated rejection at the transplant site in humanized mice but reduced the systemic burden of immunosuppression in the recipient by two orders of magnitude (*3*). Similarly, the coagulation problems that occur in xenotransplantation of vascularized organs (*4, 5*) might be resolved by using genetically modified donors. Coagulation issues in xenografts have been supposed to occur due to incompatibilities between human blood and the porcine vessel wall, with the main obstacle being the impaired activation of protein C by a heterodimer consisting of human thrombin and porcine thrombomodulin (*6*). Numerous attempts to overcome this problem have been suggested (reviewed in Cowan et al. (*7*)), but the most convincing approach would, in any case, be the transgenic expression of the human variant of thrombomodulin (hTM) in donor pigs, in particular as the molecule not only has anticoagulant but also anti-inflammatory properties (*8*). The beneficial effect of hTM has been demonstrated in early xenoperfusion studies (*9, 10*), and two models of hTM expressing pigs have been presented recently (*11, 12*). In contrast to these studies that used ubiquitously active promoters to drive hTM expression, other studies aimed at the identification of regulatory elements to restrict the abundance of a transgene to the endothelial wall (*13–15*) as the correct balance of the blood coagulation system might require not only the presence of functionally compatible key players but also their tightly controlled expression (*16, 17*). The multifaceted regulation of thrombomodulin on the transcriptional as well as posttranslational level seems to involve numerous stimulators such as shear stress, hypoxia, reactive oxygen species, free fatty acids, or inflammation (reviewed in Conway (*8*)) and suggests a *THBD* promoter to control hTM expression. Although regulatory sequences from different species have been successfully used in numerous transgenic mouse models, species-specific transcriptional initiation has been described in defined physiological processes such as the regulation of pluripotency (*18*), embryo-maternal communication (*19*) or immunology (*20*). Moreover, increasing evidence for a more significant divergence in the transcriptional regulation even between closely related species has been found (*21, 22*) and the role of specific regulatory elements has been postulated to be more relevant for expression control compared to other parameters such as epigenetic pattern, cellular environment, or the abundance of transcription factors (*23*). Consequently, transgene expression in the pig might be more efficiently initiated by porcine regulatory elements than by orthologous sequences from human or mouse.

Thus, we (i) developed and tested an expression vector for reliable transgene expression in porcine endothelial cells, (ii) generated single- and multitransgenic pigs that exhibit and inherit strong and endothelial-specific expression of hTM, and (iii), for the first time, demonstrated the potential of hTM on transgenic porcine endothelial cells to prolong clotting time in a human whole-blood coagulation assay.

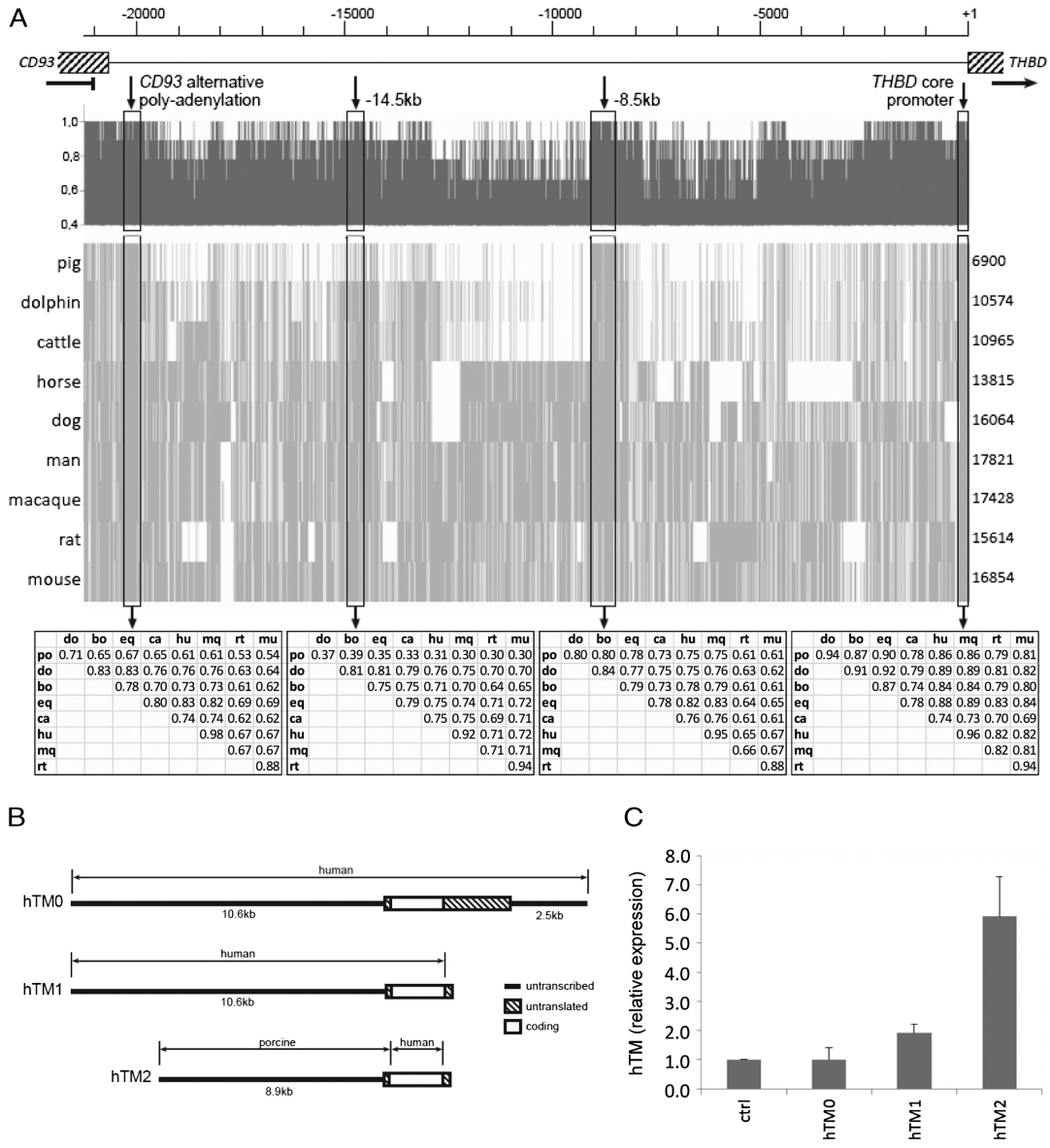
## RESULTS

### Interspecies Comparison of THBD Regulatory Elements

As fundamental characteristics of promoter regions should be conserved among related species, we examined the thrombomodulin locus of human, macaque, mouse, rat, dog, horse, cattle, dolphin, and pig to identify the most essential regulatory elements (Fig. 1A; **Figure S1, SDC,** http://links.lww.com/TP/A885). For the region between the thrombomodulin encoding *THBD* gene and the upstream *CD93* gene, multispecies alignments revealed huge differences in the intergenic length, ranging from less than 7 kb to almost 18 kb between the respective species. Nonetheless, three regions were identified in the alignment, which showed high homology between all species, whereas the fragments in between were characterized by large gaps in one or more sequences and a reduced similarity between the remaining species. One region located closely to the 3′-UTR of *CD93* was predicted to contain another potential polyadenylation site of the gene. At the very other end of the alignment, the presumed core promoter of the *THBD* gene contained commonly conserved binding sites for numerous transcription factors (TFs) and core promoter elements. The third region 8.5 kb upstream of the *THBD* transcription start might represent an enhancer element because it contained conserved binding sites for diverse TFs as well. A fourth region 14.5 kb upstream of the *THBD* transcription start is of particular interest because eight species showed sequence homologies similar to those in the other conserved regions as well as potential TF binding sites, whereas this fragment was completely absent in the pig. Another interesting feature of the porcine sequence was the unique presence of a potential insulator segment adjacent to the novel potential polyadenylation site of *CD93*.

### Design of an Endothelial-Specific hTM Expression Vector

Based on a genomic fragment containing the human *THBD* locus, we introduced two modifications (Fig. 1B). First, we replaced the 3′-UTR and downstream region of *THBD* by a widely used polyadenylation cassette from the bovine growth hormone (*GH*) gene because the 3′-UTR of *THBD* has been described to cause mRNA instability in the presence of cytokines (*24*). Second, we replaced the 5′-UTR and the upstream region of human *THBD* by a porcine fragment containing the complete intergenic region between *CD93* and *THBD*, the transcription start, as well as the 5′-UTR. In an in vitro assay, we transfected immortalized porcine endothelial cells with the respective vectors, determined the




| | do | bo | eq | ca | hu | mq | rt | mu |
|---|---|---|---|---|---|---|---|---|
| po | 0.71 | 0.65 | 0.67 | 0.65 | 0.61 | 0.61 | 0.53 | 0.54 |
| do | | 0.83 | 0.83 | 0.76 | 0.76 | 0.76 | 0.63 | 0.64 |
| bo | | | 0.78 | 0.70 | 0.73 | 0.73 | 0.61 | 0.62 |
| eq | | | | 0.80 | 0.83 | 0.82 | 0.69 | 0.69 |
| ca | | | | | 0.74 | 0.74 | 0.62 | 0.62 |
| hu | | | | | | 0.98 | 0.67 | 0.67 |
| mq | | | | | | | 0.67 | 0.67 |
| rt | | | | | | | | 0.88 |

| | do | bo | eq | ca | hu | mq | rt | mu |
|---|---|---|---|---|---|---|---|---|
| po | 0.37 | 0.39 | 0.35 | 0.33 | 0.31 | 0.30 | 0.30 | 0.30 |
| do | | 0.81 | 0.81 | 0.79 | 0.76 | 0.75 | 0.70 | 0.70 |
| bo | | | 0.75 | 0.75 | 0.71 | 0.70 | 0.64 | 0.65 |
| eq | | | | 0.79 | 0.75 | 0.74 | 0.71 | 0.72 |
| ca | | | | | 0.75 | 0.75 | 0.69 | 0.71 |
| hu | | | | | | 0.92 | 0.71 | 0.72 |
| mq | | | | | | | 0.71 | 0.71 |
| rt | | | | | | | | 0.94 |

| | do | bo | eq | ca | hu | mq | rt | mu |
|---|---|---|---|---|---|---|---|---|
| po | 0.80 | 0.80 | 0.78 | 0.73 | 0.75 | 0.75 | 0.61 | 0.61 |
| do | | 0.84 | 0.77 | 0.75 | 0.75 | 0.76 | 0.63 | 0.62 |
| bo | | | 0.79 | 0.73 | 0.78 | 0.79 | 0.61 | 0.61 |
| eq | | | | 0.78 | 0.82 | 0.83 | 0.64 | 0.65 |
| ca | | | | | 0.76 | 0.76 | 0.61 | 0.61 |
| hu | | | | | | 0.95 | 0.65 | 0.67 |
| mq | | | | | | | 0.66 | 0.67 |
| rt | | | | | | | | 0.88 |

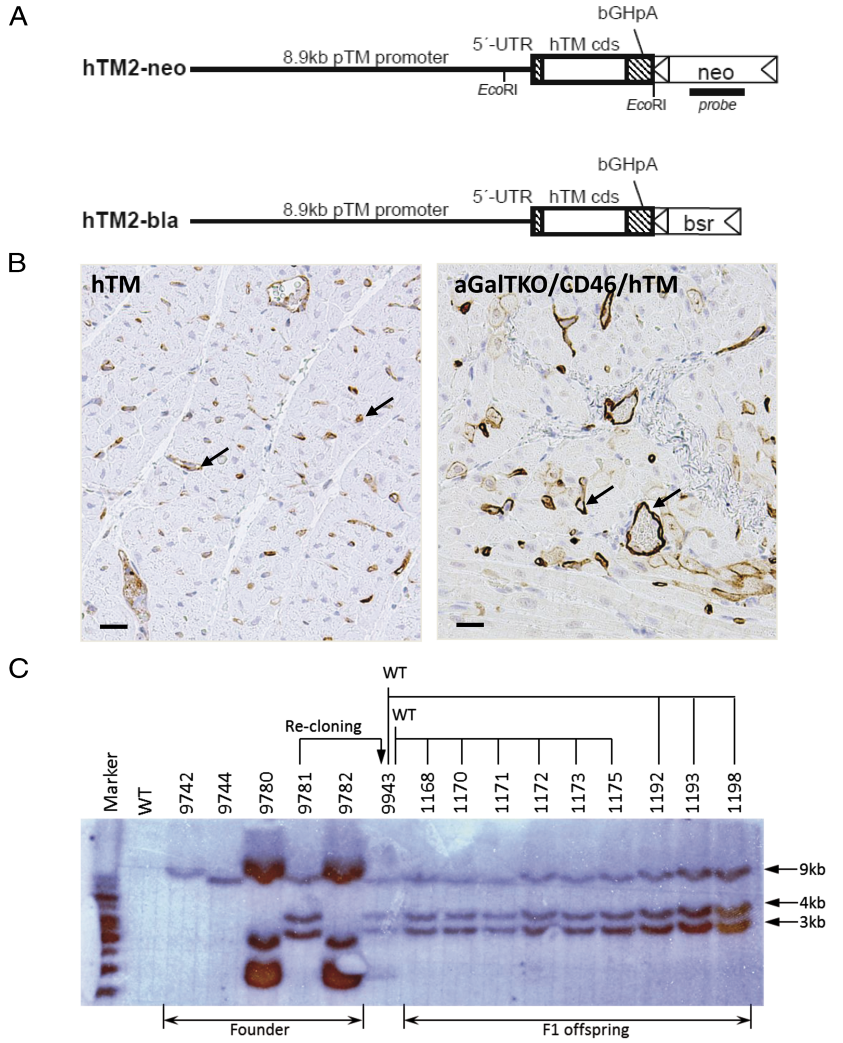
| | do | bo | eq | ca | hu | mq | rt | mu |
|---|---|---|---|---|---|---|---|---|
| po | 0.94 | 0.87 | 0.90 | 0.78 | 0.86 | 0.86 | 0.79 | 0.81 |
| do | | 0.91 | 0.92 | 0.79 | 0.89 | 0.89 | 0.81 | 0.82 |
| bo | | | 0.87 | 0.74 | 0.84 | 0.84 | 0.79 | 0.80 |
| eq | | | | 0.78 | 0.88 | 0.89 | 0.83 | 0.84 |
| ca | | | | | 0.74 | 0.73 | 0.70 | 0.69 |
| hu | | | | | | 0.96 | 0.82 | 0.82 |
| mq | | | | | | | 0.82 | 0.81 |
| rt | | | | | | | | 0.94 |

**FIGURE 1.** Evaluation of *THBD* regulatory sequences. (A) From nine mammalian species, a 21-kb alignment of the intergenic regions between the *CD93* and *THBD* genes was generated. Four conserved regions (*boxed*) have been identified by a homology plot (*upper panel*), a density plot (*middle panel*, with *white bars* indicating gaps in the respective species), as well as the accumulation of potential conserved TF binding sites (see **SDC,** http://links.lww.com/TP/A885). The calculation of similarity matrices (*lower panel*) for these regions indicated strong evolutionary conservation among the examined species with the exception of the −14.5-kb region that was lacking in pig but strongly conserved among the other species. The shown homology values between pig and the other species for this region result from alignment artifacts. (B) Three different hTM constructs were compared. hTM0 consisted of a completely human genomic fragment; in hTM1, the *THBD* 3′-UTR and downstream region were replaced by a polyadenylation cassette from the bovine *GH* gene and hTM2 contained the modified 3′-end as well as the regulatory region upstream of the porcine *THBD* gene. (C) PEDSV.15 cells transfected with the constructs hTM0, hTM1, and hTM2 were stained with the anti-hTM antibody 6980-100 and analyzed by flow cytometry. Relative expression was determined by comparing the number of hTM-positive cells obtained with each construct with background staining found in mock transfected PEDSV.15 cells (ctrl). Data represent mean values ±SD obtained in three independent experiments.

proportion of hTM expressing cells by FACS analysis, and clearly found the porcine promoter to drive a stronger expression than the human fragment (Fig. 1C; **Figure S2, SDC,** http://links.lww.com/TP/A885).

## Establishment of hTM Expressing Pigs

Primary fibroblasts from a wild-type pig and kidney cells from an alpha1,3-galactosyl transferase–deficient (aGalTKO) (*25*) and CD46 transgenic (*26*) pig were nucleofected with




D

| Generation of | | Transferred embryos | Embryo transfers | Litters | Piglets (stillborn) | Cloning effciency [%]* |
|---|---|---|---|---|---|---|
| hTM | founder | 345 | 4 | 3 | 8 (2) | 2.7 |
| | recloning | 368 | 5 | 2 | 2 (1) | 1.3 |
| aGalTKO/ CD46/hTM | founder | 298 | 4 | 1 | 3 (0) | 3.5 |
| | recloning | 2491 | 20 | 8 | 27 (24) | 2.4 |

*number of offspring born per SCNT embryos transferred, including only recipients that delivered

**FIGURE 2.** Generation of hTM-transgenic pigs by SCNT. (A) The hTM2 construct was linked to a neomycin (neo) resistance cassette for transfection into wild-type cells and to a blasticidin S (bsr) resistance cassette for transfection into aGalTKO/CD46 cells. Localizations of the *Eco*RI restristion sites and the neo-specific probe for Southern blotting are indicated. (B) Transgenic founders were examined for hTM expression by immunohistochemical detection of hTM (*brown color*) in vascular endothelial cells in the cardiac tissue of hTM and aGalTKO/CD46/hTM founder pigs. Bars=20 μm. (C) Southern blot examination of transgenic pigs carrying the hTM2-neo vector with the neo-specific probe on *Eco*RI-digested genomic DNA. Marker is 1 kb ladder (Thermo Scientific); WT is wild-type control; 9742, 9744, 9780–9782 are founder animals; 9943 was recloned from primary cells of founder 9781; and 1168–1198 are transgenic offspring that were delivered from two independent litters after mating 9943 to wild-type sows. The approximate sizes of the transgene-carrying fragments are indicated on the right side. (D) The cloning and recloning efficiencies of SCNT experiments are within our long-term experience.

the hTM2 expression vector that has been linked to a neomycin or a blasticidin S resistance cassette, respectively (Fig. 2A). After growing cells under selection, stable cell clones were pooled and used for somatic cell nuclear transfer (SCNT) to generate hTM or aGalTKO/CD46/hTM pigs according to Richter et al. (*27*). First, a total of eight hTM transgenic founder pigs were generated and five were examined for hTM expression in various organs either at the day of birth or at the age of 1 month. All founders showed strong and endothelial-specific expression of the transgene in heart and kidney, and three of them showed additionally strong expression on vascular endothelial cells in the liver and lung. Southern blot analysis suggested that the five founders represent four different integration patterns of the construct (Fig. 2C). Primary kidney cells from an animal with a high and endothelial-specific expression in the heart (Fig. 2B, left panel) were chosen to reproduce the founder by SCNT. Two delivered animals were raised to fertility, and one of them was mated to wild-type sows. The inheritance of the transgene integration pattern (Fig. 2C) and its expression pattern to vital and fertile offspring over, as of now, two generations indicates that the transgene does not interfere with animal health or reproductive traits. Second, a total of three aGalTKO/CD46/hTM founders were generated and examined for hTM expression at an age of either a few days or 1 month. Corresponding to the findings for the hTM transgenic pigs, all aGalTKO/CD46/hTM animals showed strong and endothelial-specific hTM expression in the heart and kidney, and two of them also revealed high hTM abundance on endothelial cells of the lung and liver. A total of nine aGalTKO/CD46/hTM animals have been reproduced by SCNT from primary cells of a highly expressing founder with positive hTM immunoreactivity on vascular endothelial cells of cardiac tissue specimens (Fig. 2B, right panel). The efficiencies of cloning and recloning hTM-transgenic animals (Fig. 2D) reflect our long-years' experience (*28*).

For a more detailed analysis of transgene expression and its biological function, individuals of both transgenic lines were examined at the histological, cellular, molecular and physiological levels. For both the hTM and the aGalTKO/CD46/hTM lines, several animals were examined. As the results were consistent within the lines, data from no. 1198 are shown as representative for the hTM line and data from no. 1259 are shown as representative for the aGalTKO/CD46/hTM line.

## Expression Analysis of hTM

The expression of hTM in tissue specimens and cultured endothelial cells was investigated using immunochemical methods.

In cardiac tissue sections of both lines, hTM immunoreactivity was exclusively present on the endocardium and in vessels of large as well as small caliber, displaying congruent abundance patterns with the endothelial cell marker von Willebrand factor (Fig. 3). In the kidney, hTM expression was present in the endothelia of intertubular capillaries and larger vessels but was not detectable in glomerular endothelia. The lungs of hTM and aGalTKO/CD46/hTM pigs displayed diffuse endothelial hTM immunoreactivity, whereas in the liver, endothelial hTM expression was detected on the endothelia of large vessels but not on those of the sinusoids (see **Figure S3, SDC,** http://links.lww.com/TP/A885).

The expression of the transgene on cultured aortic endothelial cells was investigated in more detail with FACS, immunofluorescence, and Western blot analyses. Immunocytochemistry verified the endothelial nature of the cultivated cells by positive CD31 staining and showed broad although unevenly distributed hTM expression (Fig. 4A). The finding of prevalent hTM expression was confirmed in flow cytometric analysis as at least 90% of the cells coexpressed CD31 and hTM in both transgenic lines (Fig. 4B). The presence of different expression levels was verified at a more quantitative level, as CD31-positive cells from aGalTKO/CD46/hTM pigs revealed two clearly distinct populations regarding the expression levels of hTM. In contrast, cells from hTM pigs showed a more homogenous population of cells that coexpressed CD31 and hTM. Cultivated endothelial cells were also used to extract protein for Western blot analysis. A single and distinct band corresponding to the expected size of hTM (116 kDa) was detected with the hTM-specific antibody (Fig. 4C).

## Biological Function of hTM on Porcine Endothelial Cells

In a first approach we tested whether hTM facilitated the cleavage of protein C in the presence of human thrombin in a biochemical assay (Fig. 5A). We found clear evidence that the amount of activated protein C (APC) in the supernatant of hTM or aGalTKO/CD46/hTM cells increased with the concentration of protein C in a dose-dependent manner, whereas the turnover of protein C remained at the basal level for any of the examined concentrations upon exposure to control cells lacking hTM.

In a further experiment, we tested whether hTM also possesses the ability to prevent blood coagulation/clotting in a more physiologic assay that models the processes at the interface of human blood and porcine vessel wall (*29, 30*). Aortic endothelial cells from wild-type, hTM, aGalTKO/CD46, and aGalTKO/CD46/hTM pigs were grown on microcarrier beads and tested for their potential to prolong the clotting time of freshly withdrawn, non-anticoagulated human blood. While clotting time in the presence of aGalTKO/CD46 aortic endothelial cells was slightly but significantly prolonged (100.9±20.5 min, $P<0.01$) as compared to the wild-type control (62.3±15.5 min), the effect of hTM either on the wild-type background or in the triple-transgenic combination (aGalTKO/CD46/hTM) was more pronounced, resulting in a three- to four-fold increase in clotting time (176.1±13.5 min, $P<0.001$ for hTM and 190.8±14.0 min, $P<0.001$ for aGalTKO/CD46/hTM) compared to wild-type endothelial cells (Fig. 5B). As the increased coagulation time in the presence of hTM-expressing cells might also result from the cytoprotective properties of the transgene product, we conducted another set of whole blood coagulation experiments and took samples at distinct time points to evaluate the coverage of the beads with endothelial cells (see **Figure S4, SDC,** http://links.lww.com/TP/A885). Albeit cells being lost during the experiment, we did not observe differences between cells that express hTM and those that do not and, thus, conclude that the main course of action of the hTM transgene was by its control of thrombin regulation. The effective anticoagulatory properties of hTM were further illustrated by the formation of thrombin–antithrombin III

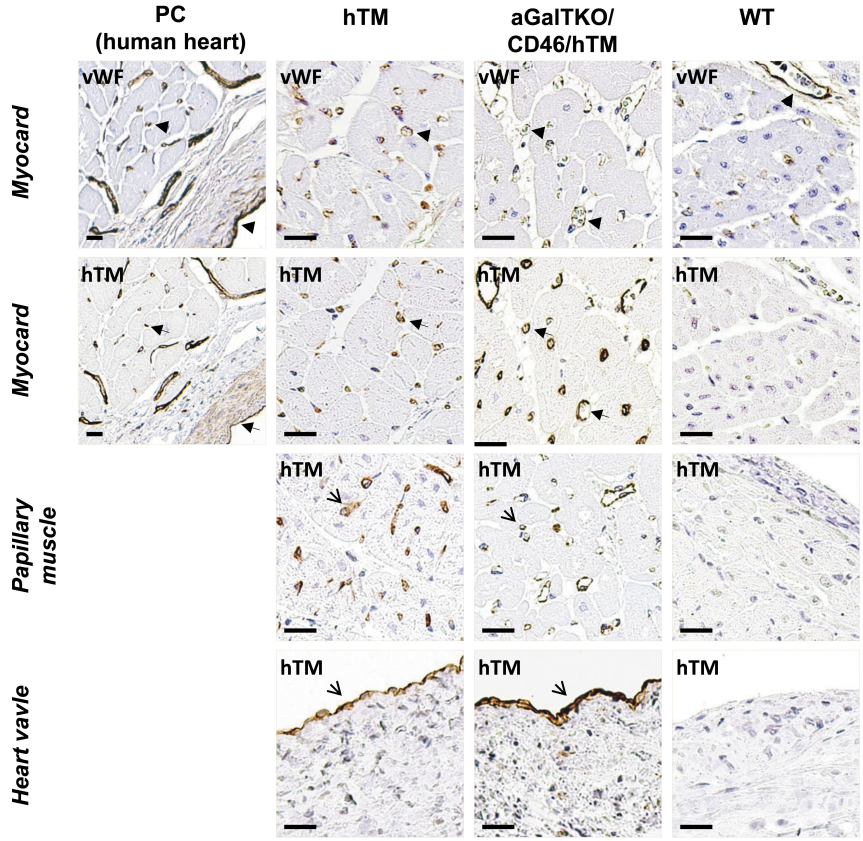



**FIGURE 3.** Immunohistochemical detection of hTM in the cardiac tissue of a wild-type control pig (WT), an hTM pig, a GalTKO/CD46/hTM pig, and in human positive control tissue (PC). Sequential sections of left ventricular myocardia were stained for the endothelial-specific von Willebrand factor (vWF) and hTM. In human and porcine cardiac tissue specimen, vWF was restricted to endocardial and vascular endothelial cells (*brown color*, *arrowheads*, *top row*). Staining of hTM corresponded to that of vWF in hTM and aGalKO/CD46/hTM pigs (*brown color*, *arrows*, *second top row*), whereas no staining was seen in WT pigs. Positive hTM immunoreactivity (*brown color*) was also detected in the capillary endothelia in the papillary muscle and in endocardial cells of the left atrioventricular heart valve of hTM and aGalTKO/CD46/hTM pigs but not in WT pigs. Paraffin sections. Bars=20 μm.

(TAT) complexes (Fig. 5C). While wild-type and aGalTKO/CD46 cells caused an immediate and steady increase of TAT until coagulation occurred, hTM transgenic cells kept TAT levels constantly low in the first phase of the experiment. Moreover, although slightly increasing afterward, in these groups, TAT did not reach the maximum levels that were seen in the wild-type or aGalTKO/CD46 groups.

## DISCUSSION

Advancements in the production of genetically modified donor pigs are a major reason for the progress that has been made in xenotransplantation research in recent years. Many different transgenic pigs have been described (*31, 32*) and many more will be produced in the future. The biotechnological production process itself does not represent a main hurdle any more, but the relatively long generation time of the pig and economic aspects of housing are still challenging factors. Thus, the detailed design of transgenic approaches and their thorough evaluation is of importance for efficient production of novel donor pig models. Consequently, we made use of state-of-the-art in silico analysis tools to estimate the potential of regulatory elements of the *THBD* gene and compared different vector systems in vitro to determine the most promising strategy for the generation of transgenic pigs and, later on, put effort in comprehensive characterization at the molecular, cellular, histological, and physiological levels.

Regarding the regulatory properties of *THBD*, the conserved regions found in multispecies alignments are indicative of common regulatory mechanisms of TM expression in mammals, but the diverse length of the examined sequences as well as the lack of certain segments in distinct species in otherwise highly conserved regions might also indicate unique properties in individual species. In the absence of detailed promoter studies in the literature, the regulative properties of *THBD* remain unclear, but the relatively short intergenic region between *THBD* and the upstream *CD93* in the pig allowed the usage of its entire length to control transgene expression.

The fact that hTM is not expressed on all endothelial cells and the distinct expression pattern of hTM in heart, kidney, lung, and liver strictly mirrors the data that have been published on a mouse model with a *lacZ* reporter

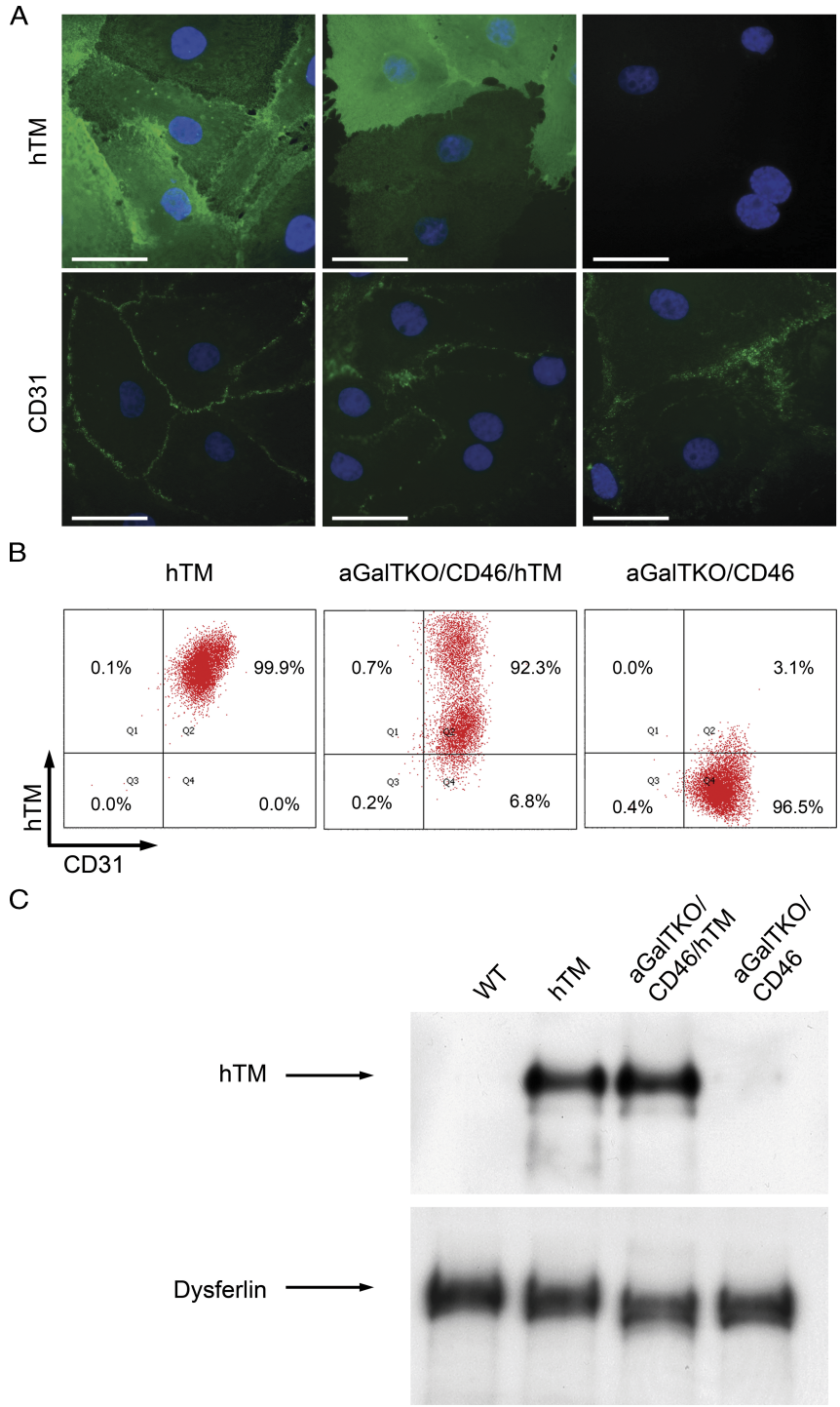



**FIGURE 4.** Expression of hTM on endothelial cells from hTM and aGalTKO/CD46/hTM pigs. (A) Immunocytochemistry showed hTM on paraformaldehyde-fixed endothelial cells from hTM and aGalTKO/CD46/hTM pigs, whereas hTM expression was absent on aGalTKO/CD46 cells. CD31 staining confirmed the endothelial origin of the cells. Bars=20 μm. (B) FACS analysis revealed a strong coexpression of CD31 and hTM on hTM and aGalTKO/CD46/hTM endothelial cells. (C) Immunoblot analysis showed a band with the correct size (116 kDa) demonstrating the expression of hTM in hTM and aGalTKO/CD46/hTM endothelial cells but not in wild-type or aGalTKO/CD46 cells.

knockin in the murine *Thbd* locus (*33*), suggesting that TM regulation is similar among mammalian species and further indicating that the transgene expression resembles the entire physiologic regulation of TM. This would be of relevance as one might assume that species-specific incompatibilities in blood coagulation have to be restored to a tightly regulated

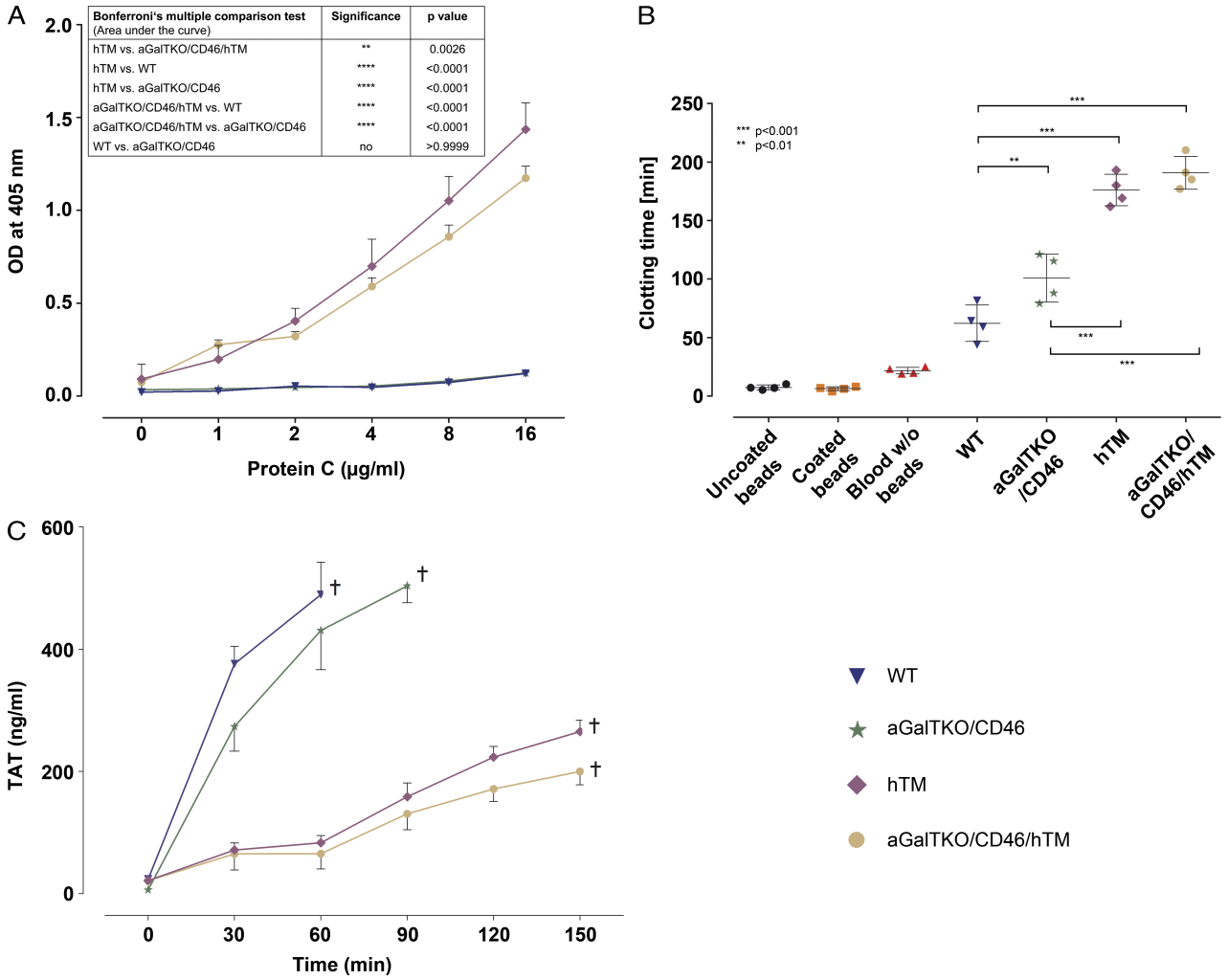



**FIGURE 5.** Biological function of the hTM transgene. (A) The ability of porcine endothelial cells to activate protein C in the presence of human thrombin was evaluated in a biochemical assay. The abundance of APC was detected colorimetrically. hTM transgenic endothelial cells revealed clear and concentration-dependent APC production when the amount of protein C was increased, whereas control cells (aGalTKO and WT) did not show any protein C turnover. Data are plotted as mean values, and standard deviations are indicated when they were above 0.025 $OD_{405}$. The inset shows the significance of area under the curve differences between the groups. Four independent experiments were performed for each group. Significance test was performed using one-way analysis of variance with Bonferroni correction. (B) Measurement of the clotting time after incubation of freshly withdrawn, non-anticoagulated whole human blood with wild-type or genetically modified endothelial cells cultivated on microcarrier beads. Microcarriers with or without collagen coating, incubated with blood, showed a strong procoagulant property with a short clotting time. Microcarriers covered by confluent hTM or aGalTKO/CD46/hTM endothelial cells showed a strong anticoagulant effect compared to wild-type and aGalTKO/CD46 PAECs. Four independent experiments were performed for each group. Significance test was performed using one-way analysis of variance with Bonferroni correction. (C) Formation of thrombin–antithrombin III (TAT) complexes was determined as a fluid phase coagulation parameter (thrombin generation) in EDTA plasma samples taken from whole blood coagulation assays at regular time intervals of 30 min until coagulation occurred (indicated by "†"). TAT is indicated as mean value (± standard deviation when it was >10.0 ng/mL). Three independent experiments were performed for each group.

physiological level, which is in contrast to other transgenic donor pigs for xenotransplantation that aim at immunological rejection processes, where the functional properties of a transgene may be less subject to transcriptional regulation. Ongoing transplantation studies using aGalTKO/CD46/hTM donors (B. Reichart and D. K. C. Cooper, personal communication) will have to prove the relevance of our strategy in nonhuman primate (NHP) models.

Albeit such transplantation experiments into primates being the most stringent tool for the evaluation of genetically modified donor organs, they suffer from their tremendous costs and the challenging experimental setup that is attributed to the complexity of the NHP system. Thus, the comprehensive examination of given transgenes under constant regimens in vivo at experimental numbers that are sufficient for scientific conclusions is limited to a small

number of transgenic combinations, and thus, they have to be selected by preevaluation in less complex systems. Here, we used an in vitro assay that exploits the natural anticoagulant properties of endothelial cells in combination with whole, non-anticoagulated human blood to test the efficacy of the hTM transgene product in coagulation control, either alone or in combination with the aGalTKO/CD46 transgenic background. Our coagulation assay confirmed the beneficial effect of hTM either on the wild-type or on the aGalTKO/CD46 background. These findings illustrate the suitability of the whole blood coagulation assay for the evaluation of clotting mechanisms in xenotransplantation approaches because it facilitates the analysis of several transgene combinations and, moreover, resembles the immediate interaction of porcine endothelium and human blood, also potentially avoiding wrong conclusions from in vivo transplantation models due to differences in the coagulation systems between NHP and man (*34*).

In summary, we developed a novel genetically modified pig for xenotransplantation and present evidence for its potential to overcome the incompatibilities in the coagulation systems between pig and man.

## MATERIALS AND METHODS

### Pig Housing

Animal experiments have been carried out according to the guidelines of the responsible authority (Regierung von Oberbayern, approval no. 55.2-1-54-2531-54).

### Bioinformatic Analysis

Sequences were fetched from the Ensembl genome browser (www.ensembl.org) and prepared in BioEdit (*35*). Mobile genetic elements were removed by RepeatMasker (*36*), and multiple alignments were done by ClustalW2 (*37*) and by using the Genomatix ElDorado/Gene2Promoter and GEMS Launcher software packages (Genomatix, Munich, Germany). The latter was also used to assign binding sites of common transcription factors.

### Vector Construction

Genomic fragments of the human and porcine *THBD* genes were excised from the BACs RP4-753D10 and CH242-263H13, respectively. The fragments were combined with other genetic elements using a two-step polymerase chain reaction, Cre-mediated recombination, and other conventional cloning procedures.

### Vector Evaluation

The immortalized porcine endothelial cell line PEDSV.15 (kindly provided by Prof. J. Seebach, Geneva (*38*)) was used for transfection and analyzed for hTM expression by flow cytometry.

### Founder Pig Generation

Transgenic pigs were established according to Richter et al. (*27*) and Klymiuk et al. (*39*) using the hTM2 expression vector and raised up to an age of 2.5 months. Genotyping was carried out as described elsewhere (*40*). Founders with a high-level expression of hTM were reestablished by SCNT and raised for breeding purposes.

### Necropsy, Histopathology, and Immunohistochemistry

For histological examination, cardiac tissue samples (including left and right heart ventricles, papillary muscles, and heart valves) and specimens of liver, kidney, and lung were taken from hTM-transgenic pigs and aGalTKO/CD46/hTM pigs. Control tissues were obtained from age-matched male wild-type pigs.

### Expression Analysis of Aortic Endothelial Cells

Porcine aortic endothelial cells (PAECs) were isolated from the aorta of transgenic pigs as described earlier (*38*) with minor modifications. Colocalization studies of hTM were performed with the endothelial-specific marker PECAM-1 (CD31). Cultivated cells were then analyzed either in a multicolor flow cytometric assay, immunocytochemistry, or Western blot analysis.

### APC Assay

The measurement of APC was performed with confluent PAECs on a 96-well plate.

### Coagulation Assay

The coagulation-inhibiting effects of the different genetically modified porcine endothelial cells were monitored in vitro using PAECs grown on microcarrier beads and whole, non-anticoagulated human blood as previously described (*29, 30*). Details about endothelial cell culture on microcarriers and the coagulation assay are described in the Supplemental Material. (see **SDC,** http://links.lww.com/TP/A885). Human blood was drawn from healthy volunteers.

For further details on materials and methods, see the Supplemental Material (see **SDC,** http://links.lww.com/TP/A885).

## ACKNOWLEDGMENTS

*The authors thank Eva-Maria Jemiller and Tuna Güngör for the excellent technical support.*

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