



# Genetically encoded Ca<sup>2+</sup>-sensor reveals details of porcine endothelial cell activation upon contact with human serum

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## Funding information

Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 310030\_182264; Deutsche Forschungsgemeinschaft, Grant/Award Number: CDC127

## Abstract

The activation of the endothelial surface in xenografts is still a poorly understood process and the consequences are unpredictable. The role of Ca<sup>2+</sup>-messaging during the activation of endothelial cells is well recognized and routinely measured by synthetic Ca<sup>2+</sup>-sensitive fluorophors. However, these compounds require fresh loading immediately before each experiment and in particular when grown in state-of-the-art 3D cell culture systems, endothelial cells are difficult to access with such sensors. Therefore, we developed transgenic pigs expressing a Ca<sup>2+</sup>-sensitive protein and examined its principal characteristics. Primary transgenic endothelial cells stimulated by ATP showed a definite and short influx of Ca<sup>2+</sup> into the cytosol, whereas exposure to human serum resulted in a more intense and sustained response. Surprisingly, not all endothelial cells reacted identically to a stimulus, rather activation took place in adjacent cells in a timely decelerated way and with distinct intensities. This effect was again more pronounced when cells were stimulated with human serum. Finally, we show clear evidence that antibody binding alone significantly activated endothelial cells, whereas antibody depletion dramatically reduced the stimulatory potential of serum. Transgenic porcine endothelial cells expressing a Ca<sup>2+</sup>-sensor represent an interesting tool to dissect factors inducing activation of porcine endothelial cells after exposure to human blood or serum.

## KEYWORDS

antibody depletion, complement activation, genetically encoded Ca<sup>2+</sup>-sensor, porcine endothelial cell activation

## 1 | INTRODUCTION

The relevance of the endothelium as the first line of xenogeneic reaction has been recognized for some time. In the endeavor to understand

mechanisms of hyperacute rejection (HAR) in discordant xenotransplantation, it became apparent that the situation a porcine graft faces in the first minutes after transplantation into a primate can be roughly recapitulated in vitro by the challenge of porcine endothelial cells

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(ECs) with human serum.<sup>1,2</sup> Moreover, the quantitative relationship of antibody deposition on the endothelial surface and activation of the complement system could be identified as major drivers of HAR.<sup>3</sup> Very soon, it became evident that the genetic modification of the donor, that is, the pig, would be the most promising approach to overcome antibody-mediated damage of the endothelium. Initially, additive transgenes expressing human complement regulatory proteins (hCRP) were introduced into the porcine genome,<sup>4</sup> aiming at suppressing the complement activation cascade that is triggered by antibody deposition. Although significant improvement was achieved by combinations of hCRP in pig-to-primate experiments,<sup>5</sup> HAR was finally overcome by eliminating the most relevant xenoepitope recognized by preformed human antibodies on porcine EC,  $\alpha$ 1,3-galactosyl-galactose (aGal), by deleting the responsible gene *GGTA1* (GTKO),<sup>6,7</sup> More recently, it was shown that expression of a human thrombomodulin (hTBM) transgene overcomes incompatibilities in the coagulation system between the human (or primate) bloodstream and the porcine endothelium<sup>8</sup> and additionally prevents inflammatory-induced activation of endothelial cells.<sup>9</sup> The combination of GTKO.hCRP.hTBM with sophisticated immunosuppression and prevention of ischemia/reperfusion injury prolonged survival of a porcine heart heterotopically transplanted into a baboon for 2.5 years.<sup>10</sup> Furthermore, it facilitated the survival of baboon recipients after fully life-supporting orthotopic heart transplantation for 6 months.<sup>11</sup> In another study, life-sustaining transplantation of porcine kidneys into macaques was achieved by a combination of only GTKO.hCRP,<sup>12</sup> whereas the hTBM transgene proved essential in cardiac transplantation.<sup>13</sup> In contrast, attempts to transplant other vascularized organs such as liver or lung trail far behind.<sup>14,15</sup> In both cases, the poor outcome has been at least partially associated with insufficiently controlled endothelial activation.<sup>16,17</sup> Thus, our general understanding of endothelial function or failure in xenografts is far from satisfactory. There is evidence that the endothelium experiences significant acute or chronic challenges even in the best possible current circumstances: (a) Although downstream activation of complement is inhibited by hCRP, the ongoing deposition of antibodies and activation of initial complement components on the surface of the vessel walls might still activate EC.<sup>18</sup> (b) The direct activation of EC by different leukocyte types<sup>19-21</sup> suggests that additional cellular players deserve attention. (c) The presence of pro-inflammatory cytokines was sufficient to activate endothelial cells in the absence of human or primate cells or serum.<sup>22</sup> (d) The protective function of Corline-Heparin Conjugate on GTKO.hCRP.hTBM pig EC indicates space for further improvement of the xenograft endothelium.<sup>23</sup> Finally, the diversity of endothelial function and its dynamic regulation has stimulated the definition of endothelial subtypes, their characterization by high-end tools, as well as their comparative analysis on a high-density data basis.<sup>24</sup>

Considering these aspects, it is highly relevant to promote research to better understand the status of xenograft endothelium. Due to the difficulties in assessing vessel structures *in vivo*, improved tools for *in vitro* examination have been developed, such as the establishment of endothelial-coated vessel-like structures under shear stress, that are perfused either under constant<sup>25</sup> or pulsatile flow.<sup>26</sup> Further attention has been paid to the dynamic processes occurring

during angiogenesis<sup>27,28</sup> or the communication of endothelial cells with each other<sup>29</sup> or with other cell types.<sup>30</sup> Interestingly, the analysis of these complex 3D-like structures was limited to a post-experimental single-point readout. This is somewhat surprising, as the quantification of intracellular calcium levels by  $\text{Ca}^{2+}$ -sensitive probes is an established tool and many different probes are commercially available. Although the loading of tissue such as murine aortas with fluorescent  $\text{Ca}^{2+}$ -sensitive probes was informative,<sup>31</sup> the examination of three-dimensional endothelial structures *in vitro* is apparently not compatible with the short-term interference of  $\text{Ca}^{2+}$ -probe loading.

As  $\text{Ca}^{2+}$ -sensitive proteins might be a valuable alternative, we describe here the generation of pigs expressing Case12, a genetically encoded fluorescent  $\text{Ca}^{2+}$ -sensor based on green fluorescent protein,<sup>32</sup> and present evidence that Case12 sensitively detects activation of EC by biologically relevant substrates. It is of note that only complement-inactivated human serum, but not antibody-depleted serum, triggered calcium influx into the cytoplasm of porcine EC, confirming that antibody depletion alone is sufficient to reduce the activation of endothelial cells.

## 2 | MATERIALS AND METHODS

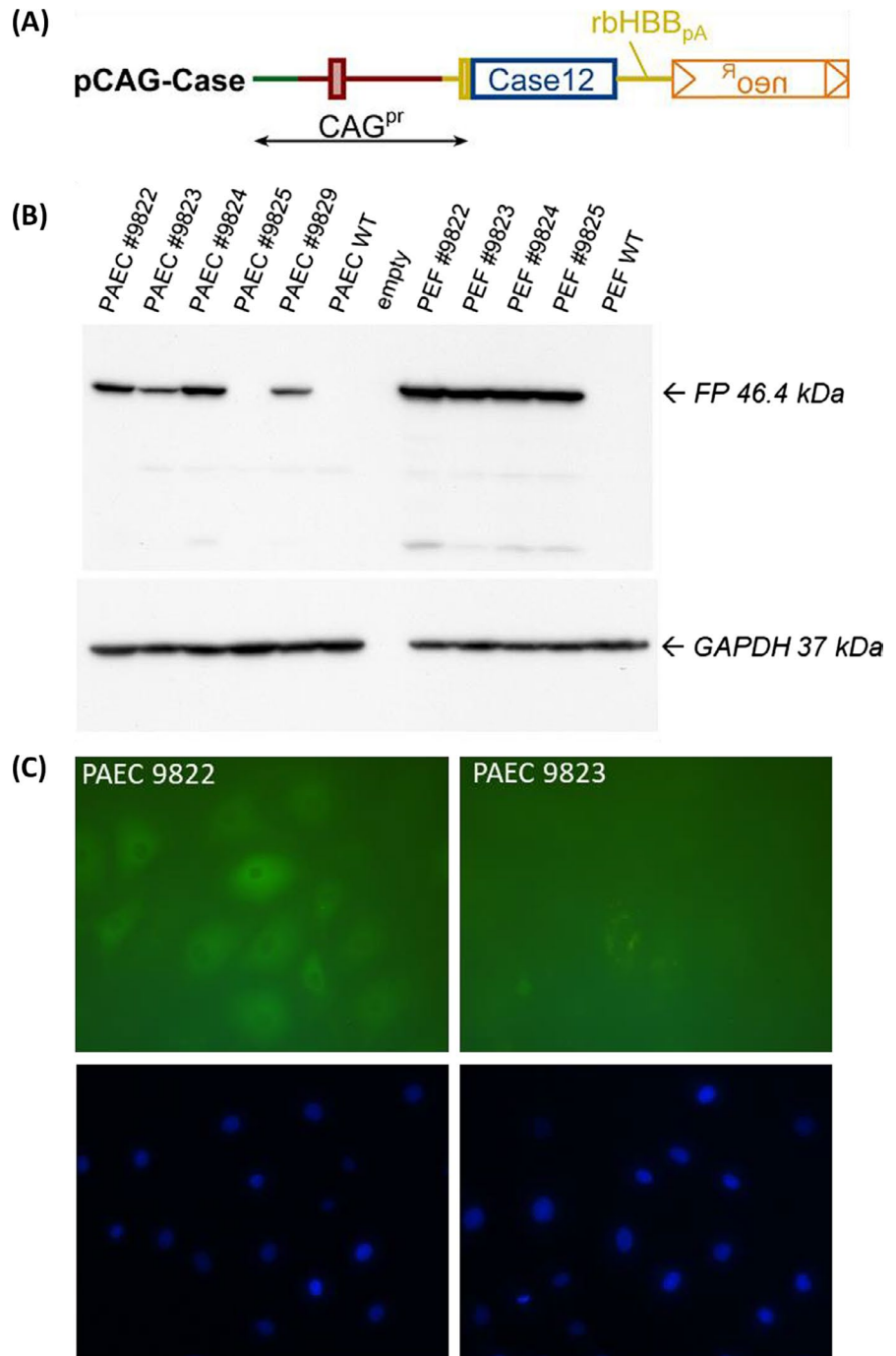
### 2.1 | Ethical statement

All animal work was carried out with the permission of the responsible authority (Regierung von Oberbayern, AZ55.2-1-54-2531-86-10) and in accordance with §15 TSchG German Animal Welfare Law. Animal experiments included the generation of genetically modified animals by somatic cell nuclear transfer. Human blood was taken from healthy volunteers.

### 2.2 | Generation of case12-pigs

For ubiquitous expression of the synthetic  $\text{Ca}^{2+}$ -sensor, we used the previously described CAG-MCS-pA/ neo plasmid<sup>33</sup> and introduced the coding region of Case12 gene (Evrogen) via the BamHI/NotI sites in the multiple cloning site (Figure 1A). The generation of genetically modified primary cells was performed as previously described.<sup>34</sup> In brief, the plasmid was verified by Sanger sequencing and prepared endotoxin-free; the CAG-Case12/neo element was excised from the backbone and nucleofected into primary fetal fibroblasts. Nucleofected cells were seeded and kept under G418 selection.<sup>35</sup> Somatic cell nuclear transfer (SCNT) was performed according to our routine procedures<sup>36</sup> and activated embryos were transferred to synchronized foster sows. Pregnancies were monitored and birth was introduced as described before.<sup>33</sup> Transgene expression was detected by Western blotting. Endothelial and fibroblast cells were lysed in lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 10 mM NaF, complete protease inhibitor (Roche)], and protein concentration was determined by BCA assay. Equal amounts of total protein were separated by 8% SDS-PAGE under reducing

**FIGURE 1** Generation of the Case12 pig model. (A) A ubiquitous CAG promoter has been used to drive the expression of the Case12 coding region. Positive selection was achieved by a floxed neo cassette. (B) Expression of the Case12 protein was detected by Western blotting primary endothelial and fibroblast cells, showing different abundance for distinct founder animals. (C) Fluorescence microscopy revealed Case12 signal even without stimulation in cells from highly expressing founders (left) but not in low expressing founders (right) (Zeiss filter Set 9: ex BP450-490/em LP 515)



conditions and blotted to PVDF membrane. CASE12 and GAPDH were detected using rabbit-anti-TAG(CGY)FP 1:20 000 (AB121, Evrogen) and rabbit-anti-GAPDH 1:3500 (#2118 Cell Signaling) and horseradish peroxidase-coupled polyclonal goat anti-rabbit antibodies (Jackson ImmunoResearch). Bound antibodies were visualized using ECL reagent (RPN2106; GE Healthcare).

### 2.3 | Primary cells

Fibroblast cultures were established from ear samples taken at an age of 3 weeks as described elsewhere.<sup>35</sup> At an age of 5 months,

transgenic pigs were sacrificed and aorta samples were taken for cultivation of primary endothelial cells as described elsewhere.<sup>8</sup> In brief, endothelial cells were isolated with 0.1% collagenase II (Life Technologies) and cultured on culture dishes coated with 0.1% gelatin in DMEM (Life Technologies) containing 10% endothelial cell supplements MV (Promocell) and 10% FCS (Life Technologies). Fixation of cells was performed in 4% paraformaldehyde in PBS for 5 min. Case12 fluorescence is known to have an excitation maximum of 491 nm and an emission maximum of 516 nm. Visualization was performed in a Zeiss Axiovision (Carl-Zeiss) using the standard green filter with an excitation window of 470/40 nm and an emission window of 525/50 nm.

## 2.4 | Human serum, heat inactivation, and immunoabsorption

Human blood was collected from healthy volunteers into polypropylene tubes containing glass beads (S-Monovette, Sarstedt, Germany) and allowed to clot for 30 min at room temperature. After centrifugation at  $2000 \times g$  for 10 min at 4°C, the supernatant was collected, stored in aliquots of 1 mL at  $-80^{\circ}\text{C}$ , and only thawed once for experimental purposes. In the present study, sera from 3 different donors with different blood groups were used both individually and pooled. Details are given in the respective figure legends. Complement was inactivated by heating the sera at  $56^{\circ}\text{C}$  for 30 min. Anti- $\alpha\text{Gal}$  antibodies were depleted as described previously.<sup>37</sup> In brief, polypropylene mini-columns (Poly Prep, Bio-Rad Laboratories) were packed with 3 mL of PAA-Bdi-Sepharose (kind gift of Prof. Nicolai Bovin, Moscow, Russia). After flushing the columns with 50 mL of PBS, they were rinsed with 25 mL of 0.1M Glycine-HCl, pH 2.3, and equilibrated with 50 mL of PBS. Incubation with 20 mL of a 5% bovine serum albumin solution was performed at room temperature for 1 h to block non-specific binding sites. The prepared columns were then immediately used for immunoabsorption of 7 mL of human serum. Sera were allowed to run through the columns under gravity flow. The effluent samples were collected in 1-mL fractions. Immunoabsorption columns were then regenerated by using 25 mL of 0.1M Glycine-HCl pH2.3.

## 2.5 | Anti- $\alpha\text{Gal}$ ELISA

The ELISA for detection of human IgM and IgG antibodies specific for the Bdi epitopes was performed as described previously.<sup>38</sup> In brief, ELISA plates (96-well NUNC MaxiSorp, NUNC AB) were coated by overnight incubation at 4°C with 100  $\mu\text{l}$  per well of PAA-Bdi (from Prof. Bovin, Moscow), in 0.1 M carbonate buffer, pH 9.6. The plates were then washed with PBS, pH 7.4, containing 0.02% Tween 20. Test sera and an internal standard normal human serum were added at a dilution of 1:40 in bovine serum albumin (BSA)-Tween-20. BSA-Tween-20 instead of serum was used as blank. The plates were then incubated on a shaker for 90 min at  $37^{\circ}\text{C}$ . Anti-Gal IgM was detected by monoclonal mouse antibody (mAb) clone AF6 and IgG with clone 8a4 (both from Abingdon), used at 1:4000 in BSA-Tween-20. Biotinylated goat anti-mouse IgG1 antibody (1:2000; Southern Biotechnology Associates, Inc) and subsequently streptavidin-alkaline phosphatase conjugate (1:1000; Amersham Life Science) were used to detect the bound antibodies. Optical densities (OD) were read at 405 nm (Infinite M1000 spectrophotometer, Tecan).

## 2.6 | CH50 assay

The activity of the classical pathway of the complement was analyzed using standard hemolytic complement (CH50) assay with

sheep erythrocytes (Biomerieux). Sheep red blood cells were washed in veronal buffered saline (VBS++) at 1:30 dilution until the supernatant was clear. The erythrocytes were then diluted to achieve the density of  $10^9$  cells/ml and incubated with rabbit anti-sheep erythrocyte antibody (S1389; Sigma-Aldrich) for 20 min at  $37^{\circ}\text{C}$ . After washing away the unbound antibodies, the sensitized erythrocytes were resuspended in Alsever's solution (A3551; Sigma-Aldrich) and stored at  $4^{\circ}\text{C}$  overnight. The following day, the cells were washed again, and the density was adjusted to  $10^8$ /ml. Sera were then added to a transparent 96-well microplate (Nunc) and incubated with the sensitized erythrocytes at  $37^{\circ}\text{C}$  for 60 min. The reaction was stopped with PBS. As control, erythrocytes were incubated in veronal buffer (DGVB++) and the reaction was stopped with water (T100, 100% lysis) or PBS (T0, 0% lysis, background). Optical density of free hemoglobin was measured at 412 nm (Infinite M1000 spectrophotometer, Tecan). Lysis percentage was calculated as follows: 
$$\frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{T0}})}{(\text{OD}_{\text{T100}} - \text{OD}_{\text{T0}})} \times 100.$$

## 2.7 | Cell activation assays

Porcine cells cultured on gelatin-coated 6-channel  $\mu$  slides (ibidi) were stimulated by adding ATP or human serum in different concentrations (as indicated in the different experiments) after washing twice with HEPES. Changes in intracellular calcium, indicated by an increase of the Case12 signal, were measured using a fluorescence detection system (Till Photonics), which emitted light at 480 nm and detected the fluorescence signal (at about 505 nm, arbitrary units) by using a digital camera. Images of areas ( $240 \mu\text{m} \times 320 \mu\text{m}$ ) were stored every 500 ms (ATP) or 125 ms (serum) for 75 s. Cells were counted as cells with an increased calcium level when the mean value of the ratio (fluorescence after stimulation/baseline fluorescence; calculated by the software for individual cells) increased by at least 5 au. The percentage of cells showing an increased calcium concentration (% responding cells) and the maximal response (maximum fluorescence—baseline fluorescence) was calculated and taken as rate of cell responsiveness. Data were corrected for bleaching by subtracting the changes of fluorescence obtained in control experiments where nothing was added to the cells.

# 3 | RESULTS

## 3.1 | Generation of Case12 transgenic pigs

Two SCNT/ET experiments using Case12 transgenic fibroblasts as donor cells resulted in pregnancies, delivering a total of 12 cloned Case12 transgenic piglets. Primary fibroblasts were isolated from the ear and primary aortic endothelial cells were isolated from the ascending aorta. Expression of Case12 was confirmed in protein extracts from the cells by Western blotting (Figure 1B) and by the basic fluorescence of genetically modified PAEC at an unstimulated stage

(Figure 1C). The amount of Case12 differed between the respective founder animals, reflecting the general diversity of transgene expression in additive gene transfer. Activation experiments were conducted in different cell lines, but as the founder 9822 showed highest expression levels of Case12 and thus allowed the most sensitive detection of  $\text{Ca}^{2+}$ -influx into the cytoplasm, the following data are shown from this cell line as a representative for genetically encoded  $\text{Ca}^{2+}$ -sensing.

### 3.2 | Activation response of Case12 PAEC to different stimuli

The principal function of Case12 as a  $\text{Ca}^{2+}$ -sensor was proved by stimulating transgenic PAEC with biologically relevant components. First, we challenged cells with different concentrations of ATP (Figure 2A) and revealed a dose-dependent response reaching a saturation plateau between 100 and 300  $\mu\text{M}$ . The procedure was repeatable several times, albeit with a shrinking signal intensity (Figure 2B). Interestingly, the PAEC did not react to histamine (Figure 2A). This is in contrast to primary fibroblasts, in which both ATP and histamine induced profound  $\text{Ca}^{2+}$ -influx into the cells (Figure 2C), although the increase of fluorescence signal was much slower in the latter.

More importantly, however, not all cells responded in the same manner to ATP stimulation, but revealed signal courses that were rather sequential and graduated (Figure 3A, Video S1, Figure S1). Apparently, within a given window of examination, cells typically responded in the following manner: Initially, a driver cell reacted and generally showed the brightest and most sustained signal, and then, the other cells reacted with some delay but did not reach the same fluorescence intensity as the driver cell. Often cells showed  $\text{Ca}^{2+}$ -influx one after the other, as in a serial connection. Of note, the differential fluorescent signaling was confirmed when the signal intensities of single cells were measured quantitatively (Figure 3B). While most of the cells reacted within a very short time-frame to ATP, the peak altitude they reached was apparently different and the signal decline was variable, revealing different slopes and occasionally also a fluctuating decline of fluorescence.

### 3.3 | Effect of manipulated human serum on PAEC activation

Transgenic porcine EC reacted profoundly but transiently to ATP, with the signal in single cells returning to baseline level mostly within a few seconds (Figure 3B). This was in contrast to the stimulation of Case12-transgenic EC with human serum, the standard challenge applied to EC in the xenotransplantation context (Video S2; Figure 4), where many cells exhibited elevated calcium levels for longer than the observation period. The difference between cells was pronounced, showing strongly reacting cells with a much higher fluorescent signal, as compared to stimulation with ATP (Serum 1/5:

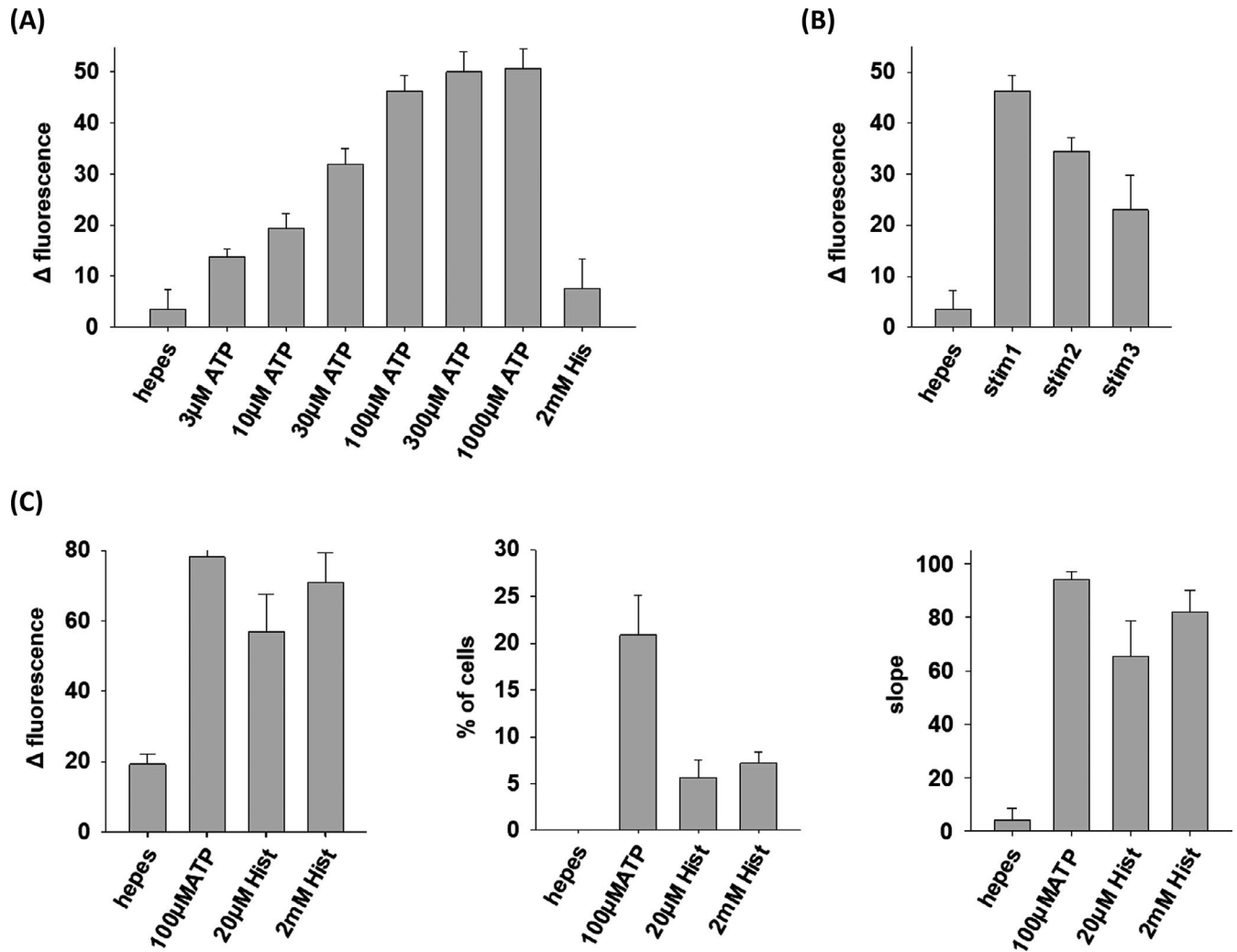
mean amplitude  $77 \pm 3\text{au}$ , individual amplitudes up to 130 au,  $n = 10$  vs 100  $\mu\text{M}$  ATP: mean amplitude  $38 \pm 4$  au, individual amplitudes up to 80 au,  $n = 15$ ). On the other hand, a significant proportion of cells displayed only very weak, if any, reaction to human serum, which was confirmed by quantitative single-cell analysis. This difference in responses may be attributed to the more complex nature of stimuli in human serum, compared to a defined single molecular component such as ATP.

To investigate which components within serum are the main drivers of EC activation, the serum was treated either by heat inactivation to eliminate complement-mediated cytotoxicity (Figure 5A), or by depleting anti-aGal antibodies (Figure 5B), which are the dominant activators of the complement system in HAR. In standard dilutions of 1/10 human serum in culture medium, activation of EC in the presence of heat-inactivated serum was similar to untreated serum, whereas the response to antibody-depleted serum (with or without heat inactivation) was significantly lower, based on the mean fluorescence intensity (Figure 5C). When examined at cellular resolution, it was evident that in the latter case only a minority (approx. 20%) of the cells showed any response, whereas almost all cells responded to either ATP, untreated serum, or heat-inactivated serum. Stimulation of Case12 cells with higher concentrations of human serum produced a similar picture (Figure 5D), albeit with some minor changes: First, heat inactivation showed a reduction in fluorescence intensity, but not in the number of cells responding, and second, the overall stimulatory potential of antibody-depleted serum was reduced, but to a lesser extent than in the 1/10 serum dilutions.

## 4 | DISCUSSION

Our study illustrates that genetic modification of pigs is not only relevant for tailoring donor animals for xenotransplantation, but might also have value for producing reporter pigs to gain more detailed insight into physiological and pathophysiological processes. Although one would nowadays consider generating such models by using state-of-the-art technologies such as gene editing,<sup>39</sup> conventional additive gene transfer still serves the purpose. The considerable effort required to examine founder animals for their expression potential can be ameliorated by the use of a ubiquitously active transgene promoter that facilitates initial characterization at a cellular level. Further, the chosen promoter also allows the examination of  $\text{Ca}^{2+}$ -activation in other cell types. Although some of the more recent genetically modified  $\text{Ca}^{2+}$ -sensors might provide higher sensitivity or lower background signal,<sup>40,41</sup> the Case12 sensor employed here fulfilled the expectations for exploring activation of pig primary endothelial cells.

A prominent finding was that not all PAEC behaved equally upon stimulation with ATP and they failed to react upon histamine application. A simple explanation for this might be the sequential loss of specific receptors during in vitro culture, but a similar diversity in the activation potential has also been shown for endothelial



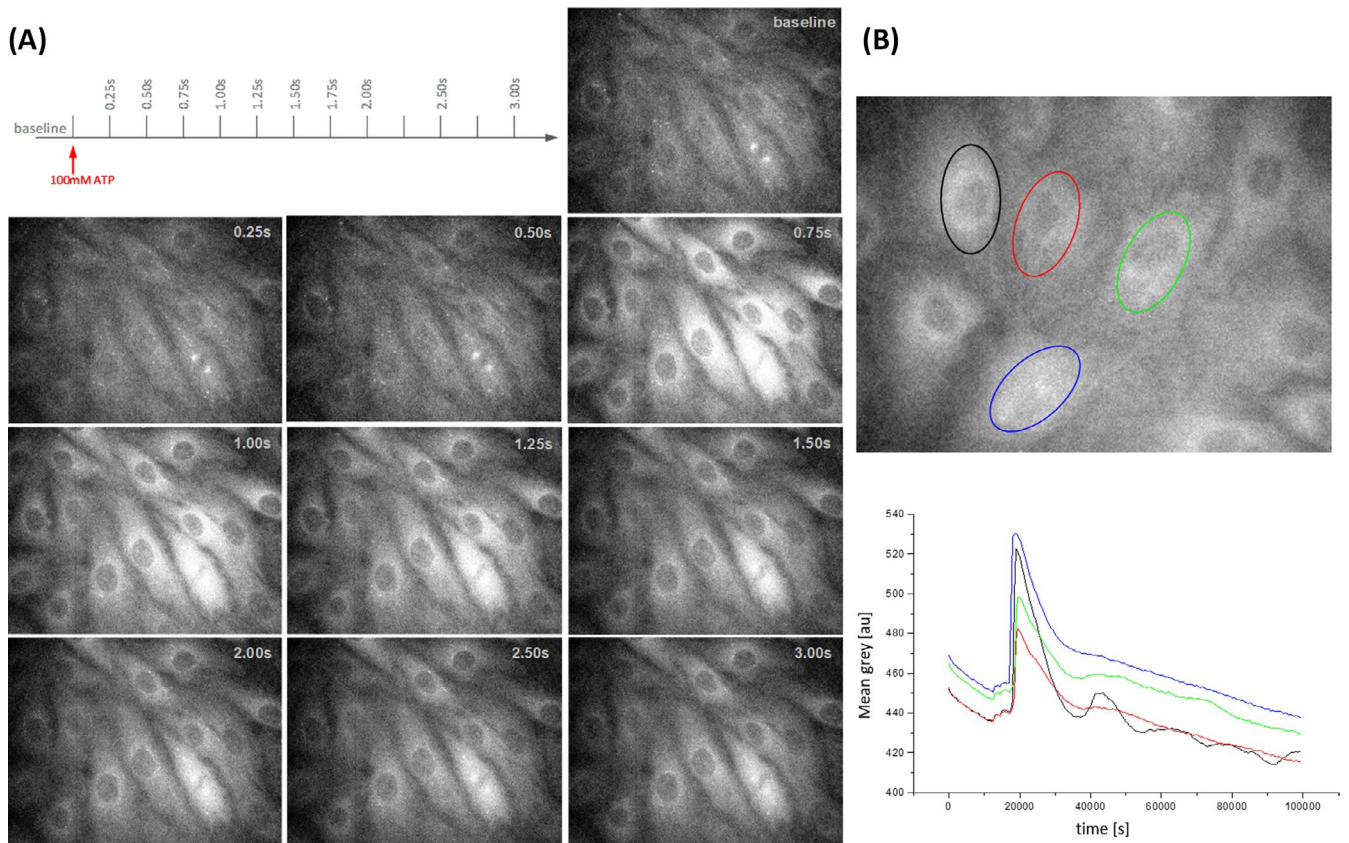
**FIGURE 2** Stimulating Case12 cells with biologically active components. (A) In PAEC, ATP showed a concentration dependent stimulation up to a saturation plateau reached at 100-300μM ATP, whereas stimulation with histamine did not result in Ca<sup>2+</sup>-influx. (B) Repeated stimulation of PAEC with 100mM ATP (stim1, stim2, stim3) resulted in a sequential decrease of the calcium influx into the cells. (C) In contrast, fibroblasts were stimulated by both, ATP and histamine, albeit for the latter the reaction appeared slower, indicated by a significantly lower slope of the signal. The slope was calculated at the inflexion point of the time course of the cellular calcium rise

cells in ex-vivo vessel systems.<sup>31</sup> The apparent diversity of EC activation is consistent with the current interpretation of “endothelial cells” as an umbrella term for cell (sub)types coating the luminal side of a vessel. It has become evident that EC diversity not only refers to the different vessel types in distinct tissues, but also to differences between endothelial cells in a local context. A substantial clue was the initial discrimination of EC by the expression of classical markers such as TBM.<sup>42</sup> Since then, endothelial subtypes have been discriminated at a broader molecular level by single-cell transcriptome profiling.<sup>24</sup> Although the consequences of this unexpected diversity and the underlying regulatory circuits remain elusive, this is in line with the increasing acknowledgment of diversity of other cell types and their Ca<sup>2+</sup>-mediated signal transduction, such as epithelial cells.<sup>43</sup>

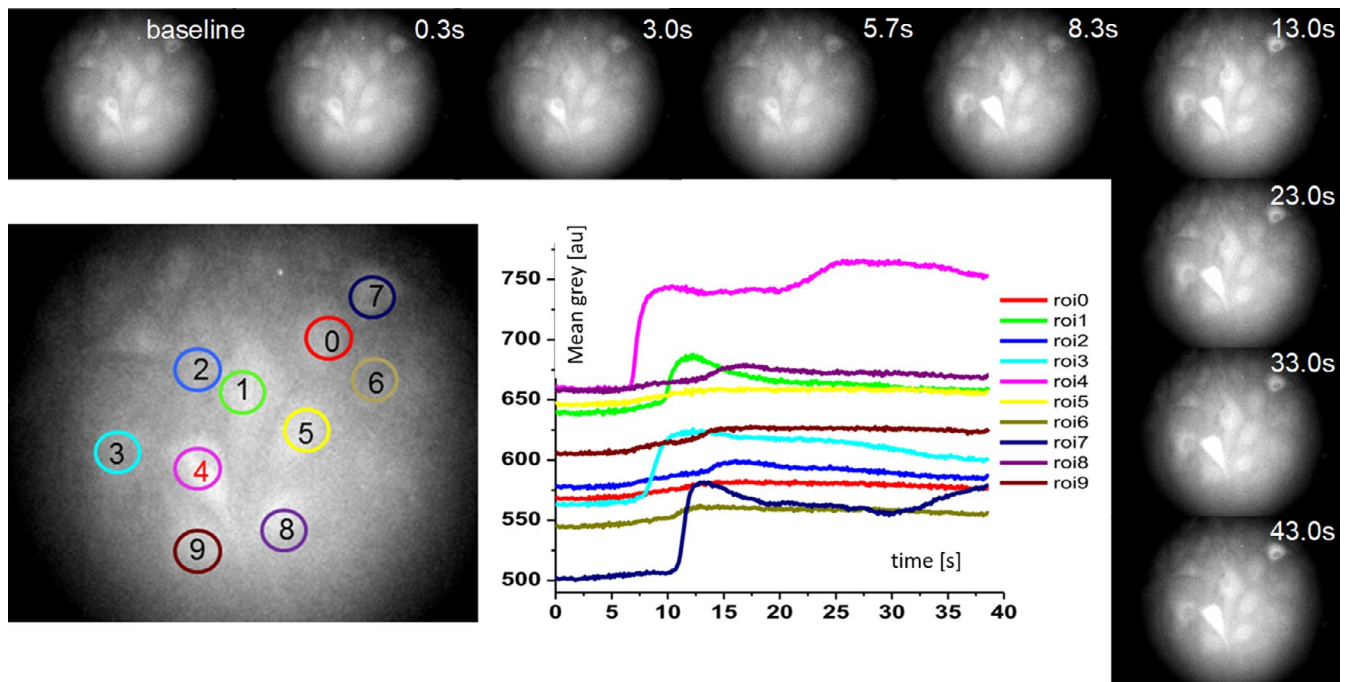
These divergent properties of individual EC also cast doubt on the popular use of immortalized endothelial cell lines, including those derived from different endothelial sources in the pig<sup>44-47</sup>

Although such cells might carry essential markers of EC and match hallmarks of endothelial function, they lack the diversity of primary cells. Furthermore, the immortalization process itself and extended in vitro cultivation will impact endothelial physiology. On the other hand, the use of immortalized cells in high-throughput assays is attractive, and in such assays it might be useful to equip them with a genetically encoded Ca<sup>2+</sup>-sensor. From a long-term perspective, however, we propose that isolating large batches of different primary cell populations from genetically modified pigs will better resemble the in vitro counterpart of the in vivo situation.

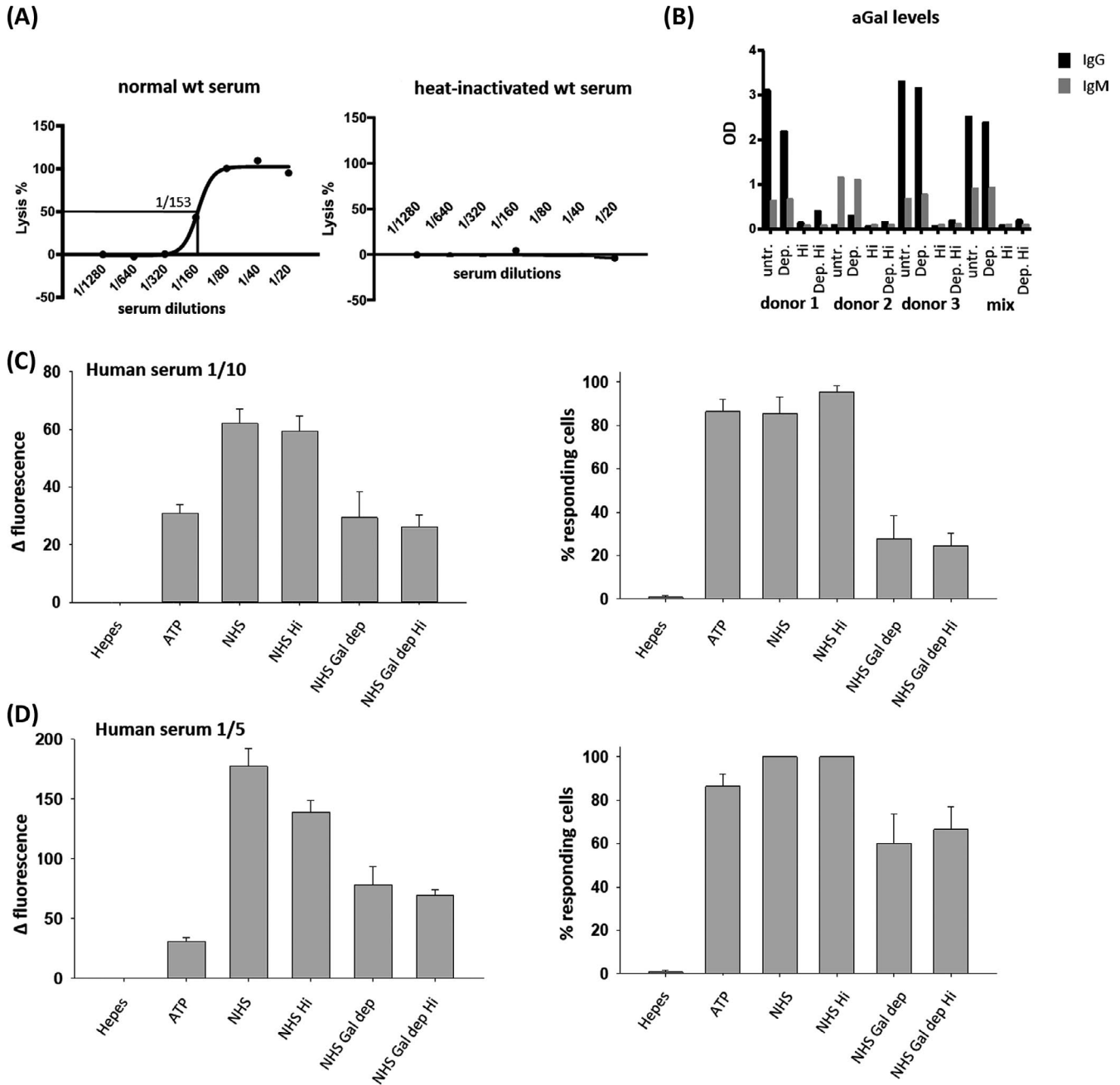
Another relevant aspect of our study was the finding that human serum had a much more pronounced effect on individual EC than ATP, which might be related to the complex composition of biologically active agents in the serum, compared to the unambiguous structure of ATP. The sustained activation of PAEC over minutes, however, is remarkable, when the fast, but transient Ca<sup>2+</sup>-influx after ATP stimulation is taken into account. Even more



**FIGURE 3** Time course of Case12-PAEC stimulation by ATP. (A) Upon administration of ATP to the culture medium, pictures were taken at regular intervals. (B) When specifying signals from single cells, it appeared that the increase of the signal took place within a relatively short interval, whereas the peak heights reached were different and the decline of the signal followed different courses



**FIGURE 4** Stimulation of Case12-PAEC with human serum. Both the overall time lapse and the single-cell signals revealed a diverse pattern of EC response on human serum, spanning highly responding and long sustained signals to almost no reaction



**FIGURE 5** Modification of human serum. (A) Cytotoxicity of serum was completely abolished by heat inactivation and (B) antibody depletion reduced the levels of anti- $\alpha$ Gal IgM and IgG to a level < 5%. (C) Stimulation with 10% serum revealed that only antibody-depleted serum reduced the overall fluorescence intensity as well as the proportion of reacting cells. (D) When serum concentration was increased to 20%, the number of cells reacting on antibody-depleted serum increased, whereas the fluorescence intensity decreased, compared to untreated human serum

surprising was the finding that antibody deposition on EC alone, but not the abundance of complement in the medium alone, is sufficient to activate endothelial cells. The reduced activation of EC in the presence of inactivated complement at higher serum concentrations supports the idea that the alternative and lectin-based pathways might play a relevant role in endothelial activation,<sup>18</sup> but we have to consider that a content of 20% serum in the culture medium is unusually high for in vitro experiments and might, thus, contribute some artifacts. We interpret our findings such that the

classical, antibody-mediated activation of the complement system is the dominating factor in EC activation when challenged with human serum.

Although our data give insight into the process of endothelial cell responses to xenorelevant stimulation, it would be interesting to examine the impact of existing and proven approaches to prevent antibody-mediated rejection, such as the GTKO.hCRP.hTBM genetic modifications. In this context, however, it would not only be necessary to combine the genetically encoded  $Ca^{2+}$ -sensor with the



triple modification, but it would be important to analyze such cells in 3D-culture assays under continuous or pulsatile flow, ideally not only with plasma, but also with whole blood. Eventually, such an innovative, complex, and high-throughput enabling setup may greatly facilitate the further improvement of vascularized xenogeneic organs or tissues.

In summary, we present here a powerful tool for evaluating effects that activate the porcine endothelium. In a proof-of-concept, we report that antibody depletion alone, but not complement inactivation, is sufficient to significantly reduce the activation status of endothelial cells in the presence of human serum.

## ACKNOWLEDGEMENTS

This work was supported by funding from the German Research Foundation (CRC127: xenotransplantation from bench to bedside). Work performed by RS and RR was funded by the Swiss National Science Foundation (310030\_182264). We thank Prof. Nicolai Bovin from the Shemyakin and Ovchinnikov Institute for Bioorganic Chemistry, RAS, Moscow, Russia, for providing anti-Gal immunoabsorption material and coating conjugates for ELISA.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTION

AW designed the study, performed genetic modification of primary cells, cultivated endothelial cells, and performed activation assays. PK and EMJ performed activation assays. RS and RR performed antibody depletion, complement standard activity test, and anti-aGal ELISA. AB performed work on pigs and isolated tissue for primary cell cultivation. MK and BK performed SCNT and ET. EK provided Western blot data. BR and CK supported design of the study. EW supported design of the study and contributed to writing of the manuscript. NK designed the study, designed and constructed the modification vector and wrote the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Wuensch A, Kameritsch P, Sfriso R, et al. Genetically encoded Ca<sup>2+</sup>-sensor reveals details of porcine endothelial cell activation upon contact with human serum. *Xenotransplantation*. 2020;00:e12585. <https://doi.org/10.1111/xen.12585>