FULL PAPER *Immunology*

# Porcine Aortic Endothelial Cell Genes Responsive to Selected Inflammatory Stimulators

Hye-Jung YEOM[1,3)]*, Kum-Joo SHIN[3)]*, Jun-Sub KIM[1)], Seung-Jun KIM[1)], Sukmook LEE[3)], Saswati PAUL[1)], Jung-Won HAN[2)], Curie AHN[3)], Je Kyung SEONG[4)], Junho CHUNG[2,3)]** and Seung Young HWANG[1)]**

[1)]*Department of Biochemistry, Hanyang University & GenoCheck Co. Ltd., Sangrok-gu, Ansan, Gyeonggi-do, 426–791,* [2)]*Department of Biochemistry and Molecular Biology,* [3)]*Cancer Research Institute and Transplantation Research Institute, Seoul National University College of Medicine, Seoul, 110–799 and* [4)]*Laboratory of Developmental Biology and Genomics, College of Veterinary Medicine, Seoul National University, Seoul, 151–742, South Korea*

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ABSTRACT. Use of porcine tissues has been suggested as a promising solution for severe shortage of transplantable human organs. The immediate hurdle for xenotransplantation is acute immune/inflammatory vascular rejection of the transplant. Because endothelial cells play a key role in the initiation and the amplification of inflammation, alteration of gene expression in human endothelial cells, by various inflammatory stimulators has been studied extensively. However, transcriptional changes induced by human and other inflammatory stimulators in porcine endothelial cells have thus far not been studied. In this study, we treated porcine endothelial cells with human tumor necrosis factor (TNF)-α, porcine interferon (IFN)-γ, $H_2O_2$ and lypopolysaccharide (LPS) and profiled transcriptional change at 1 hr, 6 hr and 24 hr, using pig oligonucleotide 13K microarray. We found that mRNA species such as chemokine (C-X-C motif) ligand 6 (CXCL6) and Cathepsin S were significantly induced in porcine endothelial cells, as was previously reported with human endothelial cell. We also found that mRNA species including secreted frizzled-related protein 2 (SFRP2), radical S-adenosyl methionine domain containing 2 (RSAD2), structure specific recognition protein 1 (SSRP1) also were highly overexpressed in porcine endothelial cells. This result shows clues to understand underlying mechanisms of xenotransplantation rejection and the highly responsive porcine genes may serve as novel targets to be regulated for improving the function of grafted porcine donor organs.

KEY WORDS: gene expression, inflammation, microarray, porcine aortic endothelial cell, xenotransplantation.

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Xenotransplantation offers a possible solution for severe shortage of transplantable human organs. The promise of porcine pancreatic islet transplantation in humans as a less invasive therapy to restore and maintain normoglycemia without the risk of hypo- or hyper-glycemia is now undergoing a clinical trial [7–10, 13, 19, 27]. The immediate hurdle for xenotransplantion is hyperacute rejection induced by the alpha-gal epitope and has been overcome by genetic removal of 1,3-galactosyltransferase [30]. As the expression level of alpha-gal epitope is low in the pancreatic islet, the current trial is being conducted with the pancreatic islets of wild type pig.

Another hurdle for xenotransplantation is acute vascular rejection (AVR), in which the endothelial cell plays a central role [3, 14, 27]. In xenotransplantaion with porcine tissues, porcine endothelial cells become activated by human cytokines such as TNF-α. Gene expression changes induced by human cytokines in human endothelial cells have been extensively studied [1, 6, 24, 29]. In this study we established porcine endothelial cell lines that lack surface expression of VCAM1 and thus mimic endothelial cells in quiescent resting status. These cells were treated with human TNF-α, porcine IFN-γ or nonspecific potent immuno-stimulators, $H_2O_2$ and lipopolysaccharide (LPS). The gene expression alternations induced after these treatments were studied using pig oligonucleotide 13K microarray and quantitative real-time PCR. In an effort to find molecules unique to the porcine endothelial cell activation process and the underlying mechanism, we compared our results with results from previous studies with human endothelial cells [31, 32, 36, 37]. We found that some mRNA species are uniquely overexpressed in the activated porcine endothelial cells but not in human cells. These species may have potential to serve as targets for development of therapeutic agents tailored to xenotransplantation.

* These authors have equally contributed to this work

**CORRESPONDENCE TO: HWANG, S. Y., Department of Biochemistry, Hanyang University & GenoCheck Co. Ltd., Sangrok-gu, Ansan, Gyeonggi-do 426–791, South Korea.
e-mail: syhwang@hanyang.ac.kr

Chung, J., Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, Yongon Dong 28, Chongno Gu, Seoul 110–799, South Korea.
e-mail s: jjhchung@snu.ac.kr

## MATERIAL AND METHODS

*Isolation of porcine aortic endothelial cells*: Porcine aortic endothelial cells (PAECs) were isolated as described previously [17]. Briefly, fresh aortas were removed from specific pathogen-free (SPF) Minnesota miniature pigs maintained at Seoul National University (Seoul, South Korea). The aortas were thoroughly washed with cold Hank's balanced salt solution (HBSS) (Invitrogen, San Diego, CA, U.S.A.) and incubated with 0.5 mg/m*l* collagenase P (Roche Diagnostics, Mannheim, Germany) at 37°C for 10 min. Detached cells were isolated by flushing, and

cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS) (Invitrogen), 15 mg/m*l* endothelial cell growth supplement (Sigma, St. Louis, MO, U.S.A.), 10 U/m*l* heparin (Sigma), 5 mM hydrocortisone (Sigma), 5 mg/m*l* insulin (Sigma) and penicillin/streptomycin (Invitrogen) at 37°C in 5% $CO_2$.

*Establishment of immortalized PAEC lines*: The primary PAECs were transfected with a pSV3-neo plasmid containing genes for neomycin resistance and SV40 large T antigen using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After two days, transfected cells were selected in a medium with 1 mg/m*l* G418 (Invitrogen). After 2 weeks, individual colonies were picked and screened for the surface expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) using flow cytometry. Five porcine endothelial cell lines were established and maintained in DMEM containing 10% FBS and 100 mg/m*l* of G418.

*Treatment of PAEC and HUVEC with inflammatory stimulators*: Human umbilical vein endothelial cells (HUVEC, from Cambrex, Baltimore, MD, U.S.A.) were maintained in EGM2 MV endothelial cell media (Cambrex), Cells at passages 8–15 were used for this study. PAEC and HUVEC were treated with 400 $\mu$M of $H_2O_2$, 20 *n*g/m*l* of human TNF-$\alpha$, 100 *n*g/m*l* of porcine IFN-$\gamma$, or 10 $\mu$g/m*l* of LPS for indicated times and harvested for further analysis.

*Flow cytometry*: PAECs were trypsinized and resuspended in a FACS buffer (PBS with 1% BSA and 0.02% sodium azide) and incubated with anti-PECAM-1 (Serotec, Raleigh, NC, U.S.A.) or anti-VCAM-1 antibody (Sigma) at 37°C for 1 hr. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies at room temperature for 30 min and washed. Their fluorescent intensity was measured with a flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.).

*RNA isolation*: Porcine endothelial cells ($7 \times 10^6$) were seeded in a 100 mm tissue culture dish. After 24 hr, cells were treated with indicated stimulators for 1, 6, and 24 hr. RNA was prepared using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Quantity and integrity of prepared RNA was determined with a spectrophotometer, and a Bioanalyzer (Agilent Technologies, CA, U.S.A.), respectively.

*Generation of pig oligonucleotide 13K microarray chip*: A total of 13,297 oligonucleotide probes (pig genome Array-Ready Oligo Sets™, Version 1.0) were purchased from Operon Biotechnologies Inc. (Huntsville, AL, U.S.A.). These probes represent 10,665 *Sus scrofa* genes with a similarity to known human and mouse transcripts or 3' expressed sequence tag (EST). They were originally designed by using The Institute of Genome Research (TIGR) Gene Index Database SsGI Release 5.0. To verify chip quality and effective normalization, we also spotted control samples in each block. In this pig oligonucleotide 13K chip, each of 24 blocks consists of 24 columns and 24 rows and contains 568 genes.

*Hybridization*: The microarray experiments were performed as described previously [16]. Briefly, each RNA (30 $\mu$g) of PAEC cultured in the absence or presence of four inflammatory stimulators was labeled with Cyanine(Cy3)- or Cyanine(Cy5)-conjugated dCTP (GE healthcare, Piscataway, NJ, U.S.A.) during reverse transcription reaction using a reverse transcriptase, SuperScript ll (Invitrogen). The labeled cDNAs were mixed and hybridized simultaneously to the pig oligonucleotide 13K chip. To control the gene-specific dye bias, we performed dye-swap experiment for all samples as described previously [35]. Processed slides were scanned with an Axon 4000B Scanner (Axon Instruments, CA, U.S.A.) with excitation at 532 and 635 nm wavelengths for the Cy3 and Cy5 dyes, respectively. The scanned images for each slide were analyzed using the GenPix pro 5.1 Software (Axon instruments).

*Microarray data processing and analysis*: Microarray data were managed with GeneSpring 7.2 software (Sillicongenetics, Redwood City, CA, U.S.A.). The raw intensity data were normalized by intensity-dependent normalization in Lowess method [34] and then again by with-print-tip group normalization method for each print-tip. A total of 48 tips were used for making this pig oligonucleotide 13K chip. S-plus PLUS software (ISTECTIBCO Software, CA, U.S.A.) was used to determine the means of data from triplicate experiments. The gene expression values for each array were normalized to their respective median values. All clustering analyses were performed using standard correlations as described previously [15, 25]. Fold change filters included the requirement that the genes are present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes.

*Quantitative real-time PCR*: cDNA was synthesized from 1–2 mg of total RNA using Power cDNA synthesis kit (iNtRON Biotechnology, Seoul, South Korea). Quantitative real-time PCR was performed as described previously [18] in triplicate in 384-well plates using Prism 7900 Sequence Detection System (Applied Biosystems). Each 20 $\mu$l reaction mixture consisted of cDNA (0.2 $\mu$g total RNA), 2x Universal SYBR Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and 8 pmol of forward and reverse primers. PCR reaction was performed at 95°C for 15 min followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec, and 72°C for 30 sec. Serial dilutions of cDNA were used to generate standard curves and Ct value was converted to relative amount of each gene over GAPDH.

## RESULTS

*Establishment of immortalized PAEC lines*: Endothelial cells were isolated from aortas of a miniature pig and transfected with a plasmid containing genes for SV40 large T antigen and neomycin resistance. Among positive clones selected with G418, five clones possessed cobblestone morphology typical to endothelial cells and were PECAM-1 positive in flow cytometry analysis (Fig. 1). Then, to select cell lines with quiescent phenotype, we checked the surface level of VCAM-1 in flow cytometry analysis before and

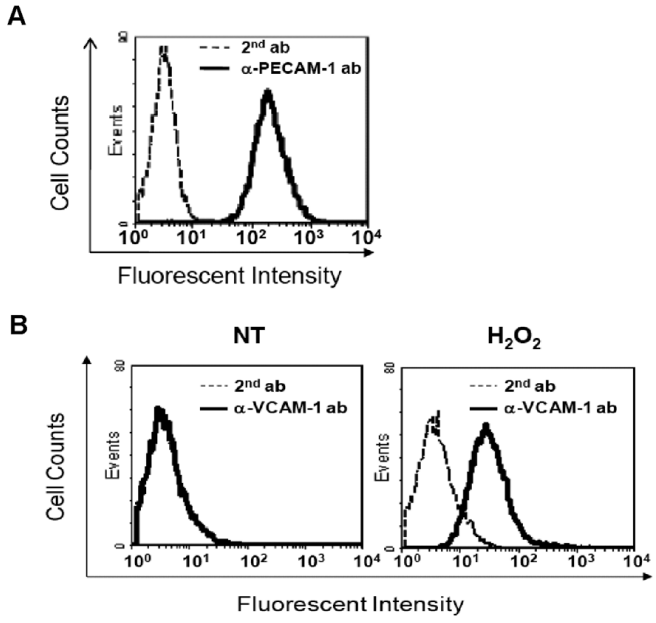



Fig. 1. Establishment of PAEC lines with quiescent phenotype. A: For confirming homogeneity of PAEC line, PAECs were incubated with anti-PECAM-1 antibody and analyzed in flow cytometry. This data are representative of 5 clones. B: PAECs were cultured in the absence or presence of 400 mM $H_2O_2$ for 24 hr. After incubation with anti-VCAM-1 antibody, the cells were analyzed in flow cytometry.

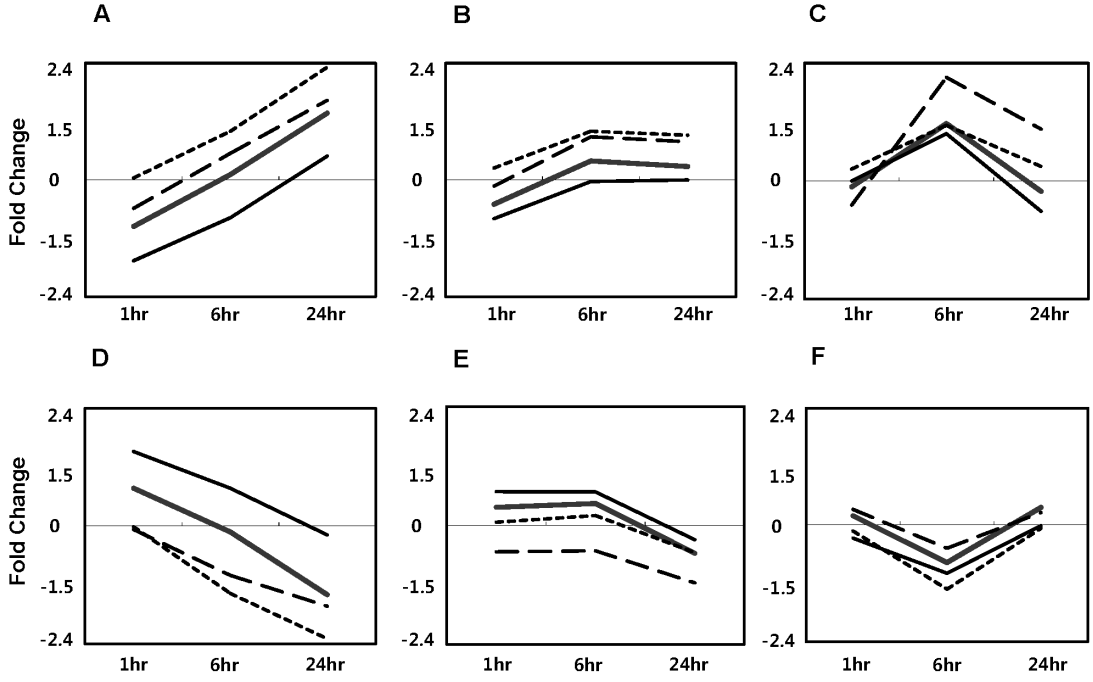
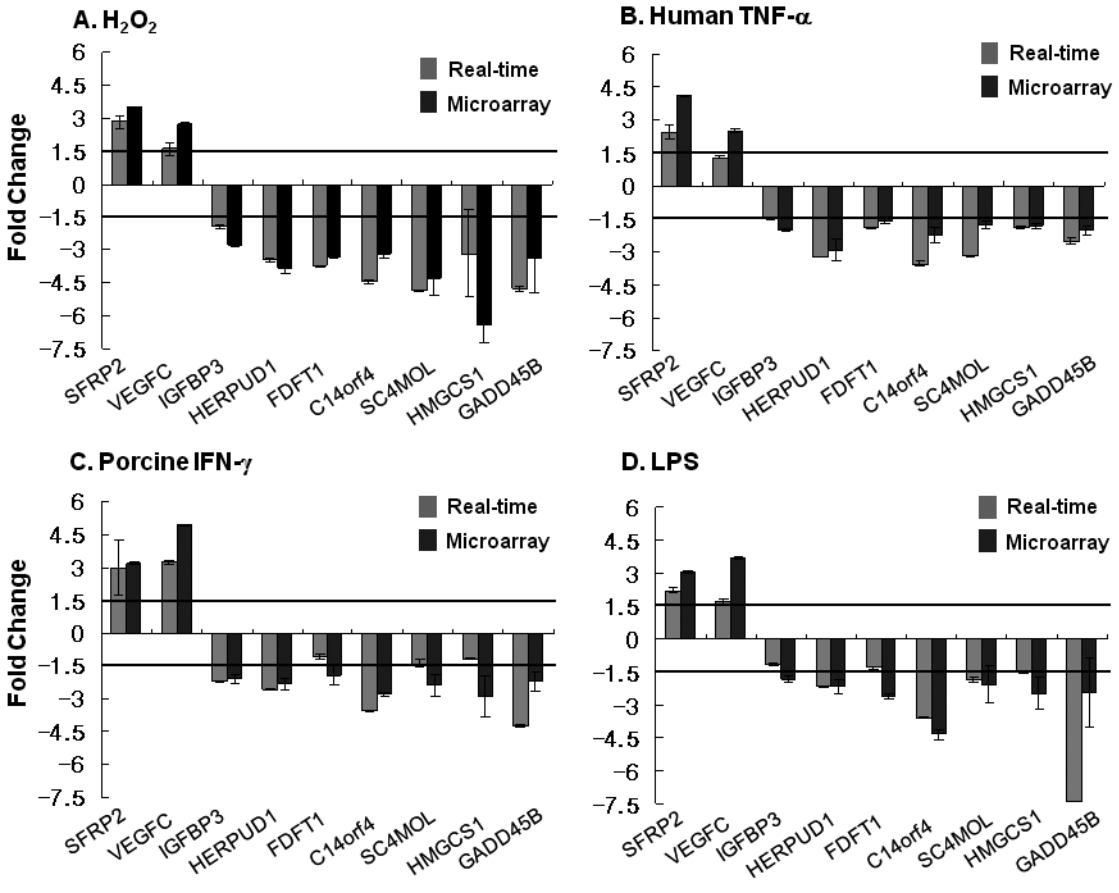



Fig. 2. Classification, by their expression patterns, of porcine genes induced by $H_2O_2$ (thick line), human TNF-α (thin line), porcine IFN-γ (dashed line) and LPS (dotted line). A: Genes that gradually increased at 1 hr, 6 hr and 24 hr, B: genes that decreased at 1 hr and increased at 6 hr and 24 hr, C: Genes that decreased at 1 hr and 24 hr and increased at 6 hr, D: Genes that gradually decreased at 1 hr, 6 hr and 24 hr, E: Genes that increased at 1 hr and 6 hr and decreased at 24 hr, F: Genes that increased at 1 hr and 24 hr and decreased at 6 hr.




**E. HVEC vs PAEC**

| | $H_2O_2$_24hr | | Human TNF-α_24hr | | LPS_24hr | |
|---|---|---|---|---|---|---|
| | VEGFC | SFRP2 | VEGFC | SFRP2 | VEGFC | SFRP2 |
| HUVEC | 0.34±0.05 | N.D | 1.35±0.09 | N.D | 2.45±0.60 | N.D |
| PAEVC | 1.58±0.32 | 2.83±0.28 | 1.30±0.02 | 2.45±0.32 | 1.69±0.09 | 2.21±0.11 |

N.D : not detectable

Fig. 3. Confirmation of gene induction by inflammatory stimulators. After treatment with $H_2O_2$ (A), human TNF-α (B), porcine IFN-γ (C), and LPS (D), nine genes differentially expressed in microarray analysis (Black bar) were chosen for validation by quantitative real-time PCR (Gray bar). (E) Expression levels of VEGFC and SFRP2 genes in PAECs and HUVECs in response to $H_2O_2$, human TNF-α, and LPS by quantitative real → time PCR (fold change compared to control). The normalized ratios of gene expression of the experimental condition over the control condition are shown for microarray and quantitative real-time PCR data. Data are expressed as the mean ± SD of samples.

after stimulation with $H_2O_2$. As shown in Fig. 1B, the expression level of VCAM-1 was negligible before stimulation but highly increased by treatment with $H_2O_2$. Five established PAEC lines were stored for further studies.

*Microarray studies of PAECs treated with inflammatory stimulators*: PAECs were cultured in the absence or presence of four inflammatory stimulators; $H_2O_2$, human TNF-α, porcine IFN-γ and LPS for 1, 6, and 24 hr. After RNA was extracted, we confirmed that the mRNA level of VCAM-1 gradually increased by the stimulation in quantitative real-time PCR analysis (data not shown). Then, cDNA was synthesized, indirectly labeled with either Cy3 or Cy5, and hybridized to 2 different slides with a dye swap method. We identified differentially expressed genes (increased or decreased two fold compared to untreated control) in response to the four inflammatory stimulators; 5,897 genes were changed by $H_2O_2$, 5,659 genes by human TNF-α, 4,131 genes by porcine IFN-γ and 4,000 genes by LPS. Next, we classified these genes into six clusters (A-F), based on their expression patterns. Each cluster included genes

Table 1. Ontology of porcine genes differentially expressed in response to inflammatory stimulators, $H_2O_2$, human TNF-α, porcine IFN-γ and LPS

| Ontologic Process | $H_2O_2$ | | TNF-α | | IFN-γ | | LPS | |
|---|---|---|---|---|---|---|---|---|
| | Cluster A | D | A | D | A | D | A | D |
| *Biological process* | 51 | 98 | 163 | 55 | 84 | 35 | 58 | 134 |
| Biological adhesion | 6 | 17 | 7 | 5 | 3 | 4 | 3 | 7 |
| cell adhesion | 6 | 17 | 7 | 6 | 3 | 4 | 3 | 7 |
| cell-substrate adhesion | 2 | 4 | 2 | 2 | 1 | – | – | 2 |
| regulation of cell adhesion | 1 | 2 | 5 | – | – | – | – | 1 |
| Biological regulation | 17 | 55 | 69 | 18 | 33 | 16 | 19 | 59 |
| regulation of a molecular function | 4 | 6 | 10 | 4 | 3 | 1 | 1 | 6 |
| regulation of biological process | 17 | 47 | 67 | 14 | 33 | 16 | 19 | 55 |
| regulation of biological quality | 4 | 22 | 13 | 5 | 2 | 3 | 2 | 14 |
| Cellular process | 45 | 116 | 140 | 44 | 71 | 29 | 47 | 116 |
| cell communication | 13 | 37 | 52 | 17 | 13 | 6 | 13 | 29 |
| cell cycle | 3 | 7 | 8 | 3 | 11 | 5 | 4 | 14 |
| cell development | 6 | 17 | 25 | 5 | 9 | 5 | 4 | 19 |
| cell growth | 3 | 7 | 6 | 2 | 2 | 1 | – | 4 |
| cell proliferation | 8 | 14 | 18 | 3 | 11 | 4 | 5 | 11 |
| cellular developmental process | 7 | 23 | 33 | 9 | 10 | 5 | 8 | 21 |
| cellular metabolic process | 29 | 65 | 96 | 24 | 47 | 18 | 35 | 75 |
| regulation of cellular process | 16 | 40 | 60 | 12 | 29 | 14 | 17 | 50 |
| Developmental process | 13 | 54 | 49 | 19 | 19 | 7 | 13 | 41 |
| Death | 6 | 14 | 23 | 3 | 8 | 4 | 4 | 16 |
| multicellular organismal development | 8 | 40 | 24 | 15 | 12 | 4 | 9 | 24 |
| Gene expression | 12 | 19 | 46 | 6 | 20 | 10 | 14 | 25 |
| regulation of gene expression | 4 | 16 | 31 | 5 | 11 | 5 | 10 | 12 |
| RNA processing | 3 | 4 | 11 | 1 | 2 | 4 | 1 | 13 |
| Transcription | 4 | 15 | 29 | 3 | 8 | 6 | 10 | 1 |
| Translation | 7 | 1 | 11 | 1 | 12 | 1 | 4 | – |
| Immune system process | 2 | 5 | 20 | 4 | 5 | 2 | 5 | 1 |
| immune effector process | 1 | 2 | 2 | – | – | – | 1 | – |
| immune response | 2 | 4 | 17 | – | 4 | 1 | 4 | – |
| Metabolic process | 32 | 75 | 104 | 28 | 54 | 21 | 40 | 81 |
| biosynthetic process | 17 | 18 | 23 | 5 | 19 | 3 | 11 | 19 |
| catabolic process | 3 | 11 | 12 | 1 | 6 | 3 | – | 9 |
| cellular metabolic process | 29 | 65 | 96 | 24 | 47 | 18 | 35 | 75 |
| nitrogen compound metabolic process | 18 | 5 | 11 | 25 | 6 | 2 | 4 | 5 |
| primary metabolic process | 32 | 66 | 98 | 5 | 52 | 18 | 35 | 75 |
| regulation of metabolic process | 4 | 25 | 35 | 5 | 13 | 8 | 11 | 29 |
| Response to stimulus | 7 | 29 | 45 | 5 | 12 | 6 | 14 | 15 |
| defense response | 1 | 5 | 13 | – | 2 | 2 | 6 | 1 |
| immune response | 2 | 4 | 17 | – | 4 | 1 | 4 | – |
| response to endogenous stimulus | 3 | 4 | 6 | 2 | 3 | – | 1 | 6 |
| response to external stimulus | 2 | 12 | 8 | 3 | – | 3 | 5 | 4 |
| response to stress | 4 | 23 | 15 | 4 | 5 | 5 | 4 | 10 |
| Total number of genes | 66 | 170 | 205 | 77 | 101 | 57 | 84 | 189 |

exhibiting expression profilies similar to each other, but distinct from the genes in other clusters. Figure 2 depicts the intensity of average fold changes of scanned signal at each time point. Among the six clusters, clusters A and D include genes showing gradual but dramatic changes in response to the stimulators, whereas genes in clusters B and E and clusters C and F show minor changes in the expression or changes recovered at 24 hr.

*Classification of genes highly responsive to the four inflammatory stimulators*: The porcine genes in clusters A and D could be classified into several groups based on gene ontology (GO) categories including associated biological process, cellular component and molecular function (Table 1). Treatment with $H_2O_2$ resulted in 236 differentially expressed genes. Of these 66 increased and 170 decreased. Treatment with human TNF-α resulted in 282 differentially expressed genes; 205 increased and 77 decreased. Treatment with porcine IFN-γ resulted in 158 differentially expressed genes; 101 increased and 57 decreased. Treatment with LPS resulted in 273 differentially expressed genes; 84 increased and 189 decreased. When we sorted differentially expressed genes in clusters A and D according to stimulators and selected 25 some genes showing the most dramatic changes (Table 2), no significant overlap was

Table 2. Porcine genes differentially expressed in response to inflammatory stimulators, $H_2O_2$, human TNF-α, porcine IFN-γ and LPS

| TIGR ID | Symbol | Description for human homologue | Mean fold change[a)] | FDR[b)] |
|---|---|---|---|---|
| $H_2O_2$ | | | | |
| **Increased in Cluster A** | | | | |
| TC182123 | MT1A | Metallothionein 1A (functional) | 5.848 | 0.043 |
| TC163874 | PSAT1 | Phosphoserine aminotransferase 1 | 5.659 | <0.001 |
| TC62905 | ESM1 | Endothelial cell-specific molecule 1 | 3.816 | <0.001 |
| TC167535 | SFRP2 | secreted frizzled-related protein 2 | 3.490 | <0.001 |
| TC168249 | CTH | Cystathionase (cystathionine gamma-lyase) | 3.777 | <0.001 |
| TC172521 | VEGFC | vascular endothelial growth factor C | 2.726 | <0.001 |
| **Decreased in Cluster D** | | | | |
| TC181598 | | 3-beta-hydroxysteroid dehydrogenase, tissue-type heart | -25.773 | <0.001 |
| TC182276 | HMGCS1 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | –6.410 | 0.078 |
| TC47292 | SCD | Stearoyl-CoA desaturase (delta-9-desaturase) | –5.917 | 0.063 |
| TC180916 | COL1A1 | Collagen, type I, alpha 1 | –5.076 | <0.001 |
| TC180931 | COL3A1 | Collagen, type III, alpha 1 | –4.975 | <0.001 |
| TC165521 | CHI3L1 | Chitinase 3-like 1 (cartilage glycoprotein-39) | –4.695 | <0.001 |
| TC165213 | NOV | Nephroblastoma overexpressed gene | –4.386 | <0.001 |
| TC163712 | SC4MOL | Sterol-C4-methyl oxidase-like | -4.274 | <0.001 |
| TC57024 | KLF2 | Kruppel-like factor 2 | –4.219 | <0.001 |
| TC182275 | HMGCS1 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | –4.080 | <0.001 |
| TC56058 | CRP | C-reactive protein | –4.049 | <0.001 |
| TC163245 | UBE2L3 | Ubiquitin-conjugating enzyme E2L 3 | –3.891 | <0.001 |
| TC165284 | VWF | von Willebrand factor | –3.861 | <0.001 |
| TC172581 | FZD1 | Frizzled homolog 1 | –3.448 | <0.001 |
| TC164833 | GADD45B | Growth arrest and DNA-damage-inducible, beta | –3.344 | <0.001 |
| TC191232 | C11orf17 | Chromosome 11 open reading frame 17 | –3.311 | <0.001 |
| TC163125 | FDFT1 | farnesyl-diphosphate farnesyltransferase 1 | –3.299 | <0.001 |
| TC188093 | ST3GAL2 | ST3 beta-galactoside alpha-2,3-sialyltransferase 2 | –3.219 | <0.001 |
| TC184129 | LMCD1 | LIM and cysteine-rich domains 1 | –3.215 | <0.001 |
| TC193041 | COL8A1 | Collagen, type VIII, alpha 1 | –3.012 | <0.001 |
| TC163712 | SC4MOL | sterol-C4-methyl oxidase-like | –2.992 | <0.001 |
| TC162856 | H1F0 | H1 histone family, member 0 | –2.924 | <0.001 |
| TC183879 | SORBS1 | Sorbin and SH3 domain containing 1 | –2.899 | <0.001 |
| TC181174 | HERPUD1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | –2.821 | <0.001 |
| TC182172 | IGFBP3 | insulin-like growth factor binding protein 3 | –2.760 | <0.001 |
| TC165819 | C14orf4 | chromosome 14 open reading frame 4 | –2.164 | 0.351 |
| Human TNF-α | | | | |
| **Increased in Cluster A** | | | | |
| TC185393 | CXCL6 | Chemokine (C-X-C motif) ligand 6 | 5.494 | 0.020 |
| TC167535 | SFRP2 | Secreted frizzled-related protein 2 | 4.099 | 0.082 |
| TC162989 | CTSS | Cathepsin S | 3.251 | 0.062 |
| TC167645 | BCL2L14 | BCL2-like 14 (apoptosis facilitator) | 3.114 | <0.001 |
| TC169144 | RSAD2 | Radical S-adenosyl methionine domain containing 2 | 3.075 | <0.001 |
| TC172521 | VEGFC | vascular endothelial growth factor C | 2.535 | <0.001 |
| TC182080 | GBP2 | Guanylate binding protein 2, interferon-inducible | 2.512 | <0.001 |
| TC178716 | PFTK1 | PFTAIRE protein kinase 1 | 2.476 | <0.001 |
| TC181218 | PPAP2B | Phosphatidic acid phosphatase type 2B | 2.316 | <0.001 |
| TC51283 | ERAP1 | Endoplasmic reticulum aminopeptidase 1 | 2.252 | <0.001 |
| TC162192 | TAP1 | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) | 1.973 | <0.001 |
| TC186448 | | EST | 1.968 | <0.001 |
| TC198894 | RNASEL | Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent) | 1.932 | <0.001 |
| **Decreased in Cluster D** | | | | |
| TC181174 | HERPUD1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | –2.920 | <0.001 |
| TC164833 | GADD45B | Growth arrest and DNA-damage-inducible, beta | –2.039 | <0.001 |
| TC182172 | IGFBP3 | insulin-like growth factor binding protein 3 | –2.018 | <0.001 |
| TC165819 | C14orf4 | chromosome 14 open reading frame 4 | –1.987 | <0.001 |
| TC165102 | LOC70447 | Nuclear localized factor 2 | –1.908 | <0.001 |
| TC164678 | BAG5 | BCL2-associated athanogene 5 | –1.873 | 0.083 |

Table 2. (continued)

| | | | | |
|---|---|---|---|---|
| TC182640 | | EST | –1.795 | <0.001 |
| TC186242 | MFNG | Manic fringe homolog | –1.721 | 0.485 |
| TC182275 | HMGCS1 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | –1.656 | 0.566 |
| TC163125 | FDFT1 | farnesyl-diphosphate farnesyltransferase 1 | –1.600 | 1.886 |
| TC163712 | SC4MOL | sterol-C4-methyl oxidase-like | –1.474 | <0.001 |
| Porcine IFN-γ | | | | |
| **Increased in Cluster A** | | | | |
| TC172521 | VEGFC | vascular endothelial growth factor C | 4.921 | <0.001 |
| TC182566 | WARS | Tryptophanyl-tRNA synthetase | 4.769 | <0.001 |
| TC164007 | SSRP1 | Structure specific recognition protein 1 | 4.301 | <0.001 |
| TC164839 | NUB1 | NEDD8 ultimate buster-1 | 4.205 | <0.001 |
| TC47457 | HLA-A | Major histocompatibility complex, class I, A | 3.936 | <0.001 |
| TC164644 | CNP | 2',3'-cyclic nucleotide 3' phosphodiesterase | 3.329 | 1.049 |
| TC167535 | SFRP2 | Secreted frizzled-related protein 2 | 3.181 | <0.001 |
| **Decreased in Cluster D** | | | | |
| TC51775 | LAMB1 | Laminin, beta 1 | –7.752 | <0.001 |
| TC163805 | C5orf13 | Chromosome 5 open reading frame 13 | –5.618 | <0.001 |
| TC194522 | LGR4 | Leucine-rich repeat-containing G protein-coupled receptor 4 | –5.181 | <0.001 |
| TC194843 | KITLG | KIT ligand | –5.051 | <0.001 |
| TC189900 | FNTB | Farnesyltransferase, CAAX box, beta | –4.202 | <0.001 |
| TC180074 | MAD2L1BP | MAD2L1 binding protein | –3.846 | <0.001 |
| TC163353 | ACOX1 | Acyl-Coenzyme A oxidase 1, palmitoyl | –3.717 | <0.001 |
| TC191162 | FNDC3B | Fibronectin type III domain containing 3B | –3.413 | <0.001 |
| TC188063 | RBM16 | RNA binding motif protein 16 | –3.077 | 0.046 |
| TC174838 | CKAP5 | Cytoskeleton associated protein 5 | –3.021 | <0.001 |
| TC168892 | SCC-112 | SCC-112 protein | –2.985 | <0.001 |
| TC173289 | MYCT1 | Myc target 1 | –2.907 | <0.001 |
| TC162923 | FSTL1 | Follistatin-like 1 | –2.833 | <0.001 |
| TC165819 | C14orf4 | chromosome 14 open reading frame 4 | –2.859 | <0.001 |
| TC194253 | DDX3X | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked | –2.786 | <0.001 |
| TC170299 | C1orf109 | Chromosome 1 open reading frame 109 | –2.674 | 0.087 |
| TC181174 | HERPUD1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | –2.514 | <0.001 |
| TC164833 | GADD45B | Growth arrest and DNA-damage-inducible, beta | –2.199 | 0.803 |
| TC182172 | IGFBP3 | insulin-like growth factor binding protein 3 | –2.078 | 8.611 |
| TC163125 | FDFT1 | farnesyl-diphosphate farnesyltransferase 1 | –1.923 | 4.790 |
| TC182275 | HMGCS1 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | –1.823 | <0.001 |
| TC163712 | SC4MOL | sterol-C4-methyl oxidase-like | –1.298 | <0.001 |
| LPS | | | | |
| **Increased in Cluster A** | | | | |
| TC185393 | CXCL6 | Chemokine (C-X-C motif) ligand 6 | 13.720 | 0.026 |
| TC169144 | RSAD2 | Radical S-adenosyl methionine domain containing 2 | 5.978 | 0.073 |
| TC164007 | SSRP1 | Structure specific recognition protein 1 | 4.752 | <0.001 |
| TC181218 | PPAP2B | Phosphatidic acid phosphatase type 2B | 4.566 | <0.001 |
| TC172521 | VEGFC | vascular endothelial growth factor C | 3.702 | <0.001 |
| TC167535 | SFRP2 | Secreted frizzled-related protein 2 | 3.024 | <0.001 |
| **Decreased in Cluster D** | | | | |
| NP300692 | TOP2B | Topoisomerase (DNA) II beta 180 kDa | –10.246 | <0.001 |
| TC163155 | PTBP1 | Polypyrimidine tract binding protein 1 | –9.901 | 0.014 |
| TC166047 | SLC26A11 | Solute carrier family 26, member 11 | –9.346 | <0.001 |
| TC162684 | TAGLN | Transgelin | –8.547 | 0.036 |
| TC62435 | COL1A2 | Collagen, type I, alpha 2 | –8.475 | 0.069 |
| TC163805 | C5orf13 | Chromosome 5 open reading frame 13 | –8.436 | 0.058 |
| TC168626 | POLR3C | Polymerase (RNA) III (DNA directed) polypeptide C (62 kD) | –7.813 | <0.001 |
| TC192166 | | EST | –6.944 | 0.074 |
| TC162651 | VIM | Vimentin | –6.410 | <0.001 |
| TC162821 | PKM2 | Pyruvate kinase, muscle | –5.714 | 0.069 |
| TC173990 | SULT1E1 | Sulfotransferase family 1E, estrogen-preferring, member 1 | –5.650 | <0.001 |
| TC161952 | RAB1A | RAB1A, member RAS oncogene family | –5.348 | <0.001 |
| TC166961 | WAC | WW domain containing adaptor with coiled-coil | –5.076 | <0.001 |
| TC182660 | UROD | Uroporphyrinogen decarboxylase | –4.950 | <0.001 |
| TC163977 | SNRPA | Small nuclear ribonucleoprotein polypeptide A | –4.854 | 0.059 |

Table 2. (continued)

| | | | | |
|---|---|---|---|---|
| TC187351 | ERBB2IP | Erbb2 interacting protein | –4.808 | <0.001 |
| TC181080 | ACTA2 | Actin, alpha 2, smooth muscle, aorta | –4.717 | <0.001 |
| TC190486 | | EST | –4.424 | <0.001 |
| TC175756 | | EST | –4.156 | <0.001 |
| TC163821 | BCKDK | branched chain ketoacid ehydrogenase kinase | –4.118 | <0.001 |
| TC164700 | PUM2 | Pumilio homolog 2 | –4.076 | <0.001 |
| TC163125 | FDFT1 | farnesyl-diphosphate farnesyltransferase 1 | –2.621 | <0.001 |
| TC164833 | GADD45B | Growth arrest and DNA-damage-inducible, beta | –2.441 | <0.001 |
| TC165819 | C14orf4 | chromosome 14 open reading frame 4 | –2.350 | <0.001 |
| TC182172 | IGFBP3 | insulin-like growth factor binding protein 3 | –1.826 | 1.001 |
| TC181174 | HERPUD1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | –1.820 | <0.001 |
| TC182275 | HMGCS1 | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | –1.346 | 0.995 |
| TC163712 | SC4MOL | sterol-C4-methyl oxidase-like | –1.029 | 3.093 |

a) Numbers represent fold change in mRNA expression in PAECs.
b) FDR: False Discovery Rate.

observed, suggesting that there is no common activation pathway.

*Validation of microarray data using quantitative real-time PCR*: In order to further confirm the reliability of our microarray results, we performed quantitative real-time PCR for nine genes that significantly changed in microarray analysis in response to $H_2O_2$, human TNF-α, pIFN-γ and LPS. The mRNA levels of secreted frizzled-related protein (SFRP2), chemokine (C-X-C motif) ligand 6 (CXCL6) and vascular endothelial growth factor C (VEGFC) increased in total RNA samples prepared at 24 hr after the treatments and those of insulin-like growth factor binding protein 3 (IGFBP3), homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1), farnesyldiphosphate farnesyl transferase 1 (FDFT1), chromosome 14 open reading frame 4 (C14orf4), sterol-C4-methyl oxidase-like (SC4MOL), 3-hydroxy-3-methylglutaryl -Coenzyme A synthase 1 (HMGCS1) and growth arrest and DNA-damage-inducible, beta (GADD45B) decreased (Fig. 3). These results from quantitative real-time PCR were remarkably similar to those of microarray experiments (Fig. 3).

*Secreted frizzled-related protein 2*: The mRNA level of SFRP2 in porcine endothelial cells increased more than 2–4 folds in microarray experiment by all treatment. Quantitative real-time PCR experiment confirmed that mRNA level of SFRP2 increased more than two-fold by human TNF-α, $H_2O_2$ and LPS treatments in Fig 3. But in parallel quantitative real-time PCR experiments, the expression of SFRP2 was not detected in HUVEC while it could be amplified in other human cell lines such as MCF7 by the same PCR primers (Fig. 3E).

## DISCUSSION

Pig endothelial cells play a key role in xenogeneic immune responses. Endothelial cell activation leads to thrombosis and infiltration of inflammatory cells, ultimately resulting in graft rejection [4, 5]. However, the interactive mechanism between human inflammatory stimulators and PAEC during the process of activation has not been studied so far. The porcine endothelial cell line [176] previously established by us showed a high level of VCAM-1 expression on cell surface without any stimulation, suggesting activated phenotype. Therefore we established five new porcine endothelial cell lines from aorta, all of which lacked surface expression of VCAM-1. Of these, we used one clone for the cDNA microarray experiment. The PAECs were stimulated with 4 inflammatory activators. Human TNF-α and porcine IFN-γ was selected as representatives of human and porcine proinflammatory cytokines, respectively. LPS, a non-specific, but potent immunostimulator, was used as a positive control. $H_2O_2$ was also included since PAEC is more susceptible to $H_2O_2$ in its activation than human endothelial cells [20].

All these stimulants induced the overexpression of VCAM-1, confirming that activation condition was successfully established. We examined gene expression profiles using pig oligonucleotide 13K chips. The genes in groups cluster A and D (Fig. 2) showing dramatic changes could be classified ontologically as associated with biological processes such as cell adhesion, cell communication, immune response, and response to stress (Table 1). In group A, the most prominently overexpressed gene in PAEC by human TNF-α was chemokine (C-X-C motif) ligand 6 (CXCL6). It was also the gene most highly induced by LPS in PAEC. And in human endothelial cells, it was reported to be one of the mRNA species highly overexpressed by LPS [22]. Radical S-adenosyl methionine domain containing 2 (RSAD2) was induced more than three-fold not only by human TNF-α but also by LPS. RSAD2 or viperin was reported as a cytoplasmic antiviral protein induced by type I interferons that inhibit infection of various human cells with viruses such as human cytomegalovirus and hepatitis C virus [11, 25]. Cathepsin S was induced more than three-fold by human TNF-α. It plays a major role in the degradation of the invariant chain associated with the major histocompatiblity complex and thus affects antigen presentation [2]. Its

overexpression by TNF-α in human cervical smooth muscle cells was previously reported [33]. Bcl-2-like 14 (BCL2L14) was induced more than three-fold by TNF-α. In humans, it was also known as Bcl-G and there are 4 alternatively spliced transcription variants encoding Bcl-G. These are pro-apoptotic members of Bcl-2 family but their role in endothelial cell activation has not been reported hitherto. Structure specific recognition protein 1 (SSRP1) was overexpressed more than four-fold by both porcine IFN-γ and LPS. It has been reported to regulate transcription elongation but its physiological role remains largely elusive [210]. SFRP2 was reported to be involved in Wnt signaling pathway possibly playing a role in cell differentiation, embryonic development and in carcinogenesis, but its function is still largely unknown [12, 23].

The most interesting mRNA species identified in this study is secreted frizzled-related protein 2 (SFRP2). It was induced more than two-four folds in porcine endothelial cells by all treatment. We confirmed, by quantitative real time-PCR analysis, that its overexpression is induced in porcine endothelial cells by all the stimulators (Fig. 3). It is to be noted that SFRP2 has never been reported to be overexpressed in human endothelial cells during the activation process. We confirmed in these studies that its overexpression was not induced in human endothelial cells after stimulation (Fig. 3E). SFRP2 might be involved in the Wnt signaling pathway, possibly playing a role in cell fate, polarity, proliferation, tissue patterning, and apoptosis. However, for the most part, its function is unknown. These observations demonstrated that the porcine endothelium had a transcriptional response to inflammatory stimuli that was different from that of human endothelium. Though SFRP2 is related to Wnt signaling and there is no direct relation between Wnt signaling and xenotransplantation, this over expression of SFRP2 in 1, 6 and 24 hr may interfere other pathway and act an anti-xenotransplantation.

In summary the present study shows that the activation of porcine endothelial cells is accompanied by the overexpression of several genes, some of which are not overexpressed in human endothelial cells during the activation process. These specific genes and their products could hold the key for understanding xenotransplantation-specific immune reactions and for development of drugs tailored to assist xenotransplantation.

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