ORIGINAL ARTICLES

Sequence alignment analysis of proteins involved in platelet-endothelial cell interaction identifies molecular incompatibilities between *Homo sapiens* and *Sus scrofa*

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ABSTRACT

Background: Platelets play a vital role in acute humoral xenograft rejection (AHXR), presenting as microvascular thrombosis in the graft and/or consumptive coagulopathy in the recipient. Adhesion and aggregation of primate platelets to the activated vascular endothelial cells through sequential binding of ligands on endothelial cells and subendothelial matrix ultimately trigger a complex biological process of prothrombotic signaling cascades. Increasing evidence suggests that the molecular incompatibilities in effector molecules across species may partially contribute to dysregulated microvascular thrombosis in xenografts.

Method: We selected amino acid sequence of candidate proteins from the NCBI database with keywords: platelet-endothelium interaction, platelet adhension, platelet aggregation, and subendothelial matrix ligands. Pair-wise amino acid alignments were made using the Emboss Needle method. Emboss needle created optimal global alignment of the amino acid sequences of human genes and pig genes using ClustalW2.

Results: Most of the proteins involved in platelet-EC interaction in *Homo sapiens* share high sequence similarity with their homologues in *Sus scrofa*. Cytokines that potentially induce endothelial damage (such as CD40L, TNF-α) were highly conserved between *Homo sapiens* and *Sus scrofa*. Some endothelium-derived cytokines (such as IL-8, CCL2, CCL5) that can induce platelet activation or enhance aggregation share high sequence similarity between *Homo sapiens* and *Sus scrofa*. Some regulators that potentially transduce inhibitory signaling to control platelet activation or complement activation have relatively poor sequence identity between *Homo sapiens* and *Sus scrofa*, and some even lack their homologues in *Sus scrofa*.

Conclusion: These characteristics of sequence similarity of proteins involved in platelet-EC interaction indicate the molecular incompatibilities between humans and pigs. This study provides clues for explanation of excessive platelet activity in pig-to-primate xenotransplantation model.

Key Words: Endothelial cells, Molecular incompatibility, Platelets, Sequence alignment analysis, Pig-to-primate xenotransplantation

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1. INTRODUCTION

Acute humoral xenograft rejection (AHXR, also known as acute vascular rejection) now poses a major hurdle to long-term xenograft survival when hyperacute rejection is overcome by using multitransgenic pigs on an α1,3-galactosyltransferase gene-knockout (GT-KO) background.[1–7] AHXR is a complex, multifactorial and muticellular scenario, which is generally linked to the action of anti-donor antibodies,[8–12] complement,[13–15] and recipient immune components, such as platelets,[16–19] T cells,[20] natural killer (NK) cells,[21,22] macrophages,[23,24] and neutrophils.[4,25] This biological process is mainly characterized by the development of thrombotic microangiopathy, with endothelial cell (EC) swelling, apoptosis, and necrosis.[26] It is becoming clear that AHXR results in functional impairment of a xenograft, and is inhibiting progress towards the clinical application of organ xenotransplantation.

Platelets, primarily recognized for their role in hemostasis and thrombosis, have been increasingly recognized as important mediators of AHXR, especially in the pathogenesis of microangiopathy.[27,28] In pig-to-primate xenotransplantation, circulating primate platelets are frequently recruited to the activated vascular ECs, mainly following the deposition of natural and/or elicited anti-donor antibodies on the EC surface.[29] A multi-step process that involves the adhesion of specific platelet-EC surface receptors with endothelial and subendothelial matrix proteins (such as collagens and von Willebrand factor), spreading of adherent platelets over the exposed subendothelial surface, and platelet aggregation, initiates the activation and procoagulant function of platelets.[30,31] The activated platelet plugs provide the surface for the assembly of coagulation factors, and support thrombin generation.[32–34] Excessive activation of platelets and coagulation cascades trigger the formation of platelet-rich microthrombi and the development of thrombotic microangiopathy.[35]

Xenogeneic molecular incompatibilities, i.e., protein-protein interactions between donor organs and recipients (such as receptor/ligand pair), may damage normal signaling transduction. Molecular incompatibilities between pigs and primates are currently perceived as the most problematic factors in the case of pig-to-primate xenotransplantation especially in the coagulation cascade.[36,37]

One well-demonstrated evidence of molecular incompatibility involves thrombomodulin. Although porcine thrombomodulin is able to recognize and bind human thrombin, the resulting complex is a weak activator of both human protein C and thrombin-activatable fibrinolysis Inhibitor.[38] In contrast, porcine von Willebrand factor has been reported to be a strong agonist for human platelet GPIb receptors, resulting in robust human platelet activation.[39] In order to control the biological effects of these incompatibilities, the generation of genetically-modified pigs has been suggested as an additional strategy to prolong xenograft survival.[40–42]

Although there has been increasing awareness of the contribution of molecular incompatibilities to xenograft failure, systematic analysis of molecular incompatibilities in the pig-to-primate context has not yet been declared. In addition, molecular incompatibilities of some proteins such as TFPI or h-DAF are still controversial.[43–45] In the present study, we systematically analyzed the amino acid sequence of proteins involved in platelet-EC interaction between humans (*Homo sapiens*) and pigs (*Sus scrofa*) by sequence alignment, aiming to explore the potential target proteins responsible for dysregulated platelet activity.

2. MATERIALS AND METHODS

2.1 Data set selection

Initially, a set of candidate proteins and their amino acid sequences (available in GenBank) was retrieved and examined. We selected candidate human protein sequence from NCBI database (`http://www.ncbi.nlm.nih.gov/` 10 july 2016) with keywords: platelet-endothelium interaction, platelet adhension, platelet aggregation, and subendothelial matrix ligands. If a protein encoded by a gene has isoform(s), the isoform with maximum sequence was representative of the gene. Sequence of proteins in this study can be found in supplements.

2.2 Alignment

We made pair-wise amino acid alignments using the Emboss Needle method (European Molecular Biology Laboratory, `ftp://emboss.open-bio.org/pub/EMBOSS/`). Emboss needle created optimal global alignment of the amino acid sequences of human proteins and pig proteins using ClustalW2. The following parameters were used to obtain suitable alignment results: Matrix: BLOSUM62, GAP OPEN: 10, GAP EXTENDED: 0.5, OUTPUT FORMAT: pair, END GAP PENALTY: false, OPEN GAP OPEN: 10 and END GAP EXTEND: 0.5. The BLAST program with options "-task blastp-short" was used to search for similarities of amino acid sequence of functional domain in human proteins against the porcine protein sequence. From the BLAST results, a description associated with each BLAST alignment was parsed to find the origin of the corresponding homologous protein sequence.

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3. RESULTS

Sequence alignment analysis of platelet receptors involved in platelet-EC interaction

Platelet-EC interaction is the first step in the biological process of platelet activation in pig-to-primate xenotransplantation. To identify the molecular incompatibilities associated with platelet-EC interaction between *Homo sapiens* and *Sus scrofa* species, we retrieved amino acid sequence of proteins that are potentially involved in platelet-EC interaction from the United States National Center for Biotechnology Information (NCBI) database using keywords: platelet-endothelium interaction, platelet adhesion, platelet aggregation, and subendothelial matrix ligands. If a protein encoded by a gene has isoforms, we took the isoform with maximum sequence length as being representative of the gene. After data set selection, the full sequence or functional domain sequence of each proteins from *Homo sapiens* and *Sus scrofa* species were subjected to pair-wise amino acid alignments (see Figure 1).

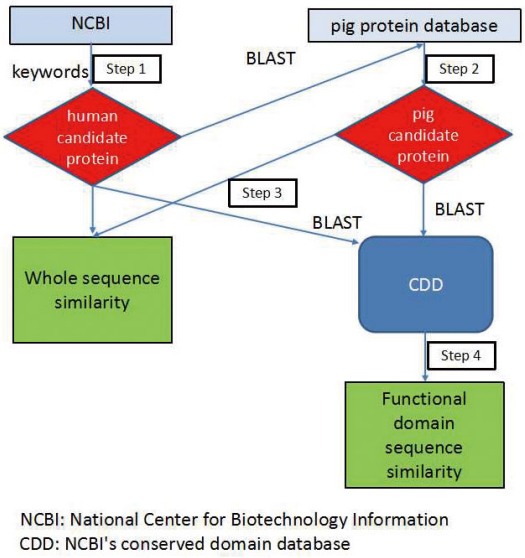

Figure 1. A flow chart of the full analysis algorithm
Step 1: Select candidate human protein sequence from NCBI database with keywords. Step 2: From the BLAST results, a description associated with each BLAST alignment was parsed to find the origin of the corresponding homologous protein sequence. Step 3: Make pair-wise amino acid alignments using the Emboss Needle method Emboss. Step 4: The BLAST program with options "-task blastp-short" was used to search for similarities of amino acid sequence of functional domain in human proteins against the porcine protein sequence.

Platelet-EC interaction is mediated by the binding of receptors (presented on the membrane surface of platelets) to their ligands within the extracellular matrix of injured vascular ECs. The well-known receptors in this process are glycoprotein (Gp) bV/IX, GpVI, integrin $\alpha_2\beta_1$, and integrin $\alpha_{IIb}\beta_3$.[46,47] We further analyzed the sequence identity of these receptors between *Homo sapiens* and *Sus scrofa* species. Among total 78 candidate genes, 66 genes in *Homo sapiens* share high sequence identity (ranking from 70% to 100% identity) with their homologues in *Sus scrofa* (see Figure 2A). Detailed analysis of the interaction domains of these receptors (selected platelet glycoproteins) revealed more conserved sequence identity between *Homo sapiens* and *Sus scrofa* species (see Table 1 and Figure 2B).

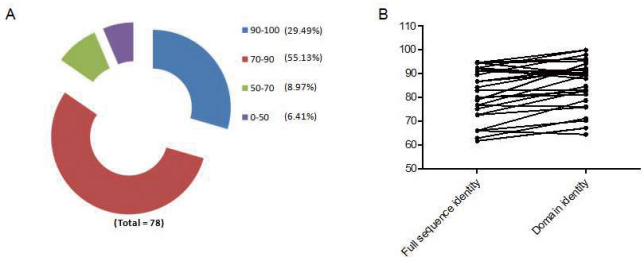

Figure 2. Sequence alignment analysis of platelet receptors involved in platelet-EC interaction (A) Frequency distributions of percent difference in full sequence alignment analysis between *Homo sapiens* and *Sus scrofa* orthologs for 78 platelet receptors. (B) Comparison of full sequence identity with domain sequence identity between *Homo sapiens* and *Sus scrofa* platelet receptors.

Sequence alignment analysis of endothelial ligands involved in platelet-EC interaction

We then analyzed the sequence identity of endothelial ligands that mediate platelet-EC interaction. Among a total 108 candidate genes, 94 genes in *Homo sapiens* share high sequence identity (ranking from 70% to 100% identity) with their homologues in *Sus scrofa* (see Figure 3A). Furthermore, sequence alignment analysis showed that nearly all the interaction domains shared >80% sequence identity between *Homo sapiens* and *Sus scrofa* (see Table 2 and Figure 3B).

Taken together, our data demonstrated that high sequence identity exists in endothelial ligands between *Homo sapiens* and *Sus scrofa*. Thus, this characteristic of high sequence similarity probably paves the way for molecular interaction between recipient platelets and donor endothelium.

Sequence alignment analysis of platelet-derived factors that contribute to EC activation

Upon activation, platelets release a variety of proteins that can influence the metabolic, adhesive, and apoptotic properties of vascular ECs.[48,49] Sequence alignment analysis of proteins and platelet-microparticle components released

from platelets (a total of 169 candidate genes) identified 9 genes showing 0-50% identity, 23 genes showing 50%-70% identity, 82 genes showing 70%-90% identity, and 55 genes showing 90%-100% identity (see Figure 4).

Table 1. Amino acid sequence alignment of platelet receptors between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
ITGA1	NP_852478.1	XP_013840217.1	83(1025/1229)	vWFA	96.69
				Int_alpha	92.31
ITGA2	NP_002194.2	NP_001231201.1	94(1109/1181)	vWFA	92.27
				Int_alpha	90.74
ITGA2B	NP_000410.2	NP_999163.1	87(907/1039)	Int_alpha	87.27
				Int_alpha	94.23
ITGA4	NP_000876.3	XP_003133565.1	94(969/1034)	Int_alpha	96.23
ITGAL	NP_001107852.1	XP_005653056.1	79(929/1181)	vWFA	75.37
				Int_alpha	84.62
ITGAM	NP_000623.2	XP_003124540.4	88(1010/1152)	vWFA	81.25
				Int_alpha	89.36
ITGAV	NP_001138471.1	NP_001077401.1	93(973/1049)	Int_alpha	98.18
ITGB1	NP_002202.2	NP_999133.1	98(778/798)	vWFA	95.13
				Integrin_B_tail	89.77
ITGB2	NP_000202.3	NP_999073.1	90(691/769)	vWFA	92.07
				Integrin_B_tail	57.69
ITGB3	NP_000203.2	NP_999167.1	97(762/788)	vWFA	92.69
				Integrin_B_tail	89.29
ITGB4	NP_000204.3	XP_013834729.1	93(1703/1824)	vWFA	89.95
				Integrin_B_tail	86.9
ITGB6	NP_000879.2	NP_001090892.1	97(764/788)	vWFA	93.65
				Integrin_B_tail	92.77
ITGB7	NP_000880.1	XP_013841173.1	92(734/800)	vWFA	91.84
				Integrin_b_cyt	88.1
GP1BA	NP_000164.5	XP_013836717.1	67(448/665)	LRRNT	59.38
				TPKR_C2	67.74
GP1BB	NP_000398.1	NP_001135457.1	86(178/207)	TPKR_C2	89.36
				LRRNT	91.43
GP5	NP_004479.1	XP_003132649.1	82(465/569)	TPKR_C2	73.08
				LRR_RI	81.09
GP6	NP_001077368.2	XP_005656014.2	32(219/694)	Ig	75.9
				Ig	82.95
GP9	NP_000165.1	NP_001135461.1	75(133/178)	TPKR_C2	64.44
CD36	NP_000063.2	XP_013835246.1	94(442/472)	CD36	82.96

From our analysis, TIMP metallopeptidase inhibitor-3, bone morphogenetic protein-4, peptidylprolyl isomerase A, angiopoietin-1, crystalline-α B, insulin-like growth factor-1, vascular endothelial growth factor (VEGF), Notch-2, CD40L, and TNF-α bear highly-conserved sequence identity between *Homo sapiens* and *Sus scrofa*, indicating the possibility of overcoming molecular incompatibility between species to functionally mediate signaling that contributes to vascular EC activation and/or apoptosis in xenograft (see Table 3).

Consistent with our study, human CD40L has been demonstrated to interact with porcine CD40 and activate porcine ECs.[50] Human TNF-α has also been demonstrated to induce porcine EC activation and immune-mediated microvascular injury.[51–53]

Furthermore, we also identified some proteins in our candidate list potentially bearing the ability to protect ECs from activation or apoptosis (according to previous studies) (see Table 4). However, sequence alignment analysis showed that these proteins had relatively poor sequence identity (see Table 4).

Taken together, most of the chemokines or ligands released from recipient platelets or other cell types that may contribute to vascular EC activation have high sequence identity between *Homo sapiens* and *Sus scrofa*. However, some potentially protective factors exhibit poor sequence identity. These characteristics of sequence similarity may contribute to persistent EC activation in xenotransplantation.

Table 2. Amino acid sequence alignment of endothelial ligands between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
VWF	NP_000543.2	NP_001233150.1	91(2568/2813)	VWD	87.8
THBS1	NP_003237.2	NP_001231465.1	99(1159/1170)	TSP_C LamG TSP_1	100 97.96 96.23
LAMA1	NP_005550.2	XP_013847226.1	43(1328/3075)	Laminin_N Laminin_B	90.32 80.77
LAMA2	B-NP_000417.2	XP_013848026.1	39(1207/3123)	Laminin_II LamG	95.62 94.57
LAMA3	NP_937762.2	XP_003482090.2	47(1583/3345)	Laminin_N Laminin_B	94.05 88.28
LAMA4	NP_001098676.2	XP_013848215.1	95(1742/1830)	Laminin_II LamG	89.06 93.98
LAMB1	NP_002282.2	XP_005667793.1	97(1738/1786)	Laminin_N EGF_CA	98.23 95.92
LAMB2	NP_002283.3	XP_013837118.1	94(1693/1802)	Laminin_N EGF_CA	97.78 91.84
LAMB3	NP_001121113.1	XP_013845124.1	58(743/1281)	Laminin_N	86.4
LAMB4	NP_001304975.1	XP_013835308.1	81(1500/1854)	Laminin_N EGF_CA	88 87.5
LAMC1	NP_002284.3	NP_001258644.1	98(1581/1609)	Laminin_N Laminin_B EGF_CA	99.17 97.62 93.48
LAMC2	NP_005553.2	XP_013835383.1	83(1079/1298)	Laminin_B EGF_CA	94.44 93.33
LAMC3	NP_006050.3	XP_003480667.2	51(800/1576)	Laminin_N Laminin_B	94.25 80.33
COL10A1	XP_006715396.1	XP_013848229.1	92(626/681)	C1q Collagen	92.65 81.82
COL11A1	NP_542196.2	XP_001929407.5	85(1550/1820)	COLFI LamG	98.68 71.43
COL12A1	XP_011533736.1	XP_013848273.1	64(1997/3120)	vWFA	98.17
COL13A1	XP_011537594.1	XP_013845872.1	77(585/761)	Collagen	100
COL14A1	XP_006716714.1	XP_013851967.1	97(1735/1797)	vWFA LamG	93.94 91.84
COL15A1	XP_011516516.1	XP_013842218.1	51(714/1389)	Endostatin-like Collagen	91.02 88.1
COL16A1	NP_001847.3	XP_013854553.1	92(1501/1631)	LamG Collagen	90.11 91.3
COL17A1	NP_000485.3	XP_013839164.1	89(1359/1533)	Collagen	94.12
COL18A1	NP_569711.2	XP_013845682.1	35(605/1755)	Endostatin-like Collagen	90.64 44.9
COL19A1	XP_011533739.1	XP_003121320.3	88(1045/1185)	LamG Collagen	79.78 90.48
COL1A1	NP_000079.2	XP_013836466.1	60(871/1464)	COLFI Collagen	95.73 53.33
COL21A1	XP_011513226.1	XP_013833223.1	95(912/957)	vWFA LamG Collagen	92.76 92.9 91.67
COL23A1	XP_006714996.1	XP_013842985.1	75(490/651)	Collagen	98.28
COL26A1	NP_001265492.1	XP_013850943.1	76(348/460)	EMI	97.1
COL27A1	NP_116277.2	XP_013849239.1	44(824/1861)	COLFI	97.99
COL28A1	XP_011513660.1	XP_013835173.1	81(957/1189)	vWFA Collagen	85.39 72.55
COL3A1	NP_000081.1	NP_001230226.1	96(1401/1467)	COLFI VWC	93.99 100
COL4A1	NP_001836.3	XP_013836142.1	93(1558/1669)	C4 Collagen	99.1 96.61
COL5A1	B-NP_000084.3	NP_001014971.1	98(1805/1841)	LamG Collagen	97.4 100
COL6A1	NP_001839.2	XP_005659104.1	38(391/1028)	vWFA Collagen	95.48 96.61
COL7A1	XP_011531639.1	XP_013837111.1	92(2700/2946)	vWFA Collagen	94.55 86.44
COL8A1	NP_001841.2	XP_001926478.1	97(725/744)	C1q Collagen	99.26 100
COL9A1	XP_011533731.1	XP_003121321.2	95(883/932)	LamG Collagen	92.31 93.33
COLGALT1	NP_078932.2	XP_003123541.1	97(604/623)	Glyco_transf_25	97.31
COLGALT2	XP_011507634.1	XP_003482786.1	91(601/659)	Glyco_transf_25	97.31
COLQ	NP_005668.2	XP_013836825.1	96(438/458)	Collagen	98.31

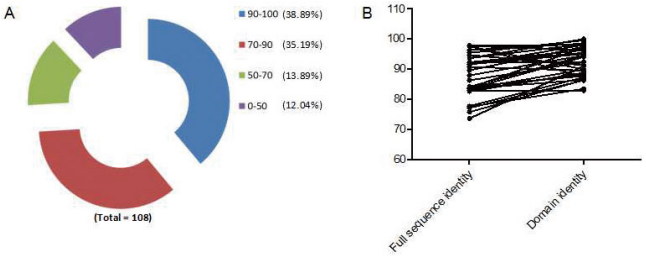

Figure 3. Sequence alignment analysis of endothelial ligands involved in platelet-EC interaction (A) Frequency distributions of percent difference in full sequence alignment analysis between *Homo sapiens* and *Sus scrofa* orthologs for 108 endothelial ligands. (B) Comparison of full sequence identity with domain sequence identity between *Homo sapiens* and *Sus scrofa* endothelial ligands.

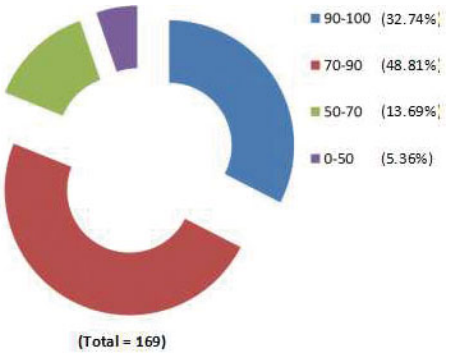

Figure 4. Sequence alignment analysis of platelet-derived factors that contribute to EC activation. Frequency distributions of percent difference in full sequence alignment analysis between *Homo sapiens* and *Sus scrofa* orthologs for 169 platelet-derived factors.

Table 3. Amino acid sequence alignment of platelet-derived factors between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
TIMP1	NP_003245.1	NP_999022.1	91(188/207)	NTR_like	84.66
TIMP2	NP_003246.1	NP_001139457.1	99(218/221)	NTR_like	98.36
TIMP3	NP_000353.1	XP_003126121.3	100(211/211)	NTR_like	100
BMP4	NP_001193.2	XP_005660027.1	99(404/409)	TGFb_propeptide	97.46
BMP7	NP_001710.1	XP_005673101.1	99(425/431)	TGFb_propeptide	97.98
PPIA	NP_001287910.1	XP_013841254.1	99(104/105)	cyclophilin	99.02
ANGPT1	NP_001137.2	NP_999124.1	99(493/498)	FReD	98.15
CRYAB	NP_001276736.1	XP_005667377.1	98(171/175)	alpha-crystallin-Hsps_p23-like	98.81
IGF1	NP_000609.1	XP_005664255.1	97(149/153)	IlGF_like	100
NOTCH2	NP_001186930.1	XP_013852682.1	49(1199/2471)	EGF_CA	89.19
CD40LG	NP_000065.1	NP_999291.1	91(237/261)	TNF	88.71
CCL5	NP_001265665.1	XP_013845402.1	43(68/158)	Chemokine	74.19
SELP	NP_002996.2	NP_999243.1	66(550/830)	CLECT	76.47
VEGFA	NP_001020537.2	XP_013833429.1	64(299/471)	PDGF	97.59
WNT5A	NP_001243034.1	XP_013837252.1	95(362/380)	wnt	100
IL1A	NP_000566.3	XP_013843310.1	83(226/272)	IL1	64.29
IL1B	NP_000567.1	NP_999220.1	75(203/270)	IL1	68.03
IFNB1	NP_002167.1	NP_001003923.1	81(151/187)	IFab	61.22
IFNG	NP_000610.2	NP_999113.1	75(124/166)	IFN-gamma	59.4
HBEGF	NP_001936.1	NP_999464.1	93(194/208)	PHA02887	100
HIF1A	NP_001230013.1	NP_001116596.1	93(790/851)	PAS HIF-1a_CTAD	100 100
TNF	NP_000585.2	NP_999187.1	92(215/233)	TNF	90.15
IL17A	NP_002181.1	NP_001005729.1	84(130/155)	IL17	82.28
IL17B	NP_055258.1	XP_013850776.1	75(171/228)	IL17	97.62
IL17D	NP_612141.1	XP_013834169.1	77(166/215)	IL17	82.93
CXCL4	NP_001502.1	XP_005666809.1	74.77	Chemokine	80.95
CXCL1	NP_002610.1	NP_999041.1	45.87	Chemokine	72

Table 4. Amino acid sequence alignment of candidates potentially bearing the function to protect endothelial cells between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
CD74	NP_001020330.1	NP_998939.1	65.2(193/296)	MHCassoc_trimer	68.66
				TY	35.71
IL-R antagonist	NP_776213.1	NP_999427.1	73.4(141/192)	IL1	38.46
CCL26	NP_006063.1	NP_001009579.1	41.11	Chemokine	47.37
Urocortin	NP_003344.1	XP_005663105.1	48	CRF	45.95

Sequence alignment analysis of endothelium-derived regulators that affect platelet activation

Besides activation by primary agonists, platelets can also be activated by chemokines produced by ECs. Analysis of endothelium-derived regulators (a total of 53 candidate proteins) indicated that most of these proteins (80%) in *Homo sapiens* have more than 70% sequence identity with their homologues in *Sus scrofa* (see Figure 5). EC-derived CCL2 (monocyte chemotactic protein-1, MCP-1), CXCL8 (interleukin 8, IL-8), CXCL12, CX3CL1 and CCL5 have been reported as strong activators of platelets and can promote platelet aggregation.[54–56] Analysis of these chemokines and other potential candidates showed that most of these proteins share high sequence identity between *Homo sapiens* and *Sus scrofa* (see Table 5).

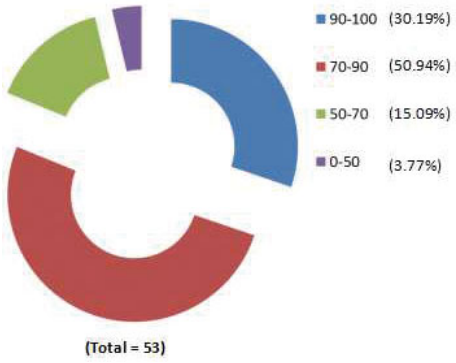

Figure 5. Sequence alignment analysis of endothelium-derived regulators that affect platelet activation. Frequency distributions of percent difference in full sequence alignment analysis between *Homo sapiens* and *Sus scrofa* orthologs for 53 endothelium-derived regulators.

Table 5. Amino acid sequence alignment of endothelium-derived regulators between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
CCL2	NP_002973.1	NP_999379.1	88(87/99)	Chemokine	79.31
CCL4	NP_002975.1	NP_998944.1	95(87/92)	Chemokine	80.7
CCL5	NP_001265665.1	XP_013845402.1	43(68/158)	Chemokine	74.19
CX3CL1	NP_002987.1	XP_013843976.1	72(292/408)	Chemokine	55.84
CXCL12	NP_001171605.1	NP_001009580.1	61(92/150)	Chemokine	96.77
IL-8	NP_000575.1	XP_003362006.1	82(84/103)	Chemokine	79.69
LEP	NP_000221.1	NP_999005.1	92(153/167)	Leptin	87.32
LGALS1	NP_002296.1	NP_001001867.1	92(124/135)	GLECT	85.48
LGALS3	NP_001170859.1	XP_013848886.1	67(173/260)	GLECT	89.47
				Bindin	75.76

Endothelium-derived regulators can also transduce inhibitory signaling to control platelet activation, and these include immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors, cell surface receptors, or small molecules. Sequence alignment analysis showed that these molecules have relatively less sequence identity between *Homo sapiens* and *Sus scrofa*, and some even lack their homologues in *Sus scrofa* (see Table 6).

Table 6. Amino acid sequence alignment of endothelium-derived inhibitory regulators between *Homo sapiens* and *Sus scrofa*

Gene name	Homo sapience	Sus scrofa	Similarity(has/ssc)	Functional domain	Sequence identity
CD46	NP_758869.1	NP_999053.1	54(229/421)	CCP	46.67
CD55	NP_000565.1	NP_998980.1	48(239/502)	CCP	33.33
CEACAM1	NP_001703.2	XP_005655946.2	61(323/527)	Ig	61.9
ESAM	NP_620411.2	XP_005667518.1	86(337/394)	Ig	74.34
F11R	NP_058642.1	NP_001121916.1	88(264/299)	Ig	77.78
PECAM1	NP_000433.4	NP_999072.1	84(620/740)	Ig	66.67
SERPINE1	NP_000593.1	NP_999075.1	93(373/402)	SERPIN	88.33
VIPR1	NP_004615.2	XP_005669452.1	92(420/459)	7tm_4	89.43
urocortin-2	NP_149976.1	XP_013837113.1	73.5(83/113)	-	
CD74	NP_001020330.1	NP_998939.1	65.2(193/296)	MHCassoc_trimer TY	68.66 35.71
IL-R antagonist	NP_776213.1	NP_999427.1	73.4(141/192)	IL1	38.46
TNFRSF 10C	NP_003832.2	-			
TNFRSF 10D	NP_003831.2	-			
G6b-B	NP_612116.1	-			

Taken together, these data indicate that most of the endothelium derived chemokines share high sequence identity between *Homo sapiens* and *Sus scrofa*, while some of the inhibitory regulators have relatively poor sequence identity. These characteristic of sequence identity may facilitate platelet activation by endothelium-derived regulators.

4. DISCUSSION

Uncontrolled activation of platelets and coagulation cascades trigger the formation of platelet-rich microthrombi and the development of thrombotic microangiopathy that is a major cause of xenograft failure. However, the study of molecular incompatibilities with regard to platelet activity in pig-to-primate xenotransplantation has been limited. In our analysis, (1) most of the proteins involved in platelet-EC interaction in *Homo sapiens* share high sequence similarity with their homologues in *Sus scrofa*; (2) nearly all the endothelial ligands, including collagen, laminin, von Willebrand factor, and thrombin, share high sequence similarity between *Homo sapiens* and *Sus scrofa*; (3) cytokines that potentially induce endothelial damage (such as CD40L, TNF-α) were highly conserved between *Homo sapiens* and *Sus scrofa*; (4) some endothelium-derived cytokines (such as IL-8, CCL2, CCL5) that can induce platelet activation or enhance aggregation share high sequence similarity between *Homo sapiens* and *Sus scrofa*; (5) regulators that potentially transduce inhibitory signaling to control platelet activation or complement activation have relatively poor sequence identity between *Homo sapiens* and *Sus scrofa*, and some even lack their homologues in *Sus scrofa*.

Sequence alignment analysis can be used to screen molecular incompatibilities of proteins between species. For example, porcine thrombomodulin has about 78% sequence similarity with human thrombomodulin, and porcine thrombomodulin is able to recognize and bind human thrombin, but the resulting complex is a weak activator of both human protein C and thrombin-activatable fibrinolysis Inhibitor.[38] Thus, from our analysis, the function of candidate targets needs to be confirmed experimentally. Furthermore, it is worthy of note that the availability, annotation, integrity of the *Sus scrofa* genome provide some obstacles that may ultimately affect the accuracy and integrity of our results.

It is reasonable for some regulators to have compatible functions across species, but abnormal expression may contribute to incompatible phenotypes. For example, coagulation is exacerbated during inflammation by the down-regulation and degradation of critical endothelial anticoagulant and antiplatelet systems. This is best illustrated by the influence of inflammatory TNF-α and IFN-γ on thrombomodulin gene expression and mRNA stability,[57–59] as well as proteolytic inactivation of endothelial protein C receptor (EPCR) by neutrophil proteinase-3.[60]

Our data provide a basic perspective for understanding molecular incompatibilities that may relate to excessive platelet activity in pig-to-primate xenotransplantation, and also provide a clue for strategies to control excessive platelet function in prevention of thrombosis. The high sequence similarity as we observed in proteins involved in platelet-EC interaction, endothelial ligands and endothelium-derived cytokines thus provide explanations to excessive platelet activation in pig-to-primate xenotransplantation. To prevent platelet activation and thrombus formation, blockade of signaling transduction

between donor endothelium and recipient platelet by targeting these candidates may be an efficient strategy. Actually, inhibition of platelet integrin GPIIbIIIa was demonstrated to reduce intravascular thrombosis and prolong survival of discordant cardiac xenografts.[61,62] Alternatively, genetic knock out of von Willebrand factor prolonged survival of porcine pulmonary xenografts.[63]

It was especially interesting that we identified several regulators that potentially transduce inhibitory signaling to control platelet activation or complement activation from previous report. However, these regulators have relatively poor sequence identity between *Homo sapiens* and *Sus scrofa*, and some even lack their homologues in *Sus scrofa*. The next step will be to experimentally verify their inhibitory potential in xenotransplantation model.

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CONFLICTS OF INTEREST DISCLOSURE

The authors have no financial conflicts of interest.

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