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Genome-Wide Association Study for Circulating Tissue Plasminogen Activator (tPA) Levels and Functional Follow-up Implicates Endothelial *STXBP5* and *STX2*

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Abstract

Objective—Tissue plasminogen activator (tPA), a serine protease, catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for endogenous fibrinolysis. In some populations, elevated plasma levels of tPA have been associated with myocardial infarction and other cardiovascular diseases (CVD). We conducted a meta-analysis of genome-wide association studies (GWAS) to identify novel correlates of circulating levels of tPA.

Approach and Results—Fourteen cohort studies with tPA measures (N=26,929) contributed to the meta-analysis. Three loci were significantly associated with circulating tPA levels (P <5.0×10^{-8}). The first locus is on 6q24.3, with the lead SNP (rs9399599, $P=2.9\times10^{-14}$) within *STXBP5*. The second locus is on 8p11.21. The lead SNP (rs3136739, $P=1.3\times10^{-9}$) is intronic to *POLB* and less than 200kb away from the tPA encoding gene *PLAT*. We identified a non-synonymous SNP (rs2020921) in modest LD with rs3136739 ($r^2 = 0.50$) within exon 5 of *PLAT* ($P=2.0\times10^{-8}$). The third locus is on 12q24.33, with the lead SNP (rs7301826, $P=1.0\times10^{-9}$) within intron 7 of *STX2*. We further found evidence for association of lead SNPs in *STXBP5* and *STX2* with expression levels of the respective transcripts. In *in vitro* cell studies, silencing *STXBP5* decreased release of tPA from vascular endothelial cells, while silencing of *STX2* increased tPA release. Through an *in-silico* lookup, we found no associations of the three lead SNPs with coronary artery disease or stroke.

Conclusions—We identified three loci associated with circulating tPA levels, the *PLAT* region, *STXBP5* and *STX2*. Our functional studies implicate a novel role for *STXBP5* and *STX2* in regulating tPA release.

Keywords

tissue plasminogen activator; genome-wide association study; meta-analysis; cardiovascular disease risk; fibrinolysis; hemostasis

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INTRODUCTION

Tissue plasminogen activator (tPA) is a glycoprotein produced mainly by vascular endothelial cells that catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for endogenous fibrinolysis and an important regulatory element in thrombosis. Circulating tPA is implicated in the progression and incidence of clinically apparent atherothrombotic cardiovascular diseases (CVD), such as myocardial infarction and stroke, and is associated, in some studies, with advanced atherosclerosis.[1–11] Recombinant tPA is approved for use in patients with acute myocardial infarction and is the only drug approved by the U.S. Food and Drug Administration for treatment of acute ischemic stroke.[5, 7]

The estimated heritability for circulating tPA level is as high as 0.67, based on family and twin studies, providing substantial evidence of genetic influences on circulating levels.[12–14] Little is known about the genetic predictors of circulating tPA. Several genetic polymorphisms within the *PLAT* gene locus have been identified, including the well-studied 311bp Alu-repeat insertion/deletion polymorphism (rs4646972).[15] In some cohorts, this Alu-repeat polymorphism has been associated with levels of circulating tPA and with CVD risk, although this finding was not seen in all cohorts studied.[4, 16, 17] Circulating levels of tPA are also associated with common polymorphisms in genes contained in the renin-angiotensin and bradykinin systems.[18, 19]

To date, there has not been a genome-wide association study (GWAS) on this circulating biomarker. We conducted a meta-analysis of 14 studies that had both tPA measurement and genome-wide genotype data in order to identify common variants that are associated with the variation in circulating levels of tPA antigen. Our study included a total of 26,929 participants who were enrolled in 14 cohorts of European ancestry with genome-wide markers. For replication, we evaluated the lead SNPs in an independent sample. We sought evidence for biological function for the lead SNPs within each locus, using human gene expression databases and RNA silencing studies in endothelial cells. We further sought to identify evidence for a role, if any, of the associated genetic variants with thrombosis-related clinical end points including apparent coronary artery disease (CAD) and stroke.

MATERIALS AND METHODS

Detailed Materials and Methods are available in the online-only Supplement.

RESULTS

Cohort Characteristics

The characteristics of a total of 26,929 participants in the 14 discovery cohorts are summarized in Supplemental Table I. The average age ranged from 45.2 years to 76.7 years. The percentage of males ranged from 38.5% to 75.3%, except for the largely female Twins UK, in which males comprised 4.8%. The BMI was similar across the cohorts, with a range of 26.1 kg/m^2 to 27.9 kg/m^2. The mean tPA level ranged from 5.06 ng/ml to 11.01 ng/ml.

Results of Primary GWAS

The *P* value results of our discovery meta-analysis for the 2,455,857 meta-analyzed SNPs are presented in Figure 1. A total of three loci reached genome-wide significance threshold of 5×10^{-8} (Table 1). For the first locus, we identified multiple SNPs (n=61) of genome-wide significance in the 6q24.3 region that harbors the *STXBP5* gene.[20] The SNP rs9399599 (within intron 26 of *STXBP5*) has the smallest *P* value of 2.9×10^{-14}. Allele T (frequency =0.54) is the risk allele, with an effect size (se) of 0.032 (0.004). As the trait was natural-logarithm transformed, this translates to an increase of 1.033 ng/ml of tPA per copy of the risk allele. The regional plot demonstrates that all significant SNPs in the region are in high LD with the lead SNP (Supplemental Figure I, Plot A). The second locus includes 7 SNPs reaching the genome-wide significance threshold; six of these SNPs lie within *POLB* while another one lies within *PLAT*, the gene that encodes tPA. The lead SNP (rs3136739, $P=1.3\times10^{-9}$) resides within intron 3 of *POLB*. The SNP within *PLAT* is a non-synonymous SNP (rs2020921, $P = 2.0\times10^{-8}$) within exon 5 of *PLAT* with the minor allele causing a tryptophan to be substituted for an arginine. Based on the 1000 Genomes project European data, these two SNPs are in LD ($r^2 = 0.5$). After re-analysis of Chromosome 8 conditioning on rs3136739, rs2020921 had a P-value of 2.1×10^{-4} and was the only SNP with a P-value < 1×10^{-3} within the 1.6 Mb region containing these two SNPs, suggesting there are two separate signals.

The third genome-wide locus includes a total of 33 SNPs lying within *STX2* in the 12q24.33 region. The lead SNP (rs7301826, $P=1.0\times10^{-9}$) resides within intron 7 of *STX2*. Regional plots for these three loci are shown in Supplemental Figure I. Summary statistics of the three lead SNPs and the *cis*-acting SNP within *PLAT* in each individual GWAS are shown in Supplemental Table III. For all four SNPs in these three loci, there was no evidence for heterogeneity across studies ($P>0.05$) (Table 1). The individual and combined effect of the three top SNPs in explaining phenotypic variance was assessed in the largest contributing study (B58C). The proportion of variance in log-transformed tPA explained by the top three loci combined was 0.75%. This comprised 0.29% variance explained by rs9399599 alone, 0.16% variance explained by rs7301826 alone, and 0.28% variance explained by rs3136739 alone.

To test for replication, genotyping was conducted in 4,487 participants from PREVEND. In the PREVEND replication cohort, none of the 3 SNPs was associated with tPA ($P<0.05$). The effect sizes were smaller: −0.001, 0.017, 0.002 compared with 0.032, 0.063, 0.027, respectively, for the 3 lead SNPs within *STXBP5*, *POLB-PLAT*, and *STX2*. After combined meta-analysis of these results with the data from the fourteen discovery cohorts, the combined meta-analysis *P* values for association for the four genome-wide associated SNPs (rs9399599, rs2020921, rs3136739, and rs7301826) each remained genome-wide significant ($P < 5.0\times \times10^{-8}$).

Association with Gene Expression

All three lead SNPs and their proxies were searched against three large eQTL sources as described in the online detailed materials and methods. eQTL results provided expression association evidence for *STXBP5* and *STX2*, but not for the chromosome 8 locus (Table 2).

SNP rs7739314 ($P < 3.1\times10^{-12}$), located ~500 bp 3' of *STXBP5*, was modestly associated with *STXBP5* expression in lymphocytes ($P < 1.6\times10^{-3}$), CD4+ lymphocytes ($P < 1.7\times10^{-4}$), and liver ($P < 0.03$), though this was not the strongest eSNP for *STXBP5* in these respective tissues. Three perfect proxy SNPs ($r^2=1.0$) for the lead *STX2* SNP (rs7301826) were strongly associated with expression of *STX2* in a wide range of blood cells and other tissues. In every case, the strongest eSNP for *STX2* was the same or a perfect proxy for the strongest SNP associated with circulating tPA level, indicating a high degree of concordance between the eQTL and association signals. tPA SNPs at the *STX2* and *STXBP5* loci were not significantly associated with expression of any other genes at those loci.

Results of Gene Silencing for *STXBP5* and *STX2* in Human Endothelial Cells

The proteins encoded by *STX2* and *STXBP5* are expressed in three types of vascular endothelial cells (HAEC, HUVEC, and HDMVEC) (Figure 2B–D). Silencing of *STX2* and *STXBP5* decreased expression of STX2 and STXBP5 proteins, respectively, in each of the three endothelial cell types (Figure 2B–D). Silencing *STXBP5* significantly decreased release of tPA, while silencing *STX2* significantly increased tPA release, in both resting and histamine-stimulated vascular endothelial cells (Figure 2A). SNP specific effects were not evaluated in the current experiments.

Association with CAD and Stroke

In a recently updated meta-analysis (based on 13 observational cohort studies and 5494 cases of CAD), a 1SD increase in tPA-antigen, adjusted for conventional cardiovascular risk factors, was associated with an odds ratio of incident CAD of 1.13 (95%CI 1.06, 1.21).[11] Since the genetic influences that we detected on tPA levels together accounted for less than 1% of phenotypic variance, and individual SNPs were associated with differences in untransformed tPA levels of less than 0.2SD, comparing homozygotes to heterozygotes, it is inherently unlikely that any of these variants would impact greatly on CAD risk, and an *in silico* look-up in previously published GWAS meta-analyses confirms this (Supplemental Table IV). The upper confidence limits in this table exclude clinically or epidemiologically important associations of the three top SNPs with cardiovascular disease, defined as either CAD or stroke.

Findings for Previously Implicated Genes

We examined for evidence of association of SNPs within a 20kb region of the *cis*-locus, *PLAT*, as well as SNPs within *ACE*, *AGT*, *AGTR1*, *BDKRB2*, and *SERPINE1*.[21, 22] For a total of 204 SNPs, 32 SNPs within *ACE*, *AGT*, *BGKRB2*, *PLAT*, *SERPINE1* have a *P* value <0.05 (Supplemental Table V). However, only three SNPs in *PLAT* (lead rs2020921, $P=5.1\times10^{-8}$) and five SNPs in *SERPINE1* (lead SNP rs2227667, $P=2.2\times10^{-5}$) remained significant after adjusting for the multiple testing (multiple testing threshold $P<2.5\times10^{-4}$). Given the correlation of SNPs within these two loci due to residual LD, the associations for each of the eight SNPs within these two loci are robust, extending evidence from the prior literature for the existence of a genetic association with plasma levels of tPA.

DISCUSSION

In a large GWAS study of over 27,000 research participants of European ancestry, we discovered a total of three loci that have not been previously reported to be associated with circulating tPA level at a genome-wide significant threshold. This is the first GWAS study that identifies a non-synonymous SNP within *PLAT* that reaches genome-wide significant threshold. eQTL examination provided strong functional evidence for associated SNPs in *STXBP5* and *STX2*, and further studies in human endothelial cells directly implicate these two genes in expression, production and release of tPA protein.

Prior candidate gene studies have not consistently noted the presence of associations between SNPs in the *PLAT* gene and circulating levels of tPA, and several studies have found no such association.[17, 23] The current study substantially extends and strengthens the prior hypothesis of a *cis*-association between SNPs in *PLAT* and circulating levels of tPA by providing evidence for a strong and genome-wide significant association of SNPs within the *PLAT* locus. We identify an association with a non-synonymous SNP rs2020921 within *PLAT*, suggesting a functional variant, and separately with SNPs in *POLB*, raising the hypothesis of an independent genetic determination of tPA in this locus. These findings suggest that the *cis*-associations are complex and may have been missed because previous mapping studies focused on mapping a narrow genomic region and were conducted in relatively smaller samples. There is little known about the functional consequences of the non-synonymous *PLAT* mutation and prediction software provides conflicting predictions of its effect (PolyPhen-2: neutral, SIFT: deleterious) therefore future functional experiments are warranted.

The associations of variants within *STXBP5* and *STX2* with circulating levels of tPA are novel findings. Syntaxins are members of a family of membrane integrated SNARE (Soluble NSF Attachment Protein Receptor) proteins that participate in exocytosis.[24] Syntaxin 4 plays a role in exocytosis of Weibel-Palade bodies in endothelial cells.[25] Our functional studies reveal that *STXBP5* and *STX2* play a role in endothelial release of tPA. Our cell culture studies strongly support a role for these two genes in regulating endothelial cell tPA expression, production and release. While these studies provide novel evidence derived from an unbiased GWAS for the role of *STXBP5* and *STX2* in regulation of tPA at the endothelial cell level, further studies are clearly warranted to examine how manipulation of the specific SNPs rather than silencing the whole gene affects the dynamics of circulating tPA level at the cellular and model organism level.

SNPs in the *STXBP5* and *STX2* loci were also reported to be associated with circulating levels of vWF in a recent study by the CHARGE Consortium.[26] Based on the 1000 Genomes data, there is moderate to strong correlation of the lead SNP associated with vWF[26] and the lead SNP we report to be associated with tPA for *STXBP5* (rs9390459, r^2=0.97, D'=1.0) and for *STX2* (rs79789987, r^2=0.63, D'=1.0). Although tPA and vWF share associations with common variants at the *STXBP5* and *STX2* loci, these relatively weak genetic associations are not a major explanation for the phenotypic correlation between these haemostatic risk factors. Both plasma components were measured in the British 1958 birth cohort (B58C), and a highly significant ($p<10^{-22}$) correlation (r=0.13) remained

between log-transformed tPA and log-transformed vWF levels, after adjustment for the top SNPs at *STXBP5* (rs9399599) and *STX2* (rs7301826). The association of identical syntaxin-coding genes with various circulating hemostatic factor levels may provide an opportunity for further investigations on these newly identified mechanisms by which these circulating hemostatic factors are implicated in thrombotic cardiovascular and metabolic diseases.

Our study was motivated in part in order to better understand the mechanism by which endogenous tPA may be implicated in clinically apparent cardiovascular disease outcomes. Our lead SNP rs9399599 in the *STXBP5* locus is associated with circulating levels of vWF[26] and with risk of venous thrombosis.[27] While elevated plasma level of vWF is a predictor of venous thrombosis, the available evidence suggests that the level of tPA is not associated with venous thrombosis.[28] Another non-synonymous SNP rs1039084 within *STXBP5* has a less significant association with vWF ($P = 1.0\times10^{-9}$) than the lead SNP in our study, but rs1039084 has a stronger association than rs9399599 with vWF in a subgroup of CHD patients.[29] For the *STX2* locus, the SNP rs7978987 has been previously reported to be associated with vWF levels[26] and with an increased risk of arterial thrombosis.[29] The *P* value for association of this SNP with tPA is 4.5×10^{-9}, similar to that of the lead SNP rs7301826 ($P = 4.1\times10^{-9}$). Two other SNPs within *STX2* (rs1236 and rs11061158) were also previously reported to be associated with CHD.[29] The former is genome-wide significant and the latter is marginally significant in our study ($P = 1.9\times10^{-9}$ and 0.048 respectively). None of the three lead SNPs in *PLAT/POLB, STXBP5* or *STX2* was found to be associated with CAD or stroke based on an *in-silico* examination of results from a large sample for CD and a moderate sized sample for stroke. However, the key function of tPA in the coagulation system and the importance of coagulation to the cardiovascular system have been well established, and the novel genes identified in our study merit further study of their potential role as intermediaries in the pathophysiology of atherothrombotic CVD.

The replication of previously reported findings for associations between genetic variation in *SERPINE1* and *PLAT* with circulating tPA levels was able to act as a “positive control” for this study and extends the evidence for the existence of a genetic association in these genes with plasma levels of tPA.

We note several potential study limitations. First, samples in our study are of European ancestry; therefore, our findings may not be generalizable to populations of different ethnicity. Second, the replication sample size is quite small, with an 80% power to detect an association at the 5% significance level. Although not particularly weak, there was still inadequate power to provide strong evidence for replication of small effects detected in the discovery study. We therefore included biological validation that includes *in vitro* cell studies. Third, due to the low frequency of the lead SNPs within the *POLB-PLAT* locus, we are not able to use a traditional LD mapping approach to refine the association signals. The lead SNP within *POLB* (rs3136739) is in perfect LD ($r^2=1$) with the non-synonymous SNP (rs2020921) within exon 5 of *PLAT*, based on the HapMap2 genotype data. However, rs3136739 has a missing rate of 10% in the HapMap2 data. Even with the most recent available 1000 genomes genotype data, however, it remains difficult to fully characterize the haplotype structure for SNPs with low minor allele frequency (~5%) based on pair-wise LD. Forth, although it is impossible to rule out that the association of the non-synonymous

mutation with circulating tPA levels is simply a confounder introduced by altered antibody binding during the tPA measurement procedure, it is unlikely after investigating all information available to us at this time. Finally, there may be distinct roles for genetic variation in regulating circulating levels in healthy individuals compared with elevated levels in individuals in whom thrombolytic activity is induced. While there is no evidence of heterogeneity of effect by cohort, our results cannot exclude the possibility of meaningful differences in effects of genetic variation on circulating levels in the overall population versus subgroups of individuals with increased thrombolytic activity.

In conclusion, by analyzing a total of 26,929 participants from 14 discovery cohorts across the United States and Europe, we provide genome-wide evidence of association of SNPs in the *STXBP5*, *PLAT* and *STX2* loci with circulating levels of tPA antigen. While our analyses do not provide evidence for association of these SNPs with clinically apparent CAD or stroke, we do provide functional evidence in endothelial cells for a novel regulatory role of *STXBP5* and *STX2* on tPA availability. The strong eQTL result for *STX2* in a wide range of tissues supports the hypothesis that associated alleles may modulate tPA levels via a functional effect on gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

CAD	Coronary artery disease
CVD	Cardiovascular disease
eQTL	Expression quantitative trait locus
eSNP	Expression single nucleotide polymorphism
GWAS	Genome-wide association study
LD	Linkage disequilibrium
MI	Myocardial infarction
SNP	Single nucleotide polymorphism
tPA	Tissue plasminogen activator
vWF	Von Willebrand factor

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SIGNIFICANCE

Tissue plasminogen activator (tPA) catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for endogenous fibrinolysis. In some but not all studies, elevated plasma levels of tPA have been associated with coronary artery disease (CAD) and other cardiovascular diseases. Through a genome-wide association study approach we provide evidence of association of genetic variants in the *STXBP5*, *PLAT* and *STX2* loci with circulating levels of tPA antigen. While our analyses do not provide supportive evidence for association of these SNPs with clinical CAD or stroke, we do provide additional functional evidence for a novel regulatory role of *STXBP5* and *STX2* on tPA availability in endothelial cells. Results from gene expression studies in various tissues support the hypothesis that associated alleles may modulate circulating tPA levels via a functional effect on gene expression. Our findings provide new insights into tPA biology and avenues for future research for the prevention and treatment of thrombosis.

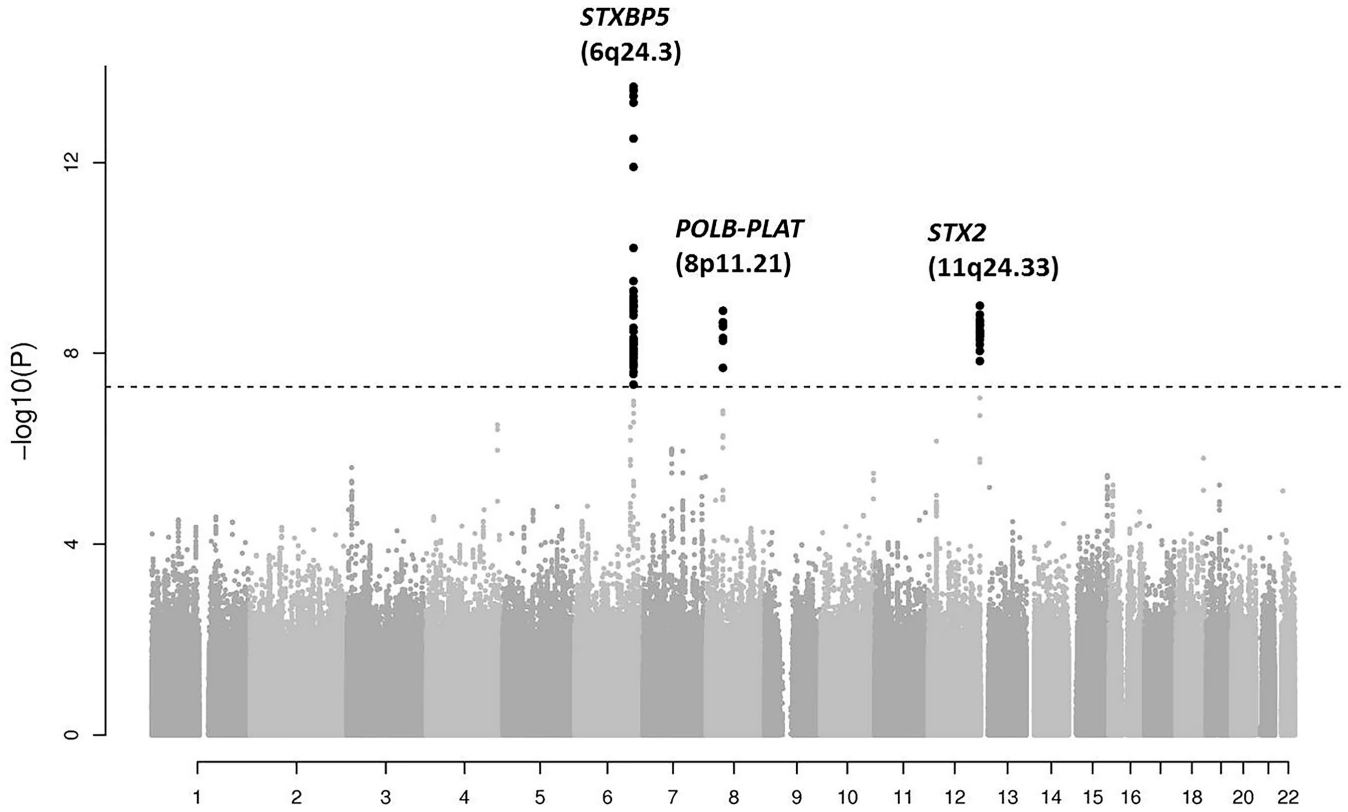

Figure 1. Manhattan plot showing the association *P*-values for the meta-analyzed SNPs in the discovery cohorts

X-axis organized by chromosome and base pair positions. Y-axis shows the -$\log_{10}$ of the association *P*-values. The horizontal dotted line marks the threshold for genome-wide significance ($P = 5.0 \times 10^{-8}$).

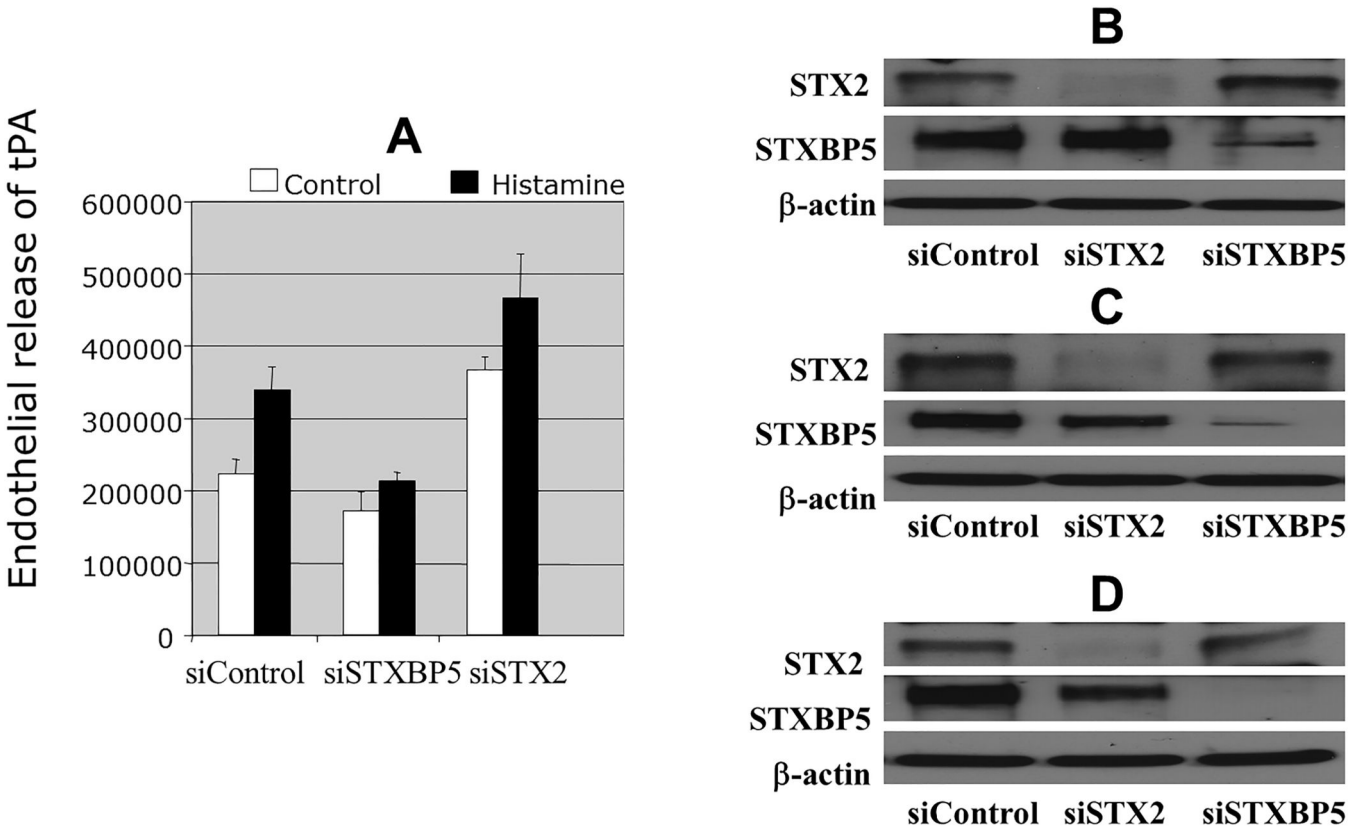

Figure 2. Effect of gene silencing on tPA release

Endothelial cells were transfected with oligonucleotides to silence *STXBP5* or *STX2*, and then treated with histamine to induce tPA release. Levels of tPA in the media were measured by ELISA. (A) Silencing *STXBP5* decreases tPA release, whereas silencing STX2 increases tPA release. (B–D) Silencing of *STX2* or *STXBP5* decreases target protein expression in human umbilical vein endothelial cells (HUVEC) (B), human aortic endothelial cells (HAEC) (C), and human dermal microvascular endothelial cells (HDMVEC) (D). Each panel includes a 3 by 3 matrix of western blot images for the 3 proteins (STX2, STXBP5, beta-actin) after 3 gene silencing approaches (siControl for control scrambled oligonucleotide, siSTX2 for siRNA directed against *STX2*, and siSTXBP5 for siRNA directed against *STXBP5*).

Table 1

Association results for four SNPs within the three significant loci with circulating levels of tPA antigen

SNP	Chr	Position	Gene	Effect Allele (freq)	β, se (ln-trans)	GM ratio* (95%CI)	*P*-value	P_{Het}**
rs9399599 (lead SNP)	6	147744992	*STXBP5* intron 26	T -> A (0.54)	0.032, 0.004	1.032 (1.024–1.041)	2.9×10^{-14}	0.61
rs2020921 (nsSNP)	8	42164122	*PLAT exon 5*	G -> A (0.95)	0.067, 0.012	1.069(1.045–1.095)	2.0×10^{-8}	0.15
rs3136739 (lead SNP)	8	42324237	*POLB* intron 3	A -> G (0.95)	0.063, 0.010	1.065 (1.043–1.087)	1.3×10^{-9}	0.33
rs7301826 (lead SNP)	12	129857054	*STX2* intron 7	C -> T (0.43)	0.027, 0.004	1.027 (1.018–1.036)	1.0×10^{-9}	0.49

* per-allele proportionate increase in geometric mean tPA

** *P* value for heterogeneity test

Table 2

eQTL for two loci with proximal gene expression in human cells and tissues.

Index SNP	Proxy eSNP† (tPA *P*, r^2)	eQTL Tissue	eQTL *P*	Strongest eSNP††	Strongest eSNP (eQTL *P*, tPA *P*, r^2†)
rs9399599 in *STXBP5*	**rs7739314** (3.1×10^{-12}, 0.97)	**CD4+ lymphocytes**	NA	rs694625	1.4×10^{-4}, 0.02, 0.17
		Lymphocytes	1.6×10^{-3}	rs620715	3.5×10^{-4}, 2.4×10^{-8}, 0.90
		Liver	0.03	rs1765028	7.8×10^{-6},4.4×10^{-6},0.44
rs7301826 in *STX2*	**rs10848205** (1.5×10^{-6}, 1.0)	**Monocytes**	3.8×10^{-274}	Same as proxy	
	rs10773819 (1.4×10^{-6} ,1.0)	**Lymphocytes**	7.5×10^{-92}	Same as proxy	
		Monocytes	1.5×10^{-80}	rs1106369	7.1×10^{-91},1.5×10^{-6},1.0
		PBMC	1.4×10^{-25}		
		Macrophage	1.8×10^{-24}	rs1106369	2.3×10^{-28}, 1.5×10^{-6},1.0
		CD4+ lymphocytes	2.9×10^{-20}	Same as proxy	
		Leukocytes	2.7×10^{-10}	Same as proxy	
		Liver	9.0×10^{-6}	Same as proxy	
		Mammary artery	4.9×10^{-3}	Same as proxy	
	rs2001483 (1.8×10^{-6}, 1.0)	**Liver**	2.7×10^{-5}	Same as proxy	

All sentinel SNPs and their proxies were searched against eQTL sources as described in the online detailed materials and methods.

† Proxy SNP is the measured SNP in highest LD with the index SNPLD measured in correlation R-square, based on HapMap2 CEU data.

†† Strongest eSNP is the one with the best eQTL *P*-value, for the same tissue and transcript as for the lead proxy eSNP.