Rare SNP rs12731181 in the miR-590-3p Target Site of the Prostaglandin F₂α Receptor Gene Confers Risk for Essential Hypertension in the Han Chinese Population

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Objective—To investigate whether rs12731181 (A→G) interrupted miR-590-3p-mediated suppression of the prostaglandin F₂α receptor (FP) and whether it is associated with essential hypertension in the Chinese population.

Approach and Results—We found that miR-590-3p regulates human FP gene expression by binding to its 3′-untranslated region. rs12731181 (A→G) altered the binding affinity between miR-590-3p and its FP 3′-untranslated region target, thus reducing the suppression of FP expression, which, in turn, enhanced FP receptor-mediated contractility of vascular smooth muscle cells. Overexpression of FP augmented vascular tone and elevated blood pressure in mice. An association study was performed to analyze the relationship between the FP gene and essential hypertension in the Han Chinese population. The results indicated that the rs12731181 G allele was associated with susceptibility to essential hypertension. Carriers of the AG genotype exhibited significantly higher blood pressure than those of the AA genotype. FP gene expression was significantly higher in human peripheral leukocytes from individuals with the AG genotype than that in leukocytes from individuals with the AA genotype.

Conclusions—rs12731181 in the seed region of the miR-590-3p target site is associated with increased risk of essential hypertension and represents a new paradigm for FP involvement in blood pressure regulation. (Arterioscler Thromb Vasc Biol. 2015;35:1687-1695. DOI: 10.1161/ATVBHA.115.305445.)

Key Words: hypertension, essential ▪ microRNAs ▪ polymorphism, single nucleotide ▪ prostaglandin F₂α receptor

Prostanoids are biologically active derivatives of arachidonic acid that is released from membrane phospholipids and then converted through cyclooxygenase metabolism. These lipid mediators play an important role in blood pressure (BP) homeostasis. Both traditional nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 selective nonsteroidal anti-inflammatory drugs can elevate BP in patients.¹ Vasodilator prostanoids, such as prostaglandin (PG) I₂ and PGE₂, are essential for regulating renal blood flow and mediating salt/water reabsorption in the kidney.²,³ Deletion of the PGI₂ receptor or the PGE₂ receptor subtype 2 (EP2) results in salt-sensitive hypertension.⁴,⁵ However, inactivation of the PGE₂ receptor subtypes 1 and 3 (EP1 and EP3, respectively) reduces chronic angiotensin-II–driven hypertension and attenuates the angiotensin-II pressor response by reducing arterial contractility.⁶,⁷ The levels of PGF₂α, a vasoconstrictor, are augmented
in circulation under the conditions that increase cardiovascular risks, for example, after chronic high-salt intake, smoking, and hypercholesterolemia. The PGF\textsubscript{2\alpha} receptor (FP) is highly expressed in distal convoluted tubules and cortical collecting ducts in the kidney and mediates water absorption. FP is also present in resistance arterioles that are involved in regulating the vasopressor response. Thus, ablation of FP induces hypotension and retards atherosclerosis in low-density lipoprotein receptor–knockout mice.

MicroRNAs (miRNAs), \textasciitilde 22 single-stranded nucleotide RNA molecules, play an important gene-regulatory function mostly by binding to the 3′-untranslated region (3′ UTR) of target genes, which is implicated in a wide range of biological processes, including cell proliferation, differentiation, and apoptosis. Single-nucleotide polymorphisms (SNPs) are commonly recognized as the most frequent variation in the genome with a rate of approximate 1 of every 300 to 500 bp. SNPs in miRNA-target sites in the 3′ UTR of genes represent a specific class of functional polymorphisms and may lead to the dysregulation of post-transcriptional gene expression by disrupting regulatory miRNA binding. An increasing number of 3′ UTR SNPs in miRNA binding sites have been found to be associated with a variety of complex human diseases, such as hypertension, cancer, Tourette syndrome, asthma, and Parkinson disease. Recently, a European genome-wide association study reported that the SNP rs12731181, located in the FP 3′ UTR, may be associated with increased risk for hypertension in individuals from southern Germany (\textit{P}=2.5\times10^{-4}), but this association was not replicated in other European populations. Thus, ablation of FP induces hypotension and retards atherosclerosis in low-density lipoprotein receptor–knockout mice.

We investigated an miR-590-3p–binding site in the 3′ UTR of the FP gene and examined the functional consequence of the rs12731181 (A→G) within this binding region. The G variant of rs12731181 affected miR-590-3p–binding affinity to the 3′ UTR of the FP gene, which in turn disrupted the post-transcriptional regulation of the FP gene and subsequently resulted in enhanced contractility of vascular smooth muscle cells (VSMCs). We also showed that the G allele at rs12731181 was associated with increased risk for essential hypertension in the Han Chinese population. These findings suggested that the rare SNP rs12731181 was a functional SNP that increased the risk of essential hypertension by upregulating FP gene expression.

Material and Methods

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

Results

SNP rs12731181 Is Located in miR-590-3p–Binding Site at the 3′ UTR of the FP Gene

Using miRNA-target prediction tools (PicTar, miRanda, and Target Scan), we discovered that the FP 3′ UTR harbored an miR-590-3p–binding site and that the SNP rs12731181 was located in the seed region of the miR-590-3p–binding site (Figure 1A). To test whether rs12731181 in the FP 3′ UTR interfered with the binding affinity of miR-590-3p to the FP gene, we used a thermodynamic model to calculate the different energy parameters and constructed the corresponding binding energy diagrams for both 3′ UTR-A (A allele) and 3′ UTR-G (G allele). As shown in Figure 1B, the dissociated target (E\textsubscript{diss}) of the A allele was greater than that of the G allele (-5.2 kcal/mol versus -6.9 kcal/mol), and the energy required for activation (\Delta E\textsubscript{a}) of the A allele was much less than that for the G allele (4.8 kcal/mol versus 6.5 kcal/mol). These results suggested that the A allele was more accessible to miR-590-3p than the G allele variant. The binding energy of the A allele (\Delta E\textsubscript{a}=−0.36 kcal/mol) was much less than that of the G allele (\Delta E\textsubscript{a}=−0.18 kcal/mol), implying that miR-590-3p had a higher binding affinity for the A allele. Structure-based energy analysis revealed a tight interaction of miR-590-3p with the A allele but not the G allele (Figure I in the online-only Data Supplement). To further validate the computational modeling, we inserted a 327-bp FP 3′ UTR sequence containing either the A or the G allele into the 3′ terminal of the Renilla luciferase gene in the PRL-TK vector (Figure IIA and IIB in the online-only Data Supplement). In accordance with the computational predictions, we observed that the miR-590-3p mimic markedly suppressed Renilla luciferase activity in both HEK293T and HeLa cells in a concentration-dependent manner (Figure IIC and IID in the online-only Data Supplement) when the construct contained an A allele in the FP 3′ UTR (Figure 1C). In addition, specific miR-590-3p inhibitor could abolish the suppressive effects of miR-590-3p on the luciferase activity regulated by the A allele of the FP 3′ UTR (Figure IIE in the online-only Data Supplement). However, we did not observe suppression of luciferase activity in constructs with the G allele in the FP 3′ UTR when miR-590-3p mimic was added (Figure 1C). We also failed to observe any regulatory effects for other candidate miRNAs (ie, miR-581, miR592, and miR-599; data not shown), which exhibited complementary sequences near the SNP rs12731181 at the FP 3′ UTR.

G Variant in the FP 3′ UTR Disrupts miR-590-3p–Mediated Transcriptional Repression of the FP Gene

The miR-590-3p mimic was found to markedly reduce endogenous FP expression in HEK293T cells with the AA genotype (Figure 1D). To further distinguish between the effects on the A and G alleles on miR-590-3p inhibitory function, 3 different FP overexpression vectors pc-FP (without 3′ UTR), pc-FP-3′ UTR(A), and pc-FP-3′ UTR(G) (Figure IIIA in the
online-only Data Supplement) were cotransfected with the miR-590-3p mimic into HEK293T cells. FP overexpression was confirmed by Western blot (Figure IIIB in the online-only Data Supplement). As shown in Figure 1E, FP-specific siRNA significantly reduced FP gene expression in all groups by targeting FP mRNA. However, the miR-590-3p mimic had little effect on FP gene expression in pc-FP transfected cells because of lack of the binding fragment (FP 3′UTR).

Consistent with our luciferase reporter assay, the miR-590-3p mimic dramatically suppressed FP gene overexpression because of pc-FP-3′UTR(A) transfection (>60% suppression) in a concentration-dependent manner (Figure IIIC in the online-only Data Supplement). Notably, the miR-590-3p mimic did not affect FP expression after pc-FP-3′UTR(G) transfection, thus indicating that rs12731181 (A→G) could perturb transcripational repression of the FP gene by disrupting miR-590-3p binding.

VSMCs With the G Genotype in the FP 3′UTR Exhibits Enhanced Cell Traction Force

miR-590-3p was expressed in cultured T/G HA-VSMCs (Figure IVA in the online-only Data Supplement) at levels comparable with those of several known functional miRNAs (Figure IVB in the online-only Data Supplement) in the vasculature.21–26 We then examined the potential miR-590-3p–mediated transcriptional regulation of the FP gene in T/G HA-VSMCs (AA genotype). Indeed, transfection with the miR-590-3p mimic markedly suppressed endogenous FP expression (Figure 2A and 2B), whereas inhibition of miR-590-3p significantly elevated FP expression in T/G HA-VSMCs in a concentration-dependent manner (Figure 2C and 2D). However, transfection with the miR-590-3p mimic did not affect the differentiation (Figure VA and VB in the online-only Data Supplement), cell cycle distribution (Figure VC in the online-only Data Supplement), or proliferation (FigureVD in the online-only Data Supplement) of T/G HA-VSMCs.
The FP receptor in VSMCs mediates PGF$_{2\alpha}$-induced vasoconstriction in vessels. To determine whether T/G HA-VSMCs with the G genotype in the FP 3′ UTR possessed higher cell contractile force by following manipulation of miR-590-3p function, cell traction force was measured in T/G HA-VSMCs transfected with miR-590-3p mimic at the indicated concentrations. *P<0.05 and **P<0.01 vs scramble miRNA mimic (miR-control); n=3. Western blot analysis of endogenous FP in T/G HA-VSMCs transfected with miR-590-3p mimic at the indicated concentrations. *P<0.05 and **P<0.01 vs miR-con; n=3. Real-time PCR analysis of relative FP mRNA expression in T/G HA-VSMCs transfected with the miR-590-3p inhibitor at the indicated concentrations. *P<0.05 and **P<0.01 vs scramble miRNA inhibitor (miR inhibitor control); n=3. Western blot analysis of endogenous FP in T/G HA-VSMCs cotransfected with pc-FP, pc-FP-3′ UTR(A), or pc-FP-3′ UTR(G) constructs and miR-590-3p or miR-con. *P<0.05 vs miR-con; n=3. Real-time PCR analysis of relative FP mRNA expression in T/G HA-VSMCs cotransfected with pc-FP, pc-FP-3′ UTR(A), or pc-FP-3′ UTR(G) constructs and miR-590-3p or miR-con. Green fluorescent protein fluorescence indicates successful transfections. Cell traction force (CTF) was quantified by measuring bead displacements according to the elasticity theory and finite element method. *P<0.05 vs miR-con; n=20 to 35.
Strains exhibiting global hFP overexpression (hFP<sup>Tg-A</sup> and hFP<sup>Tg-E</sup>) were obtained by crossing hFP<sup>Tg-stop</sup> to Ella Cre mice (Figure 3B–3D).<sup>28</sup> As shown in Figure 3E and 3F and Figure VII in the online-only Data Supplement, both aortas and mesenteric resistance arteries from hFP<sup>Tg-A</sup> and hFP<sup>Tg-E</sup> mice exhibited significantly greater contractile activity in response to PGF<sub>2α</sub> and phenylephrine when compared with those from nontransgenic mice. Moreover, hFP<sup>Tg-A</sup> and hFP<sup>Tg-E</sup> mice had significantly higher systolic BP (SBP) and diastolic BP (DBP) values than nontransgenic mice (Figure 3G). However, we did not observe significant differences of heart rates between hFP transgenic and nontransgenic mice (Figure 3H). Thus, overexpression of hFP may elevate BP by enhancing vascular contractility in mice.

**Rare SNP rs12731181 Is Associated With Increased Risk of Essential Hypertension in the Han Chinese Population**

To determine the effects of genetic variants of the FP gene on susceptibility to essential hypertension susceptibility, 6 SNPs (rs2352043, rs3753380, rs3766355, rs41292960, rs35425451, and rs12731181) were selected for analysis using the

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**Figure 3.** Overexpression of the FP receptor augments vascular tone and elevates blood pressure (BP) in mice. A, The Cre-loxP strategy for generation of human FP (hFP) transgenic (Tg) mice. B, Polymerase chain reaction genotyping of tail biopsies of offspring from the mating of hFP<sup>Tg-stop</sup> with Ella Cre mice. Number 2 indicates the hFP<sup>Tg-positive</sup> strain (mCherry excision). C and D, Western blot analysis of FP expression in different tissues from hFP<sup>Tg-A</sup> and hFP<sup>Tg-E</sup> mice. E, PGF<sub>2α</sub> dose–response curve of endothelium-containing aortic rings from hFP<sup>Tg-A</sup>, hFP<sup>Tg-E</sup>, and nontransgenic (NTG) mice. **P<0.01 (hFP<sup>Tg-A</sup> vs NTG); ##P<0.01 (hFP<sup>Tg-E</sup> vs NTG); n=7 to 8. F, Phenylephrine (PE) dose–response curve in endothelium-containing aortic rings from hFP<sup>Tg-A</sup>, hFP<sup>Tg-E</sup>, and NTG mice. *P<0.05 (hFP<sup>Tg-A</sup> vs NTG); #P<0.05 (hFP<sup>Tg-E</sup> vs NTG); n=6 to 8. Systolic BP (SBP) and diastolic BP (DBP; G) and heart rates (H) in NTG, hFP<sup>Tg-A</sup>, and hFP<sup>Tg-E</sup> mice measured by the tail-cuff method. *P<0.05 (hFP<sup>Tg-A</sup> vs NTG); #P<0.05 and ##P<0.01 (hFP<sup>Tg-E</sup> vs NTG); n=8.
Haplovie version 3.32 software program (Figure 4A). For our association study, we enrolled 3406 participants, including patients with 1779 essential hypertension and 1627 normotensive controls. We found that patients with essential hypertension exhibited higher SBP, DBP, body mass index, blood glucose, total cholesterol, total triglycerides, and a higher rate of smoking and drinking than normotensive controls (Table I in the online-only Data Supplement). The rs41292960 and rs35425451 SNPs were not detected in the Han Chinese population. Notably, the minor allele frequency of rs12731181 was 0.017 in normotensive participants; the homozygous GG genotype at rs12731181 was not identified in this study. All of the selected SNPs passed the Hardy–Weinberg equilibrium test in the normotensive population (P>0.05). Logistic regression analysis of these candidate SNPs using different models revealed that rs12731181 was statistically associated with susceptibility to essential hypertension (adjusted odds ratio=1.91 for the AG genotype; 95% confidence interval, 1.33–2.74; P=0.0004, compared with the AA genotype). Carriers of the AG genotype had significantly higher SBP and DBP than those with the AA genotype (BP: β [SE], 7.79 [2.02], P<0.0001; DBP: β [SE], 3.25 [1.32], P=0.014; Table). Sex strategic analysis also indicated that rs12731181 was significantly associated with essential hypertension and increased SBP in both men and women (Table II in the online-only Data Supplement). In addition, we observed a significant interaction for SBP between smoking and rs12731181 (P=0.027; Table III in the online-only Data Supplement). Peripheral leukocytes, as readily accessible specimen from patients, were prepared for further gene expression analysis. Interestingly, subjects that carried the risk allele G at rs12731181 had significantly higher levels of FP mRNA and protein expression in peripheral leukocytes than did carriers with the AA genotype (Figure 4B–4D). Furthermore, a positive correlation was observed between the FP mRNA level and SBP/DBP in the Han Chinese AA carriers tested (P<0.05; Figure 5A and 5B), and a significant negative correlation was found between miR-590-3p level and FP mRNA level in AA carriers (Figure 5C). These results suggested that the miR-590-3p–mediated regulation of FP gene expression was involved in BP homeostasis. We also examined relative expression of miR-590-3p in both European whites and Chinese Hans. Interestingly, we found

Table. Possible Association Between FP SNPs and Essential Hypertension in the Study

<table>
<thead>
<tr>
<th>SNPs (Region)</th>
<th>Minor Risk Allele</th>
<th>MAF (Normotensive/Hypertensive)</th>
<th>Hardy–Weinberg Equilibrium (Normotensive)</th>
<th>Essential Hypertension</th>
<th>SBP, mmHg</th>
<th>DBP, mmHg</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
<td>Dom</td>
<td>PValue</td>
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<tr>
<td>rs2352043 (5'UTR)</td>
<td>G</td>
<td>0.326/0.326</td>
<td>0.535</td>
<td>0.98 (0.88–1.10)</td>
<td>0.92 (0.79–1.07)</td>
<td>−0.29 (0.64)</td>
</tr>
<tr>
<td>rs3753380 (5'UTR)</td>
<td>T</td>
<td>0.251/0.249</td>
<td>0.211</td>
<td>0.95 (0.84–1.08)</td>
<td>0.93 (0.79–1.08)</td>
<td>−0.70 (0.68)</td>
</tr>
<tr>
<td>rs3766355 (intron 1)</td>
<td>C</td>
<td>0.452/0.446</td>
<td>0.880</td>
<td>0.97 (0.87–1.08)</td>
<td>0.91 (0.77–1.07)</td>
<td>−0.66 (0.60)</td>
</tr>
<tr>
<td>rs12731181 (3'UTR)</td>
<td>G</td>
<td>0.017/0.028</td>
<td>0.404</td>
<td>1.91 (1.33–2.74)</td>
<td>...</td>
<td>7.79 (2.02)</td>
</tr>
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</table>

The OR and P-values were adjusted for age, sex, body mass index, total cholesterol, triglycerides, blood glucose, smokers and drinkers. Add indicates additive genetic model; CI, confidence interval; DBP, diastolic blood pressure; Dom, dominant genetic models; MAF, minor allele frequency; OR, odds ratio; SBP, systolic blood pressure; and SNP, single-nucleotide polymorphism.
strikingly lower expression of miR-590-3p (≈146-fold; Figure VIIIA in the online-only Data Supplement) and FP mRNA (4–7-fold; Figure VIIIB in the online-only Data Supplement) in peripheral blood leukocytes from both normotensive and hypertensive whites compared with those from Han Chinese individuals.

**Discussion**

Hypertension is a complex disease that affects >25% of the adult population. In this study, we identified and validated a functional SNP, rs12731181, in the 3′UTR of the FP gene whose minor G allele was associated with a higher risk for essential hypertension. We also showed that the G risk allele for rs12731181 disrupted the binding of miR-590-3p, enhanced translation of the FP gene, and augmented FP-mediated contractility in VSMCs. Therefore, we propose that rs12731181 modulates the epigenetic regulation (miRNA binding) of a vasoconstriction mediator, the FP receptor, through miR-590-3p.

miRNAs are known to regulate gene expression by binding to the 3′UTR of the target gene. As few as 7 bp of a complementary sequence to the miRNA 5′ end (designated as the seed sequence) is sufficient for functional miRNA–mRNA interactions. Effective binding between an miRNA and its mRNA-target is essential for miRNA-mediated cleavage or translational repression of target genes, and SNPs within these miRNA-target sites have the potential to affect miRNA binding. The 3′UTR of the FP gene, which harbors an A allele, exhibits a perfect 7-base pairing with the 5′ end of miR-590-3p, whereas the rs12731181 G allele produces a G:U wobble pairing that disrupts miRNA-mediated repression of gene translation. Computational analysis revealed that the presence of a G allele in the FP gene diminished the binding affinity of miR-590-3p to the target RNA. Thus, rs12731181 variants could interrupt miR-590-3p binding and enhances FP gene expression, as determined by a reporter assay.

In response to shear stress, vascular endothelial cells secrete large quantities of PGF$_2\alpha$, a vasoconstrictor, that plays an important role in the maintenance of vascular tone by interacting with its receptor in the medial layer of resistance arteries. Deletion of FP markedly reduces BP in mice on a regular chow, high-fat, or high-salt diet. We confirmed that FP was a target gene for miR-590-3p and that the rs12731181 G allele significantly reduced the inhibitory effect of miR-590-3p on FP gene expression when compared with the A allele, thus resulting in increased transcription of the FP gene in VSMCs. We also found that VSMCs with the G genotype exhibited greater contractility in response to PGF$_2\alpha$ stimulation than those with the A genotype. Therefore, the G allele at rs12731181 may facilitate PGF$_2\alpha$-mediated vasoconstriction by increasing FP expression. Indeed, forced expression of the hFP in mice was associated with elevated BP concurrently with augmented arterial contraction. FP receptor mediates water reabsorption in collecting ducts and rennin secretion in juxtaglomerular apparatus in kidneys. Because we were unable to obtain live colonies characterized by VSMC-specific overexpression of hFP (by crossing hFPTg-stop with SM22Cre mice), we cannot rule out the possible contribution of some effects in renal function to elevated BP detected in hFP transgenic mice. Moreover, it would be interesting to verify BP regulatory function of miR-590-3p in vivo, such as in miR-590-3p transgenic or knockout mice. However, the sequence of the miR-590-3p-binding site at the FP 3′UTR (rs12731181 locus) is not present in rodents, despite highly conserved among primates, indicating that miR-590-3p cannot bind to the FP 3′UTR and regulate its expression in mice.

In our case–control study, we identified a strong association ($P=0.0004$) between rs12731181 and essential
hypertension in the Han Chinese population in both men and women. Moreover, carriers with the risk G allele exhibited elevated expression of FP in peripheral tissues and had significantly higher SBP and DBP than individuals with the AA genotype. We also observed a positive correlation between FP mRNA levels in circulating leukocytes and SBP/DBP in the Han Chinese population. These data seem consistent with our observations that the G allele had a greater effect on the SBP than DBP in both men and women (Table II in the online-only Data Supplement). Thus, the rs12731181 polymorphism may increase the expression of the FP gene, likely in resistance vessels, to elevate BP, particularly SBP. However, this association was not consistently observed in the European population. This inconsistency may be attributed to population stratification or genetic heterogeneity in ethnically diverse populations. Specifically, there is an obvious discrepancy in the minor allele frequency of rs12731181 between the European (CEU, 0.165–0.229) and the Han Chinese populations (CHB, 0.011–0.026) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12731181). Differences in allele frequencies at regulatory polymorphisms may account for some population differences in the prevalence of complex genetic diseases. Further, single regulatory mechanisms may contribute to the variations in expression levels between populations, suggesting that genetically determined differences in gene expression may enhance our understanding of the population differences observed in complex genetic diseases. Consistent with previous data on FP expression in human blood leukocytes (white, 5±0.7×10⁻⁴/GAPDH versus Han Chinese, 0.3±0.2×10⁻⁴/GAPDH), we also found strikingly lower expression of miRNA-590-3p and FP mRNA in peripheral blood leukocytes from both normotensive and hypertensive whites compared with those from Han Chinese individuals. Thus, the rs12731181 polymorphism in FP gene may have a more marked effect on Han Chinese individuals than European individuals.

In mice, FP disruption depresses renin–angiotensin–aldosterone system activity, suggesting that FP may modulate renin secretion by juxtaglomerular granular cells. We did not observe elevated plasma renin and angiotensin-I levels in subjects with the AG genotype when compared with those with the AA genotype. This indicates that rennin secretion, a complex process in vivo, is regulated by multiple factors, such as water/salt change and the sympathetic nervous system. In summary, rs12731181 impairs the regulatory binding of miRNA-590-3p to the FP gene, which leads to enhanced transcription of the FP gene and increased vasocoactivity. In addition, the rs12731181 G allele is a genetic risk variant of essential hypertension in the Han Chinese population.

Acknowledgments

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Disclosures

None.

References


**Significance**

In this study, we have found that the G risk allele for rs12731181 disrupts the binding of miR-590-3p on 3’UTR of FP gene, thus elevating FP mRNA expression and increasing FP-mediated contractility of vascular smooth muscle cells. Furthermore, we have identified and validated a functional single-nucleotide polymorphism rs12731181, whose minor G allele is associated with a higher risk for essential hypertension in the Han Chinese population. Our results suggest that rs12731181 polymorphism might be a novel predisposing genetic factor for essential hypertension in the Han Chinese population via modulation of FP expression.
Rare SNP rs12731181 in the miR-590-3p Target Site of the Prostaglandin F\textsubscript{2}\textalpha{} Receptor Gene Confers Risk for Essential Hypertension in the Han Chinese Population

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SUPPLEMENTAL MATERIAL

Rare SNP rs12731181 in the miR-590-3p target site of the prostaglandin F$_{2\alpha}$ receptor gene confers the risk for essential hypertension in the Han Chinese population

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**Supplemental Figures**

Figure I. Secondary structure-based energies at different stages of the binding process. Schematic of the binding energy diagram for A allele (Upper) and G allele (Lower).
Figure II. Effects of miR-590-3p on FP 3'UTR-mediated luciferase activities

(A) The FP 3'UTR fragment (containing rs12731181) in the PRL-TK vector. (B) Sequencing of the PRL-TK-3'UTR(A/G). Effects of miR-590-3p transfection on FP 3'UTR mediated luciferase activities in HEK293T (C) and HeLa cell lines (D). **P < 0.001 vs. miR-con (scramble miRNA mimic), n = 4. (E) Effects of miR-590-3p inhibitor on FP 3'UTR mediated luciferase activities in HeLa and HEK293T cell lines. **P < 0.001; n = 4.
Figure III. The rs12731181 G allele disrupted the suppressive effect of miR-590-3p on FP expression in vitro. (A) Schematic of FP overexpression constructs. pc-FP construct without the 3’UTR regulatory fragment at the 3’end of the FP cDNA; pc-FP-3’UTR(A/G), construct with the 3’UTR (either A or G variant) at the end of the FP cDNA. (B) Western blot analysis of FP protein in pc-FP, pc-FP-3’UTR(A), and pc-FP-3’UTR(G)-transfected HEK293T cells. (C) Western blot analysis of FP in HEK293T cells cotransfected with miR-590-3p mimic and pc-FP-3’UTR(A/G) at the indicated concentrations. miR-con, scramble miRNA mimic.
Figure IV. miR-590-3p expression in VSMCs.

(A) Candidate miRs (miR-590-3p, miR-581, miR-592 and miR-599) expression in T/G HA-VSMCs, HUVECs and human peripheral blood leukocytes. n =6-20. (B) Comparison of miR-590-3p expression with the expression of other known functional miRNAs in T/G HA-VSMCs. n = 4.
Figure V. Effects of miR-590-3p on VSMCs differentiation and proliferation.
(A) Effects of transfection with the miR-590-3p mimic on the expression of synthetic markers in T/G HA-VSMCs. n = 6. (B) Effects of transfection with the miR-590-3p mimic on the expression of contractile markers in T/G HA-VSMCs. n = 6. (C) Effects of transfection with miR-590-3p mimic on cell cycle progression in HA-VSMCs. n = 4. (D) Effects of transfection with miR-590-3p mimic on cell proliferation in HA-VSMCs. n = 4.
Figure VI. Effect of forced expression of FP receptor and miR-590-3p inhibitor on cell traction forces (CTFs) in T/G HA-VSMCs. (A) CTFs of the T/G HA-VSMCs transfected with control vector, pc-FP, pc-FP-3’UTR(A), or pc-FP-3’UTR(G) constructs. *P < 0.05 vs. vector, n = 20 - 35. (B) Effect of miR-590-3p inhibitor on CTFs in T/G HA-VSMCs. miR inhibitor control, scramble miRNA inhibitor, *P < 0.05 vs. miR-inhibitor control, n = 20 - 30.
Figure VII. PGF$_{2\alpha}$ dose-response curve of endothelium-containing mesenteric resistance arteries from hFPTg-A, hFPTg-E, and nontransgenic (NTG) mice. *$P < 0.05$ (hFPTg-A vs. NTG); †$P < 0.05$ (hFPTg-E vs. NTG); n = 5–6.
Figure VIII. Comparison of FP and miR-590-3p expression of peripheral leukocytes from Chinese Han with that from Caucasian.

(A) Quantitative real-time PCR analysis of the miR-590-3p expression of peripheral leukocytes from Chinese Han and Caucasian populations. *P < 0.01 vs. Chinese Han. (B) Quantitative real-time PCR analysis of the FP mRNA expression of peripheral leukocytes from Chinese Han and Caucasian populations. **P < 0.01, *P < 0.05 vs. Normotensive (same race), *P < 0.01 vs. Chinese Han.
## Supplemental Tables

Table I. Baseline characteristics of the subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normotensive (n = 1627)</th>
<th>Hypertensive (n = 1779)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44.8±13.5</td>
<td>46.3±10.6</td>
<td>0.0003</td>
</tr>
<tr>
<td>Gender, m/f</td>
<td>791/836</td>
<td>1030/749</td>
<td>7.06e-08</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
<td>23.1±2.7</td>
<td>25.1±3.1</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>111.9±10.3</td>
<td>157.5±18.6</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>69.2±6.3</td>
<td>98.0±12.7</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>GlU, mmol/L</td>
<td>5.0±1.0</td>
<td>5.5±1.3</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>4.7±0.9</td>
<td>4.8±0.9</td>
<td>1.68e-08</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.3±0.9</td>
<td>2.1±1.6</td>
<td>&lt; 2.2e-16</td>
</tr>
<tr>
<td>Smokers(n), %</td>
<td>272, 16.7%</td>
<td>467, 26.3%</td>
<td>1.36e-11</td>
</tr>
<tr>
<td>Drinkers(n), %</td>
<td>175, 10.6%</td>
<td>327, 18.4%</td>
<td>3.25e-10</td>
</tr>
</tbody>
</table>

Values are mean±standard deviation (SD). TC, total cholesterol; TG, triglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure; GLU, glucose.
Table II. Possible associations of rs12731181 with essential hypertension, systolic (SBP) and diastolic (DBP) blood pressure in male and female populations.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gender</th>
<th>Population</th>
<th>Genotype (n) (AA / AG)</th>
<th>Essential Hypertension OR (95% CI)</th>
<th>P</th>
<th>SBP (mm Hg) beta (SE)</th>
<th>P</th>
<th>DBP (mm Hg) beta (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12731181</td>
<td>Male</td>
<td>Normotensive</td>
<td>763 / 27</td>
<td>1.88 (1.12-3.17)</td>
<td>0.015*</td>
<td>7.21 (2.46)</td>
<td>0.003**</td>
<td>2.64 (1.68)</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypertensive</td>
<td>972 / 58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12731181</td>
<td>Female</td>
<td>Normotensive</td>
<td>807 / 29</td>
<td>1.89 (0.99-2.66)</td>
<td>0.016*</td>
<td>7.99 (3.21)</td>
<td>0.013*</td>
<td>3.66 (2.05)</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypertensive</td>
<td>705 / 41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval; SE, standard error. The OR and P-Values were adjusted for age, BMI, TC, TG, GLU, smokers and drinkers.
Table III. Interaction of rs12731181 polymorphism and lifestyle factors effect on hypertension, SBP and DBP risk.

<table>
<thead>
<tr>
<th>Lifestyle factors</th>
<th>Essential Hypertension</th>
<th>SBP (mm Hg)</th>
<th>DBP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA Normotensive / hypertensive OR (95% CI)</td>
<td>AG Normotensive / hypertensive OR (95% CI)</td>
<td>P beta (SE)</td>
</tr>
<tr>
<td>nonsmoker</td>
<td>1303/1235 Reference 51/74 1.55 (1.06 - 2.27) 0.167 11.88 (5.37) 0.027* 3.54 (3.45) 0.306</td>
<td></td>
<td></td>
</tr>
<tr>
<td>smoker</td>
<td>267/442 1.54 (1.26 - 1.88) 5 / 25 4.89 (1.82 - 13.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nondrinker</td>
<td>1400/1367 Reference 51/82 1.69 (1.17 - 2.45) 0.857 1.86 (6.10) 0.760 -1.11 (3.92) 0.777</td>
<td></td>
<td></td>
</tr>
<tr>
<td>drinker</td>
<td>170/310 1.64 (1.31 - 2.05) 5 / 17 3.06 (1.09 - 8.62)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The OR and P-Values were adjusted for age, gender, and BMI.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
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<tbody>
<tr>
<td>FP</td>
<td>ggaagtcggtggaacttgagg</td>
<td>gccaacactcaagtcatctc</td>
</tr>
<tr>
<td>β-actin</td>
<td>gacaggatgcagaaggaga</td>
<td>ccacatctgctggaaggtgg</td>
</tr>
<tr>
<td>GADPH</td>
<td>aaggtgaaggtcgagtcacac</td>
<td>ggggtcattgatggcaacaata</td>
</tr>
<tr>
<td>Myh11</td>
<td>ggtcaggttgggaagatga</td>
<td>ggccaggtgttttatagggggtt</td>
</tr>
<tr>
<td>Myocd</td>
<td>aagggcacaggtctctcg</td>
<td>catctgctgaactccgggttcatttg</td>
</tr>
<tr>
<td>SRF</td>
<td>tcaacctacaggtcggagtc</td>
<td>gtcttgtttgatgggtggaggt</td>
</tr>
<tr>
<td>KLF4</td>
<td>ggcgggtgatgggcaagtt</td>
<td>tgccgcagggctgccttttg</td>
</tr>
<tr>
<td>KLF5</td>
<td>tcgtcagactgacagtcagctg</td>
<td>gttgcacacccgcactgga</td>
</tr>
<tr>
<td>OPN</td>
<td>ggtgcatcaaggccatcccg</td>
<td>tccttccacggtgtcaca</td>
</tr>
</tbody>
</table>
Material and methods

Subjects
In this study, 1779 patients with essential hypertension and 1627 normotensive participants were recruited from the Hypertension Clinic and Physical Examination Departments of the Ruijing Hospital of Shanghai Jiaotong University and Shanghai Xuhui Central Hospital from March 2010 to June 2012. Twenty-one Caucasians with the AA genotype (12 patients with essential hypertension and 9 normotensive participants) were recruited from Catholic University of Rome, Italy. BP was measured for a total of three times at 10 min intervals, by trained and certified observers while the subjects were in a sitting position, according to standard protocol recommended by the American Heart Association.1 A fixed value of 10/5 mmHg was added to the observed systolic BP (SBP)/diastolic BP (DBP) for patients on antihypertensive treatment.2 Hypertension exhibited a systolic BP (SBP) ≥ 140 mmHg or a diastolic BP (DBP) ≥ 90 mmHg, or self-reported of taking an antihypertensive medication. The normotensive controls exhibited a SBP of <130 mm Hg and a DBP of <85 mm Hg, and had no previous diagnosis of hypertension or history of treatment with antihypertensive medication. None of the participants had a history of secondary hypertension, coronary heart disease, kidney failure, or drug abuse. Ethical approval was obtained from Shanghai Xuhui Central Hospital, the Ruijing Hospital of Shanghai Jiaotong University, Catholic University of Rome and the Institute for Nutritional Sciences, Chinese Academy of Sciences. All subjects provided written informed consent for the collection of blood sample and subsequent analysis.

Thermodynamic Model for Predicting miRNA-Target Interactions
To investigate the binding affinity of miR-590-3p to the target sites that harbor the rs12731181 (A/G) SNP variants, we analyzed the energies of the secondary structures of each molecule involved in the binding process using a parameter-free thermodynamic model.3, 4 Since rs12731181 is located in the seed region of the miR-590-3p targeted site, the seed site, and the flanking 30 bp region (total of 60 bp) were used to calculate the energies. $E_t$ is the local energy of the dissociated target without interaction with miRNA, $E_i$ is the local energy of the transition state when we force the binding site to be unpaired, and $E_c$ is the local energy of the miRNA and target mRNA target dimer. $E_t$ was computed using RNAFold (Vienna RNA Package), which identified the secondary structure of the target with the lowest free energy based on its sequence. To compute $E_i$ and $E_c$, we assumed that the miRNA-target binding process only affected the local structure near the proximity of the binding site.5 We used RNAcoFold to calculate the structure-associated energies from the connectivity matrix representing the secondary structure of either the intermediate or the complex product constructed. $\Delta E_{(a)}$ indicates the difference between $E_i$ and $E_t$, and $\Delta E_{(b)}$ indicates the difference between $E_i$ and $E_c$.

Quantification of Cell Traction Force (CTF)
Fluorescent microbeads-embedded elastic gels (Young's modulus, 3 kPa) were prepared using 40% acrylamide and 2% methylenebisacrylamide solutions as
The gels were treated with sulfo-SANPAH (0.5 mg/mL, Pierce) and activated by 365 nm UV for 10 min, then immediately incubated with 100 μg/mL type I collagen (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C overnight for cell culture. Human aortic smooth muscle cells (T/G HA-VSMCs, ATCC, CRL-1999) were co-transfected with an miR-590-3p mimic, FP overexpression vector and green fluorescence protein (GFP) expression vector. The presence of green fluorescence in the cells indicated successful transfection. The transfected T/G HA-VSMCs were digested after 48 h and re-seeded on the gels for CTF measurement by routine computational approaches.

**Generation of Human FP (hFP) Transgenic Mice**

The hFP^{Tg-Stop} vector was constructed according to previously described methods. A specially designed stop sequence (loxP-mcherry-SV40PA-loxP) that expresses the red fluorescent mCherry protein and prevents FP gene expression, was inserted between a CMV early enhancer/chicken β-actin (CAG) promoter and the human FP coding sequence. By crossing the hFP^{Tg-Stop} mouse strains with Cre transgenic strains, the Cre recombinase recognized and recombined the loxP sites of the hFP^{Tg-Stop} transgene, thereby removing the stop sequence and activating the FP gene (Figure 3A). The genotypes of all offspring were analyzed by polymerase chain reaction (PCR), and the PCR products were further confirmed by sequencing. For PCR analysis, mouse tail DNA (1 μg) was amplified. The PCR was performed by denaturation at 94 °C for 3 min; followed by 35 cycles each of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min. The sense primer for EIIaCre was 5′-attgcctgcaattaccggtc-3′ and the anti-sense primer was 5′-atcaacgttttcttttcgg-3′. The sense and anti-sense primers for the hFP^{Tg-Stop} transgene were 5′-acctccccctgaacctgaaa-3′ and 5′-cgatggcaaggctgtttgac-3′, respectively. The hFP^{Tg-Stop} mice were maintained by intercrossing to wild-type C57B/L mice and subjected to further PCR screening of their offspring. No abnormalities were observed among founders or their offspring.

**Isometric Tension Measurements**

The isometric tension that developed in mouse arterial rings was measured as described previously. Briefly, mouse thoracic aorta were transferred immediately to Kreb’s buffer, cleaned of periadventitial tissues while keeping the endothelium intact, and cut transversely into ring segments (3.0 mm in length) which were attached to a force transducer. Organ bath chambers were filled with Kreb’s solution, which was warmed to 37 °C and gassed with 95% O2/5% CO2. Rings of the thoracic aorta were allowed to equilibrate for 90 min at a resting tension of 3 mN, while mesenteric artery rings were allowed to equilibrate for 90 min at a resting tension of 1mN. After 90 min, the rings was treated with KCl (60 mM) and then washed with fresh Kreb’s solution to allow spontaneous contractions to develop. After the isometric tension had balanced, different doses of PGF_{2α}(Cayman) and phenylephrine (Sigma-Aldrich, St. Louis, MO, USA) were added in a cumulative manner. Studies were carried out using an eight-chamber Radnoti tissue organ bath system (ADInstruments ltd., Chalgrove, Oxfordshire, UK), and data were recorded using the Powerlab software Chart (version 7.2).
BP Determination
Tail-cuff BP was determined in mice between 8 and 10 weeks of age in a blinded manner using a computerized tail-cuff system (Visitech Systems, Cary, NC, USA) as previously described.10

Genotyping
Genomic DNA was extracted from the peripheral blood leukocytes of participants by using the proteinase K method. Genotyping was performed using the TaqMan SNP allelic discrimination method and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. The laboratory personnel performing the genotyping analyses were blinded to the subjects’ case-control status.

Vector Construction and Site-Directed Mutagenesis
A 327 bp fragment containing the rs12731181 locus in the 3’UTR of the human FP gene was amplified by PCR of cDNA from human peripheral blood leukocytes using the following primers (sense, 5’-catgtctagacttaataggacagtaaatct-3’; antisense, 5’-gaatgcggccgcatgcaaataggccaagtaaagg-3’). The PCR product was then subcloned into the PRL-TK vector (Promega, Madison, WI, USA) at the XbaI and NotI sites downstream of the Renilla luciferase open reading frame (ORF). The completed construct was termed PRL-TK-3’UTR(A). The QuickChange site-directed mutagenesis kit (Stratagene) was utilized to generate an A→G mutation at the rs12731181 site (termed as PRL-TK-3’UTR(G)) using the primer pair 5’-aatgggaggtagacacaatgaaataatgccatgggagtc-3’, and 5’-gactcccatgcatctattcgctctccctatt-3’. To investigate the effect of a G mutation at the rs12731181 site in the human FP gene in vitro, the human FP ORF was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and the 327 bp fragment of the FP 3’UTR containing either an A or G allele at the rs12731181 locus was inserted between XbaI and ApaI sites downstream of the human FP gene. All constructs were confirmed by sequencing.

miRNA mimic and Anti-miRNA inhibitor Transfection
The miR-590-3p mimic and miR-590-3p inhibitor were ordered from GenePharma Company (Shanghai, China). miRNA inhibitors are chemically-modified and optimized complementary single stranded nucleic acids designed to specifically target the miRNA and knockdown individual miRNA molecules.11 miRNA mimics were transfected into HEK239T cells, HeLa cells, and T/G HA-VSMCs for luciferase report assays and western blot assays previously described.12,13

Luciferase Reporter Assay
To perform luciferase reporter assays, HEK293T and HeLa cells were seeded in 48-well culture plates for 24 h and then transfected with firefly or Renilla constructs. Transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. All miRNAs mimics, scrambled RNAs, anti-miR590-3p inhibitors, and anti-miRNA inhibitors controls (GenePharma) were cotransfected with PRL-TK-3’UTR(A) or PRL-TK-3’UTR(G).
PGL3-Control was used as an internal control. Firefly and Renilla luciferase activities were measured at 24 h after transfection using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control (miR-con). Experiments were independently repeated three times.

**Western Blot**

Total cell protein was extracted using lysis buffer containing protease inhibitors. Protein concentrations were determined by BCA quantification using a BCA protein assay kit (Thermo). Protein samples were subjected to 10% SDS-PAGE and then transferred to a PVDF membranes (Millipore). Rabbit monoclonal anti-human FP receptor antibody (Epitomics) was used at a 1:1000 dilution. Equivalent protein loading was evaluated with an anti-β-actin antibody (1:2000 dilution; Sigma). Specific secondary antibodies labeled with horseradish peroxidase(HRP; 15000 dilution; Sigma) and an ECL detection kit (Thermo) was used to detect protein signals. The protein signals were quantified using image J 1.44p and all experiments were independently repeated three times.

**RNA Extraction and Real-Time PCR**

Total RNA was isolated from HEK293T cells or leukocytes using TRIzol (Invitrogen). Total mRNA was reverse-transcribed into cDNA by using a primer mix (oligodT/random primers) and Superscript reverse transcriptase (Takara, Shiga, Japan) according to the manufacturer’s instructions. Real-time PCR was performed in triplicate for each sample, in a 20 μL reaction volume with SYBR Green universal PCR mix (Takara). To correct for sample variation, an endogenous control, (GADPH) was amplified with the target and served as an internal reference for data normalization. The primers sequence used for real-time PCR are included in Supplementary Table IV. For the comparison of mRNA expression levels in tissues/cells from different groups (e.g. different genotypes), we normalized the expression level of the specific gene in control group (e.g. AA population) to equal 1, the relative expression levels of the gene in other groups (e.g. AG population) were presented to avoid potential variations between batches, as previously described.14

**Measurement of miRNA Levels by Real-Time PCR**

miRNA expression levels in cells and tissues were determined by stem-loop real-time PCR with primers specific for candidate miRNA. Total RNA extraction and real-time PCR were performed using the miScript Reverse Transcription Kit (GenePharma), according to the manufacture’s protocol. Endogenous small non-coding mRNA (U6) was used as an internal control. Each sample was run in triplicate, and the experiments were repeated three times. The expression levels of mature candidate miRNA relative to U6 were determined using the $2^{-\Delta\Delta Ct}$ method.

**Statistical Methods**

Comparisons between two groups were analyzed by a Student’s t-test or the $\chi^2$ test, as appropriate. The allele and genotype frequencies were determined by direct counting. The Hardy-Weinberg equilibrium test was performed for the genotype distribution in
normotensive participants. Multiple logistic regression analysis was used to detect the associations between genotypes and hypertension risk. Associations between the genotypes and clinical characteristics were assessed with a multivariate linear regression model. The Pearson’s correlation test was used to analyze the relationships between the FP mRNA levels of subjects and BP. Power calculations were performed using QUANTO power calculator (v1.2.4). All computations were performed using the R package (version 2.13.2) or GraphPad Prism software (version 5.0).

Reference


6. Tse JR, Engler AJ. Preparation of hydrogel substrates with tunable mechanical properties. Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]. 2010;Chapter 10:Unit 10 16


