

HSP70 Is Associated with Endothelial Activation in Placental Vascular Diseases

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Endothelial cell injury and activation in the placenta are features of placental vascular disease (PVD). While advances in PVD have been made, the pathogenesis of this disease is still unknown. The objective of this study was to pursue potential risk factors and signal transcription pathways involved in PVD pathogenesis. Gene expression in subjects with PVD and with normal pregnancies was compared using a two-channel microarray technique. Higher expression of HSPA6 and HSPA1A was exhibited in PVD subjects. HSPA6 and HSPA1A both encode HSP70, and, therefore, we localized HSP70 expression in placental tissue. Using quantitative polymerase chain reaction (PCR) and Western blot, we observed a significant upregulation of HSP70 in both mRNA and protein levels in placental tissue and microvascular endothelial cells of PVD subjects when compared with normal pregnancies ($P < 0.05$). HSP70 mRNA and protein expression also correlated negatively with infant birth weight ($P < 0.05$). HSP70 was expressed mainly in endothelial cells and smooth muscle cells in the placental microvessels. We therefore conclude that HSP70 may mediate endothelial activation and play a role in pathogenesis of PVD.

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INTRODUCTION

The placenta plays a pivotal role in the acceptance of the fetal-placental unit by the maternal immune system. Placental vascular disease (PVD) induces complications in human pregnancy such as preeclampsia (PE) and fetal intrauterine growth restriction (IUGR) (1). PVD may be identified antenatally by umbilical artery Doppler flow velocity waveforms (2–3). Recent studies have provided direct evidence for endothelial cell activation of placental villi and a proinflammatory cytokine response in PVD (4), however, the underlying mechanisms of pathogenesis in this vascular disease have yet to be defined.

Heat shock proteins (HSPs) are highly conserved and are found in all cell types. They may be expressed as a result

of temperature increase or of stressful environmental, pathological, or physiological stimuli (5). HSPs contribute to protein folding and guard cells against stressful insults (6,7). The HSP genes A6 and A1A both encode the protein HSP70. This frequently studied member of the HSP family has been detected in placental tissue, however, no expression level difference was observed between preterm and term pregnancies (8,9). Higher levels of HSP70 have been found in the peripheral circulation of patients with severe preeclampsia as well as in patients with peripheral and renal vascular disease (10,11).

Oxidative stress is a feature associated with PVD that occurs in the placenta. Stress induces endothelial cells to release nitric oxide leading to increased HSP70

expression (12). A previous study has suggested that increased levels of HSP70 may protect cells and tissues from ensuing noxious conditions such as hypoxia and ischemia-reperfusion (13), however, HSP70 also may bind to specific cells of the immune system with high affinity and avidity. Binding occurs through receptors TLR2 and TLR4 and is dependent on the cofactor CD14 (14,15). Following binding, rapid intracellular Ca^{2+} flux activates NF- κ B, upregulating expression of proinflammatory cytokines (16). Studies indicate that microvascular endothelium in the placental vasculature may produce the proinflammatory cytokines during PVD (1). This is supported by evidence of TLR4 mRNA upregulation in placental villi endothelium in PVD (17). Additionally, increased expression of intercellular adhesion molecule-1 by microvascular endothelial cells also is associated with PVD, indicating injury and activation of microvascular endothelial cells (18). The present study was carried out to investigate the relationship of endothelial activation, immune response, and HSP70 expression in PVD.

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MATERIALS AND METHODS

Patients

Placentas were collected upon delivery and immediately prepared from pregnant women with abnormal high-resistance umbilical artery blood flows ($n = 28$) and from women with normal pregnancies ($n = 34$). Subjects were identified by abnormal umbilical artery Doppler study (systolic/diastolic ratio greater than 3) 1 to 4 d prior to delivery (in the third trimester). Of the 28 PVD subjects, 18 were diagnosed with preeclampsia (PE), 4 with fetal intrauterine growth restriction (IUGR), and 6 with both PE and IUGR. PE is the development of hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg), in a woman whose blood pressure was previously normal after the twentieth wk of pregnancy, accompanied by proteinuria (> 300 mg/24 h). IUGR is a condition in which newborn birth weight is below the tenth percentile for a given gestational age. All PVD pregnancies were delivered by elective cesarean section. All normal pregnancies were uncomplicated, with no identifiable medical or obstetric diseases, and were delivered by elective cesarean section at term (for reasons not associated with fetal compromise). The protocol was approved by the Shandong Provincial Hospital Ethics Committee and consent forms were signed by all subjects in this study.

Human Genome Oligo Array (22 K)

Human genome oligo array (22 K) was designed by CapitalBio Corporation (Beijing, China). CapitalBio 22 K Human Genome Oligo Array comprises 21,522 70-mer oligo probes, each representing one transcript of the human genome (19). Total RNA was extracted from placental tissue using Trizol (Invitrogen, Carlsbad, CA, USA). RNA was purified using the NucleoSpin RNA clean-up kit (Macherey-Nagel, Düren, Germany). RNA optical density at 260 nm/280 nm was consistently > 1.8 . RNA samples were reverse-transcribed into single-strand cDNA,

synthesized into double-strand cDNA, and transcribed into cRNA *in vitro* using T7RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA). After reverse transcription with random primers, cRNA products were marked with Klenow enzyme.

Samples were hybridized using a hybridization solution (25% formamide, $3 \times$ standard saline citrate [SSC], 0.2% sodium dodecyl sulfate [SDS], $5 \times$ Denhart's) at 42°C overnight in a humid environment. Subsequently, slides were washed on a horizontal shaker at 42°C for 4 min, with washing solution I ($2 \times$ SSC, 0.2% SDS) followed by washing solution II ($2 \times$ SSC). Arrays were scanned using CapitalBio's confocal scanner LuxScan 10K-A (Beijing, China). An intensity-dependent lowess program in the R language package was used to normalize the two channel ratio values. Statistical data and differential analysis files were generated by using SAM software 3.0 (Stanford University, Stanford, CA, USA).

Quantitative Polymerase Chain Reaction (QPCR)

Total RNA was extracted from placental tissues and the purified microvascular endothelial cells using Trizol (Invitrogen). The first-strand complementary synthesis reaction was performed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). Amplification reactions were performed using SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa, Dalian, China) on ABI 7500 Real-Time (RT) PCR System. The primers were chosen as follows. HSP70: 5'-aggcc aacaagatcacatc-3' and 5'-tcgtcctccgctttg tactt-3'; human β -actin: 5'-ctctccagccttc ttct-3' and 5'-agcactgtgtggcgctacag-3'.

To determine a threshold, the algorithm multiplied the SD of the background reporter signal in the first few cycles (cycles 3–15) by a default factor of 10. The cycle at which this baseline level is exceeded is defined as the threshold cycle (C_T). The value stands for $2^{-\Delta\Delta CT}$. The reverse transcription reaction was performed on 2 μg total RNA according

to the manufacturer's instruction. Then one cycle of denaturation at 95°C for 10 s, and PCR reaction of 40 cycles with denaturation (15 s at 95°C), annealing (30 s at 56°C), and elongation (34 s at 72°C with a single fluorescence measurement) were carried out. PCR was performed in triplicate in a 25 μl final volume containing SYBR Premix Ex Taq, ROX Reference Dye, cDNA, forward primer, and reverse primer. PCR products were confirmed as a single product at the desired size on agarose gels.

Protein Extraction and Western Blot Analysis

Total protein was extracted from placental tissues and the purified microvascular endothelial cells using RIPA and 1% PMSF (Shenergy Biocolor, Shanghai, China). Protein concentrations were determined with a protein quantitative analysis kit (Shenergy Biocolor). Equal amounts of protein samples (50 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis (10% gel). Immobilized proteins then were transferred to a nitrocellulose membrane, blocked with 5% skim milk in TBS-T for 1 h, and subsequently probed with a rabbit polyclonal antibody against HSP70 (R&D, Minneapolis, MN, USA) at 4°C overnight. Horseradish peroxidase-linked goat anti-rabbit IgG (Dako, Glostrup, Denmark) was used as a second antibody in conjugation with Western blotting luminol reagent (Santa Cruz, CA, USA) to visualize HSP70 on autoradiography films. Films were scanned and bands quantified by ID Image Analysis Software. A rabbit polyclonal antibody against human β -actin (Abcam, Cambridge, UK) was used to normalize data to an internal standard. Relative expressive levels were evaluated by the optical density ratio.

Immunohistochemistry

Formalin-fixed, paraffin-embedded placental tissues were deparaffinized, rehydrated, sectioned, and immunostained. A rabbit polyclonal antibody

against HSP70 (1:400) was used as a primary antibody (R&D). Immunohistochemical procedures were carried out with a Histostain-plus Kits (Zymed, Carlsbad, California, USA). Immunostained sections were microwaved for 8 min in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 10 min. Sections were blocked for non-specific staining and incubated with primary and secondary antibodies. Peroxidase was introduced using a streptavidin conjugate and peroxidase reactivity visualized using the DAB Substrate Kit (Zhongshan Goldenbridge, Beijing, China). Finally, sections were counterstained with hematoxylin and mounted. Immunohistochemical staining of samples and negative controls occurred simultaneously.

Isolation of Placental Microvascular Endothelial Cells

Placentas were collected fresh with four or five placental lobes (10 g) immediately excised, finely minced into small fragments (1 × 1 mm), and stored in cold phosphate-buffered saline solution (PBS). The minced tissue suspension was passed through a nylon sieve (pore size of 75 μm), washed with cold PBS, centrifuged, and the tissue pellet transferred into pre-warmed 0.2% I-type collagenase (Sigma-Aldrich, St. Louis, MO, USA) solution in DMEM (Gibco, Carlsbad, CA, USA). Tissues and cells were incubated for 2 h at 37° C in a shaking water bath then washed three times with PBS. The mixture then was passed through nylon mesh (pore size 55 μm) to obtain a single cell suspension. Dynabeads CD31 (DYNAL, Oslo, Norway) were washed twice with PBS containing 0.1% bovine serum albumin (BSA) and added to the prepared single-cell placental tissue suspension. After a 20 min incubation at 4° C with gentle tilting and rotation, the Dynabead-endothelial cell complex was collected with a magnetic particle concentrator-1 (MPC-1). The cells were washed twice before the final rosetted

Table 1. Differentially expressed genes in PVD compared with control placentas.

| Gene name | Regulation (up(↑)/down(↓)) | Mean differential expression |
|----------------|----------------------------|------------------------------|
| <i>HSPA6</i> | ↑ | 82.285 |
| <i>HSPA1A</i> | ↑ | 6.33 |
| <i>MT1L</i> | ↑ | 8.91 |
| <i>MT1E</i> | ↑ | 8.165 |
| <i>MT1H</i> | ↑ | 8.38 |
| <i>MTIX</i> | ↑ | 5.025 |
| <i>SLC18A2</i> | ↑ | 3.205 |
| <i>SSI-1</i> | ↑ | 2.235 |
| <i>SNFT</i> | ↑ | 3 |
| <i>H1FO</i> | ↓ | 0.335 |

endothelial cells were used for further analysis. Cell purity was validated using the vWF antibody (Thermo, Waltham, MA, USA) by flow cytometry. Purity of microvascular endothelial cells was above 90%.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows 11.5. All data were expressed as mean ± SEM. The differences and correlations of clinical data with the expressions of HSP70 between the two groups were assessed using the independent Student *t* test and bivariate correlations (two-tailed). A *P* value of 0.05 or less ($P \leq 0.05$) was considered statistically significant.

RESULTS

Differential Gene Expression in PVD Samples

We began our pursuit of potential risk factors and signal transcription pathways involved in PVD pathogenesis by examining gene expression. Gene expression was compared in subjects with PVD and with normal pregnancies using a two-channel microarray technique. Ten genes exhibited significantly altered differential expression levels between PVD and control placentas (Table 1). Of these genes, *HSPA6* and *HSPA1A* showed significantly higher levels of expression in PVD placentas when compared with normal pregnancies. *HSPA6* and *HSPA1A* both encode HSP70, suggesting a potential role for this protein in PVD.

HSP70 is Specifically Upregulated in PVD

Since both *HSPA6* and *HSPA1A* encode the protein HSP70, and both were upregulated in placentas from subjects with PVD, we next examined placental tissue and microvascular endothelial cells for the presence of HSP70 mRNA. Placental tissue and microvascular endothelial cells from normal pregnancies and PVD pregnancies were examined. Expression of HSP70 mRNA was upregulated in placental tissue (2.4355 ± 0.26647 versus 1.5920 ± 0.17142, $P = 0.008$) and microvascular endothelial cells (2.9089 ± 0.44197 versus 1.8065 ± 0.21503, $P = 0.038$) from PVD subjects compared with normal pregnancies (Figure 1). The presence of HSP70 mRNA indicates that the HSP70 protein may be present in PVD pregnancies.

HSP70 Protein is Overexpressed in PVD

HSP70 mRNA was upregulated significantly in PVD pregnancies compared with controls. To confirm the presence of the protein, we examined placental tissue and microvascular endothelial cells from PVD and control pregnancies. HSP70 expression was upregulated significantly both in placental tissue (optical density ratios: 0.7758 ± 0.02826 versus 0.5671 ± 0.03841, $P < 0.001$) and in microvascular endothelial cells (optical density ratios: 0.7262 ± 0.03836 versus 0.5463 ± 0.06292, $P = 0.003$) from PVD subjects when compared with normal pregnancies (Figure 2).

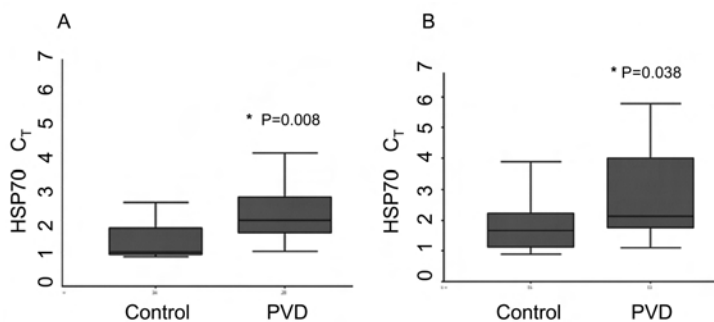


Figure 1. HSP70 mRNA are upregulated both in placental tissue and microvascular endothelial cells from patients with PVD (QPCR). (A) placental tissue (2.4355 ± 0.26647 versus 1.5920 ± 0.17142 , $P = 0.008$). (B) microvascular endothelial cells (2.9089 ± 0.44197 versus 1.8065 ± 0.21503 , $P = 0.038$).

HSP70 Expression Localizes to Endothelium and Smooth Muscle in PVD

The presence of increased HSP70 mRNA and protein in tissues from PVD pregnancies suggests that the maternal placental-fetal unit is experiencing stress. To more accurately target areas within the placental-fetal unit responding to stress, we analyzed HSP70 localization in placental tissues immunohistochemically. Protein expression-revealed HSP70 was expressed mainly in endothelial cells and smooth muscle cells in the placental microvessels of placental tissues (Figure 3).

In addition to the strong staining exhibited by endothelial and smooth muscle cells, HSP70 also was expressed in cytotrophoblasts, syncytiotrophoblasts, and intermediate trophoblasts; however, the expression was weaker (data not shown).

HSP70 Negatively Correlates with Low Birth Weight

Having shown an upregulation of *HSPA6* and *HSPA1A* genes, the presence of HSP70 mRNA and protein in PVD placentas, and localization of HSP70 to endothelial and smooth muscle cells of the placenta, we next examined whether

these factors could contribute to any negative outcomes between normal and PVD subjects. We reviewed time to delivery and infant birth weight with respect to normal and PVD pregnancies. Subjects with PVD delivered earlier and had infants with lower birth weight for gestational age (Table 2). Expressions of total HSP70 mRNA and protein negatively correlated with infant birth weight, $P = 0.012$ and $P = 0.005$, respectively (Figure 4).

DISCUSSION

Placental blood flow and vascular development are essential for normal placental function and are critical to fetal growth. Placental vascular disease may lead to incomplete conversion of the spiral arteries, resulting in undilated myometrial segments. These vessels display an abnormally high vascular resistance, which is associated with reduced uteroplacental perfusion, and which may play a negative role in fetal development.

In the current study, we examined the potential signal transcription pathways involved in the pathogenesis of PVD. Ten genes were altered significantly in PVD, among which were the heat shock proteins *HSPA6* and *HSPA1A*.

We have shown that HSP70 was expressed mainly in endothelial cells and in smooth muscle cells in the microvasculature of PVD placenta. The expression of HSP70 in mRNA and protein levels was upregulated significantly in PVD compared with normal pregnancies, indicating that endothelial response to injury may be involved in PVD.

Oxidative stress, a feature of PVD occurring in the placenta, stimulates endothelial cells to release nitric oxide, leading to increased HSP70 expression (12). Although evidence indicates that elevated HSP70 is capable of protecting cells and tissues from the subsequent noxious conditions (including hypoxia and ischemia-reperfusion) (13), HSP70 also binds selectively and with high affinity and avidity to specific cells of the immune system. This occurs through receptors TLR2 and TLR4 and is depen-

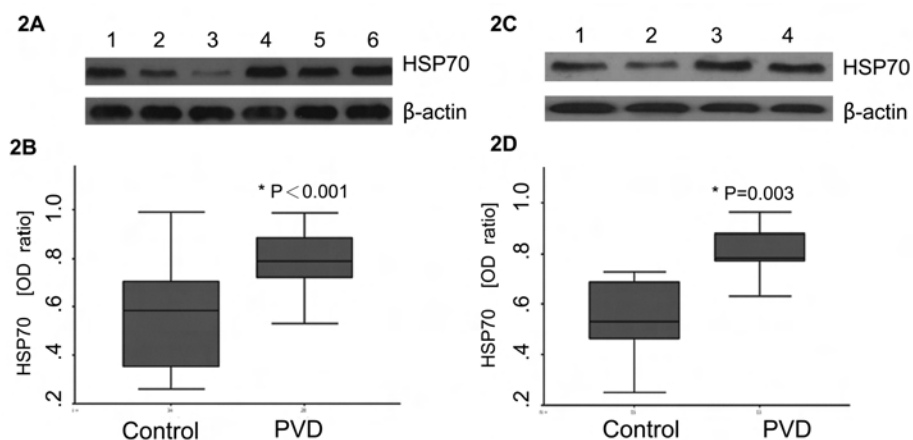


Figure 2. HSP70 protein expressions (Western blot). (A) HSP70 protein expression in placental tissue. (B) HSP protein expression in placental tissue in PVD versus control group (0.7758 ± 0.02826 versus 0.5671 ± 0.03841 , $P < 0.001$). (C) HSP70 protein expression in microvascular endothelial cells. (D) HSP protein expression in microvascular endothelial cells in PVD versus Control group (0.7262 ± 0.03836 versus 0.5463 ± 0.06292 , $P = 0.003$).

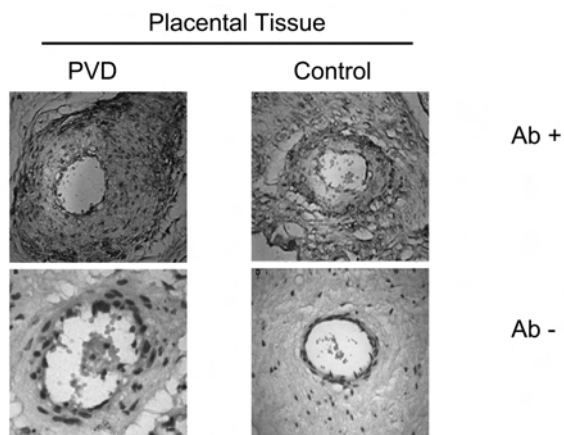


Figure 3. HSP70 expressions in tissues (immunohistochemistry). (A) HSP70 expression in PVD (magnification 40x). (B) Control in PVD (magnification 40x). (C) HSP70 expression in normal placenta (magnification 40x). (D) Control in normal placenta (magnification 40x).

Table 2. Comparison of clinical data between the study group and the control group.

| items | Study group ^a | Control group ^b | P value |
|-------------------------|--------------------------|----------------------------|------------------|
| Gestational age(weeks) | 35.3895 ± 0.53174 | 38.0526 ± 0.33772 | <i>P</i> < 0.001 |
| Infant birth weight (g) | 2619.375 ± 173.2 | 3375.385 ± 157.9 | <i>P</i> < 0.001 |
| Percentile birth weight | 73.9868 ± 2.35313 | 87.9716 ± 1.20594 | <i>P</i> = 0.002 |

^a*n* = 28.

^b*n* = 34.

dent on the cofactor CD14 (14,15). Following binding, HSP70 stimulates rapid intracellular Ca²⁺ flux, activates NF-κB, and upregulates the expression of pro-inflammatory cytokines (16). Evidence suggests that microvascular endothelium in the placental vasculature produces the

pro-inflammatory cytokines in PVD (1). TLR4 mRNA is upregulated in placental villi endothelium in PVD (17). An increase in the expression of intercellular adhesion molecule-1 by microvascular endothelial cells also is associated with PVD, indicating injury and activation of

microvascular endothelial cells (18). Combining our results with the above studies, we hypothesize that over-expression of intracellular HSP70 plays a protective role against oxidative stress, which is the manifestation of endothelial activation. In contrast, extracellular HSP70 over-expression in PVD may initiate an innate immune response that includes the production of proinflammatory cytokines through a TLR-mediated pathway, resulting in the expression of adhesion molecules in endothelial cells via NF-κB activation (16). Our observed negative correlation between total HSP70 expression and birth weight provides evidence that HSP70 may play a role in the pathogenesis of PVD.

Similarities exist between the pathogenesis of atherosclerosis and PVD (20). The inflammatory component of atherosclerosis may involve immune reactivity to HSPs (21). Atherosclerosis now is recognized as a chronic inflammatory disease of the arteries initiated by monocyte/lymphocyte adhesion to activated endothelial cells (22). In advanced atherosclerotic lesions, several cell types, including monocytes, macrophages, dendritic cells, and smooth muscle cells, overexpress HSP70 (23). Furthermore, observations have indicated that HSP70 is a specific and early marker for the cardiovascular disease process. Our results support the hypothesis that PVD and atherosclerosis may share similar pathogenic mechanisms.

In summary, our study has shown the enhanced expression of HSP70 both in placental tissues and in microvascular endothelial cells in PVD. This indicates that HSP70 may be associated with the pathogenesis of PVD and, also, it may provide an explanation for the endothelial cell activation and pro-inflammatory response previously reported in PVD. Higher expression of intracellular HSP70 provided the protective role against oxidative stress (24,25), whereas extracellular HSP70 may initiate an inflammatory response in PVD. Further work is required to understand the molecular mechanism of HSP70 on endothelial injury *in vivo* and *in vitro*

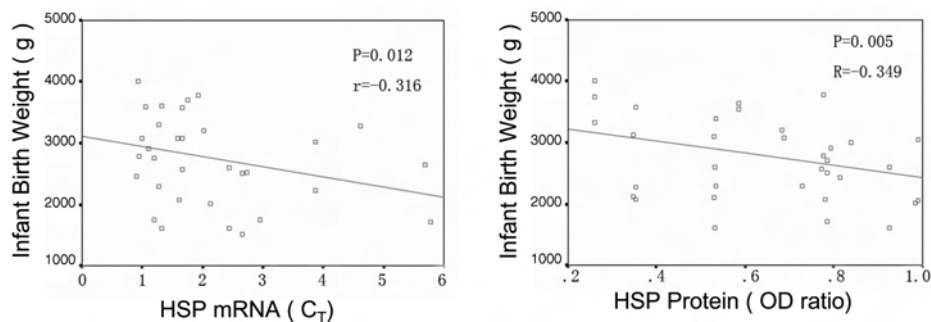


Figure 4. HSP70 negatively correlates with low birth weight. (A) Expression of total HSP70 mRNA was negatively correlated with infant birth weight (*P* = 0.012). (B) Expression of total HSP70 protein was negatively correlated with infant birth weight (*P* = 0.005).

in PVD, which will allow researchers to develop pharmacological or molecular tools for patients.

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