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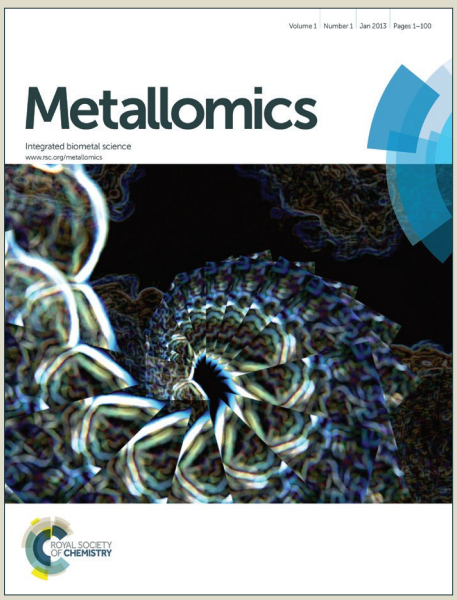

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The involvement of vimentin in copper-induced regression of cardiomyocyte hypertrophy

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Abstract

Dietary copper supplementation reverses pressure overload-induced cardiac hypertrophy. Activation of vascular endothelial growth factor receptor-1 (VEGFR-1) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase-1 (PKG-1) is required for the regression. The present study was undertaken to determine the link between VEGFR-1 and PKG-1 in copper regression of cardiomyocyte hypertrophy. Human cardiac myocytes (HCM) or primary cultures of neonatal rat cardiomyocytes were exposed to phenylepherine (PE) at a final concentration of 100 μM for 48 h to induce cell hypertrophy. Copper sulfite was added to cultures of hypertrophic cardiomyocytes at a final concentration of 5 μM copper element for 24 h to reverse cell hypertrophy. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis identified a 56 kDa copper-binding protein, vimentin, which was co-immunoprecipitated with VEGFR-1 and PKG-1. Copper supplementation increased vimentin levels and enhanced PKG-1 activity. Gene silencing using siRNA targeting vimentin prevented copper-induced elevation of vimentin, depressed the activity of PKG-1, and blocked copper-induced regression of cardiomyocyte hypertrophy. This study demonstrates that vimentin is critically involved in the VEGFR-1 mediated activation of PKG-1 signaling pathway, leading to regression of cardiomyocyte hypertrophy.

Keywords: Copper, cardiomyocyte, hypertrophy, PKG-1, vimentin

1. Introduction

Dietary copper (Cu) supplementation results in regression of cardiac hypertrophy induced by pressure overload in mouse model, as evidenced by recovery of cardiac contractile function, increase in capillary density, normalization of mitochondrial structural integrity, and reversal of increased cardiac cell size.[1] *In vitro* studies also showed that cell hypertrophy induced by hydrogen peroxide in embryonic rat cardiac H9c2 cells or induced by phenylephrine (PE) in primary cultures of neonatal rat cardiomyocytes were all reversed by addition of physiologically relevant amount of Cu (5 μM).[2, 3] In the regression of cardiomyocyte hypertrophy, vascular endothelial growth factor (VEGF) is essential.[2] The binding of VEGF to VEGF receptor-2 (VEGFR-2) causes cardiac hypertrophic growth through activating a series of signaling pathways including protein kinase B (Akt1), protein kinase C (PKC), and extracellular regulated protein kinases 1/2 (ERK1/2).[4-8] However, it was identified that in Cu-induced regression of cardiomyocyte hypertrophy, VEGF predominately activates VEGFR-1,[9-11] leading to activation of cyclic guanosine monophosphate (cGMP)-dependent protein kinase-1 (PKG-1).[9]

In cardiomyocytes, PKG-1 signaling pathway is critically involved in the regression of cardiac hypertrophy. Phosphodiesterase-5A inhibitor, sildenafil, by suppressing degradation of cGMP leading to activation of PKG-1, reverses pressure overload-induced cardiac hypertrophy in mice.[12, 13] Depression of PKG-1 by siRNA targeting PKG-1 suppressed Cu-induced regression of cardiomyocyte hypertrophy.[9] The association between PKG-1 and VEGFR-1 was defined by the observation that VEGFR-1 gene silencing using siRNA targeting VEGFR-1 inhibited PKG-1 activity and Cu-caused regression of cardiomyocyte hypertrophy.[9] Cu, through regulating

post-translational modification, suppresses the activity of VEGFR-2 in hypertrophic cardiomyocytes.[11] Thus, Cu-induced regression of cardiomyocyte hypertrophy involves overexpression of VEGF, suppression of VEGFR-2 pathway, and activation of VEGFR-1-mediated PKG-1 signaling pathway. However, it is important to understand how VEGFR-1 links to PKG-1 signaling pathway.

The link between main proteins involved in signaling transduction pathways involves signal transducing adaptor proteins. These adaptor proteins contain a variety of protein-binding modules that link protein-binding partners together and facilitate the creation of larger signaling complexes. These proteins tend to lack any intrinsic enzymatic activity themselves but instead mediate specific protein-protein interactions that drive the formation of protein complexes. In this context, the link between VEGFR-1 and PKG-1 would involve such adaptor proteins. Identification of such proteins through analysis of physical connections between these unknown adaptors and the signaling molecules, VEGFR-1 and PKG-1, would help understanding how VEGFR-1 links to PKG-1 activation.

Therefore, the present study was undertaken to identify possible adaptor proteins that critically involved in the VEGFR-1 mediated activation of PKG-1. We employed co-immunoprecipitation methods to identify proteins associated with both VEGFR-1 and PKG-1, then determined the role of the identified proteins in the link between VEGFR-1 and PKG-1 activation by deletion of the particularly interested protein. Through this process, we found that vimentin is critically involved in the link between VEGFR-1 and activation of PKG-1 signaling pathway.

2. Materials and Methods

2.1 Cultures of cardiac myocytes

HCM were purchased from ScienCell (Catalog # 6200, USA). T-75 flasks were pre-treated with poly-L-lysine (PLL) of 1 mg/mL for either 1 h or overnight, and then washed 2 times with PBS prior to cell plating. Cardiac myocyte medium (CMM) (ScienCell, USA) was used throughout.

Primary cultures of neonatal rat cardiomyocytes were isolated from 1- to 3-day-old Wistar rats and cultured according to a procedure published previously.[9] This investigation follows the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication NO. 85–23, revised 1996). The animal procedure was approved by the Institutional Animal Care and Use Committee at Sichuan University.

2.2 Experimental procedure

The model of cardiomyocyte hypotrophy was established according to a previous publication,[2] with some modifications. Briefly, primary cultures of neonatal rat cardiomyocytes or HCM were treated with phenylephrine (PE, Sigma Aldrich, USA) at a final concentration of 100 μM for 48 h in serum-free media, and then added $CuSO_4$ at a final concentration of 5 μM for additional 24 h. The cells were collected by trypsinization, suspended in PBS buffer, and counted using a hemocytometer. Protein content was measured using the Bradford method (Bio-Rad, USA) and normalized by cell number.

2.3 Co-immunoprecipitation (Co-IP) and LC-MS/MS

Cells were washed with PBS and lysed with lysis buffer. The insoluble material was removed by centrifugation. The clarified lysate was incubated with 2 μg /ml primary antibody for 16 h with constant agitation at 4 ° C. The following morning, 20-40 μL of protein G sepharaose beads were adding to the mixture and incubate under rotation at 4 ° C for 4 h. The samples underwent centrifugation at 1000 g, and the supernatant was carefully removed, and the bead pellets were washed 3 times with ice-cold PBS. To elute the immune complex, capture beads were resuspended in 50 μl of 0.1 M glycine, pH 2.5 and incubated with agitation for 10 min at 4 ℃. The supernatant was collected after centrifugation, run through detergent removal spin columns (Thermo Scientific, USA) according to the manufacturer's instructions, and analyzed using LC-MS/MS. Samples were analyzed with the LCQDuo System Ion Trap Mass Spectrometer.

2.4 Western blotting analysis of PKG-1 and vimentin.

Cells scraped in PBS were washed 3 times and lysated in 1% SDS solution. Protein samples were mixed with 5× loading buffer, boiled for 10 min at 100□ and cooled. Equal amounts of protein (50-100 μg) from each sample were separated by 10% SDS-PAGE. Proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Transferred proteins were blocked with 5% non-fat dry milk in Tris-HCl buffer solution containing Tris-HCl (50 mM), NaCl (150 mM), and Tween-20 (0.1%) (TBS-T) for 1 h at room temperature. The blots were then incubated with respective primary antibodies against PKG-1 or vimintin (Abcam, USA) in blocking solution according to the vender's recommendation. After incubation, the blots were washed with TBS-T six times for 5 min each. The blots

were incubated for 2 h with appropriate secondary antibody. After washing six times (5 min each), target proteins were visualized using chemiluminescence (Bio-Rad, USA) and analyzed by densitometry using a Quantity One Software.

2.5 Gene silencing of vimintin

Three different siRNAs targeting rat vimrntin and negative-mismatched siRNA were purchased from RiboBio (Ribo-Bio, China). The effective siRNA sequence for vimintin was as follows: 5'CAGACAGGAUGUUGACAAU dTdT3'; 3'dTdT GUCUGUCCUACAACUGUUA5'. The optimal transfection efficiency was determined from our preliminary studies testing the range from 5 to 50 nM, and we selected the condition of siRNAs with minimal cytotoxicity. After PE treatment, cardiomyocytes were transfected with 25 nM siRNA targeting rat vimintin or negative-mismatched siRNA in antibiotic-free media. Lipofectamine2000 (Invitrogen, USA) was used as the transfection reagent according to the manufacturer's instruction. After 48h transfection, cells were trypsinized and collected for further analysis as described in the experimental procedure.

2.6 PKG-1 activity

PKG-1 activity was assayed by immunoblot for PKG-phosphorylated vasodilator-stimulated protein (VASP) at serine 239 (Ser 239). VASP was characterized as a substrate of both PGK-1 and cAMP-dependent kinases (PKA).[14] Three phosphorylation sites, Ser157, Ser239, and Thr278, have been identified in VASP. Ser239 is the major PKG phosphorylation site while Ser157 is the major PKA phosphorylation site.[15] Phosphorylation at Ser239 of VASP is a useful marker for monitoring PKG activation and signaling.[16, 17] Increased PKA activity was detected in

hypertrophic cardiomyocyte.[18] Therefore, PKG-1 activity was detected by using a monoclonal antibody (CST, USA) to p-VASP (Ser 239) at 1:1000 dilution. The assay was performed after treatment with vimintin siRNA, as described previously.

2.7 Statistical analysis.

Data were obtained from three separate experiments and expressed as means ± S.E.M. A multiple factorial design was applied to this study. The data were analyzed according to the experimental design. After a significant interaction detected by the analysis of variance (ANOVA) based on the factorial design, the significance of the main effects was further determined by T-test. The level of significance was considered when $P< 0.05$.

3. Results

3.1 Identification of a 56 kDa Cu-binding protein, vimentin, as an adaptor protein linking VEGFR-1 and PKG-1

Proteins pulled down using agarose-conjugated primary antibody were identified by LC-MS/MS, as presented in supplemented table 1. Table S1A lists proteins pulled down by agarose-conjugated VEGFR-1 antibody. Table S1B includes proteins pulled down by agarose-conjugated PKG-1 antibody. Among these proteins, a 56 kDa vimentin was associated with both VEGFR-1 and PKG-1.

3.2 Effects of Cu on PE-increased PKG-1 and vimentin levels

To determine the effect of Cu on vimentin and PKG-1 protein levels, the primary cultures of neonatal rat cardiomyocytes were subjected to varying treatments, including PE, Cu, or PE plus Cu. Western blot analysis showed that PE treatment significantly increased the protein levels of PKG-1. Cu addition neither increased nor affected PE-increased PKG-1 protein levels (Fig 1A). On the other hand, both PE treatment and Cu addition significantly increased vimentin levels, but PE plus Cu treatment did not show any additive effect on vimentin (Fig 1B).

3.3 Depression of PKG-1 activity in vimentin deficient cardiomyocytes

To determine whether vimentin participates in the regression of cardiomyocyte hypertrophy, we produced vimentin-deficient cardiomyocytes using siRNA targeting vimentin. As shown in Fig 2A, gene silencing under the present condition reduced about 60% of total vimentin levels. This reduction in vimentin levels did not affect PE-induced cardiomyocyte hypertrophy, but significantly

impeded Cu-induced regression of cardiomyocyte hypertrophy, as measured by increase in total protein content (Fig 2B). Unexpectedly, vimentin reduction per se increased the protein content of cardiomyocytes (Fig 2B).

PKG-1 activities, determined by its kinetic reaction product p-VASP, were measured under varying treatment conditions. As shown in Fig 3, PE treatment increased PKG-1 activities and Cu addition helped maintaining high levels of PKG-1. Vimentin reduction significantly suppressed the increase in PKG-1 activities induced by PE or PE plus Cu treatments.

Changes in the protein levels of active ERK, p-ERK, were measured. As shown in Fig 4, PE treatment increased p-ERK levels, agreeable to previous observations,[7, 19] but vimentin reduction further increased p-ERK levels.

Discussion

PKG-1 plays a critical role in regression of cardiac hypertrophy.[12, 13] The activation of PKG-1 is associated with cardiac hypertrophic growth.[9, 13] However, in the progression of cardiac hypertrophy, the activation of PKG-1 does not overcome the overt hypertrophic signaling transduction, resulting in the ultimate hypertrophic growth. The counteraction of PKG-1 to the hypertrophic signaling becomes dominant when VEGF activation of VEGFR-2 is switched to activation of VEGFR-1.[9] This switch from VEGFR-2 to VEGFR-1 signaling pathway takes place in Cu-induced regression of cardiac hypertrophy.[9, 11] The association between VEGFR-1 and PKG-1 is essential for the regression of cardiac hypertrophy. Therefore, it becomes an important question: how does VEGFR-1 link to PKG-1 signaling pathway? The data obtained from the present study identified that vimentin is critically involved in the link between VEGFR-1 and PKG-1.

Vimentin is a type III intermediate filament (IF) protein that is expressed in mesenchymal cells. Vimentin and other IFs, along with tubulin-based microtubules and actin-based microfilaments, comprise the cytoskeleton. Because vimentin is the major cytoskeletal component of mesenchymal cells, it is often used as a marker of mesenchymally-derived cells or cells undergoing an epithelial-to-mesenchymal transition (EMT) during both normal development and metastatic progression. Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria,[20, 21] playing a significant role in supporting and anchoring the position of the organelles in the cytosol. It is accepted that vimentin is the cytoskeletal component responsible for maintaining cell integrity. However, transgenic mice that lack vimentin appeared normal and did not show functional differences.[22] It is possible that the microtubule

network may have compensated for the absence of the intermediate network. On the other hand, wounded mice that lack the vimentin gene heal slower than their wild type counterparts.[23] Vimentin is also found to control the transport of low-density lipoprotein, LDL.[24] The present study found that vimentin also regulates VEGFR-1 activated PKG-1 signaling transduction system.

Both PE and Cu treatments increased vimentin levels in cardiomyocytes. This increase appeared not relate to cardiomyocyte hypertrophy or regression of the hypertrophy. However, depletion of vimentin caused cardiomyocyte hypertrophy and blocked regression of the hypertrophy. Previous study has shown that mice lacking desmin IF develop cardiomyocyte hypertrophy.[25] The lack of vimentin IF would cause the same effect as that of the lack of desmin IF in cardiomyocytes. One of the reasons for cardiomyocyte hypertrophy induced by the lack of IFs is the reduced tension to the cells, leading to activation of hypertrophic signaling pathways.[25] In this context, we found here that ERK was activated in the vimentin deficient cardiomyocytes. ERK activation increases protein synthesis, leading to cardiomyocyte hypertrophy.[26]

As demonstrated previously, Cu-induced regression of cardiomyocyte hypertrophy depends on the activation of PKG-1.[9] The data presented here showed that lack of vimentin depressed PKG-1 activity. The link between vimentin and PKG-1 activation has been demonstrated in other cell types.[27-29] The new finding in the present study is that VEGFR-1 was linked to vimentin. VEGFR-1, one of tyrosine kinase receptors, is a transmembrane protein. The combination of ligand and tyrosine kinase receptors triggers dimerization, and then autophosphorylation of the tyrosine kinase receptor activates the intracellular signaling transduction.[30] Intracellular C-

terminus of activated tyrosine kinase receptors can bind to a diversity of proteins with Src homology-2 (SH2) domain structure, such as growth factor receptor-bound protein 2 (GRB2) and phosphatidylinositol 3-kinase (PI3K).[31-33] Previous studies have shown that Fyn, a Src kinase, possesses SH2 and Src homology-3 (SH3) protein binding domains and vimentin binds to the SH2 of Fyn in MC/9 mast cells.[34] We speculate that a protein with SH2 in cardiomyocytes would be involved in the link between VEGFR-1 and vimentin.

It was reported that zinc (Zn) supplementation prevented vimentin expression in high glucose-induced epithelial-to-mesenchymal transition and Zn was necessary for glycogen synthase kinase-3 (GSK-3β) activation.[35] The effect of Cu on vimentin expression and on GSK-3β activation would be opposite to that of Zn. The VEGFR-1/PKG-1 signaling pathway is involved in Cu-induced regression of cardiomyocyte hypertrophy.[9] Inhibition of phosphodiesterase-5 with sildenafil, which augments cGMP accumulation and PKG-1 activity, also reverses cardiac hypertrophy.[13] But, sildenafil inactivates GSK-3β via PKG-1 activation in cardiomyocytes.[37] Therefore, it was also possible that Cu addition-induced activation of PKG-1 would also cause inactivation of GSK-3β.

The expression of vimentin was increased in PE-induced hypertrophic cardiomyocytes. Nitric oxide (NO) was a negative regulator in cardiac hypertrophy.[39] It is reported that NO induces vimentin dephosphorylation in human retinal pigment epithelial cells (ARPE-19).[40] On the other hand, it was reported that vascular p-VASP (Ser239) is primarily regulated not only by cGMP pathway but also by NO.[41] Therefore, vimentin expression and function may be also regulated by NO in hypertrophic cardiomyocytes. It is worth to probe in next study.

In summary, the present study provided a further understanding of the mechanism by which VEGFR-1 mediates the regression of cardiac hypertrophy. In particular, vimentin, as an adaptor protein linking VEGFR-1 and PKG-1, is involved in Cu-induced regression of cardiomyocyte hypertrophy.

Acknowledgments

This work was supported by National Science Foundation of China (grant number: 81230004 to Y. J. Kang) and Sichuan University West China Hospital. The funding sources had no influence in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Author Contributions

All authors participated in the design of this study, analysis of the data, interpretation of the results, and review of the manuscript; RL, KB, TW, and MS carried out the experiments; YJK and RL wrote the manuscript.

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Figure legends

Figure 1. Effects of Cu on PE-increased PKG-1 and vimentin levels. (A) Western blot analysis of PKG-1 protein levels. Primary cultures of neonatal rat cardiomyocytes were treated with PE for 48 hrs followed by Cu treatment for an additional 24 hrs before harvesting. (B) Western blot analysis of vimentin protein levels under the same treatment protocol as above. The procedure was repeated three times and the data obtained from the semiquantitative analyses were the averaged density change of the three repeats. The comparison was done in relation to controls. Values are means ± SEM. * Significantly different from control group ($P < 0.05$). PE: phenylephrine; PKG-1: cyclic guanosine monophosphate (cGMP)-dependent protein kinase-1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

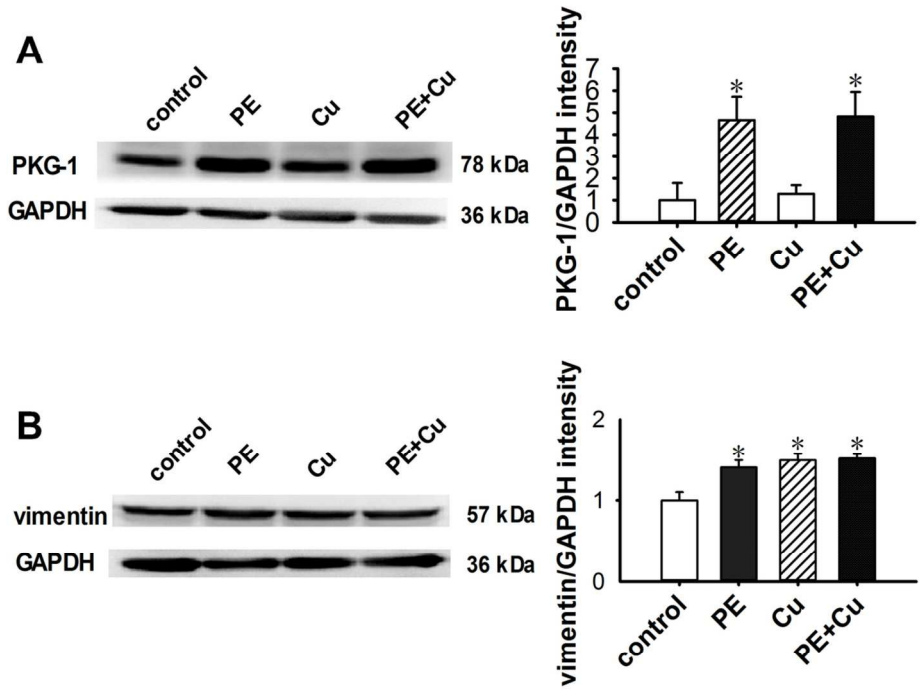
Figure 2. Effects of vimentin reduction on Cu regression of cardiomyocyte hypertrophy. (A) Immunocytochemistry staining and western blot analysis of changes in vimentin protein levels after the treatment with siRNA targeting vimentin. (B) Changes in total protein content normalized by cell number under the treatments indicated. Each data point was obtained from three independent experiments. Values are means ± SEM. * Significantly different from control group ($P < 0.05$).

Figure 3. Changes of PKG-1 activity in vimentin deficient cardiomyocytes. Western blotting analysis of p-VASP (Ser 239) was used to reflect PKG-1 activity. Each data was obtained from three independent experiments. Values are means ± SEM. *Significantly different from control group ($P < 0.05$).

Figure 4. Effects of vimentin deficiency on p-ERK levels. Western blotting analysis of p-ERK was used to reflect ERK activity. PE group was positive control. Each data

point was obtained from three independent experiments. Values are means ± SEM. *

Significantly different from control group ($P < 0.05$).



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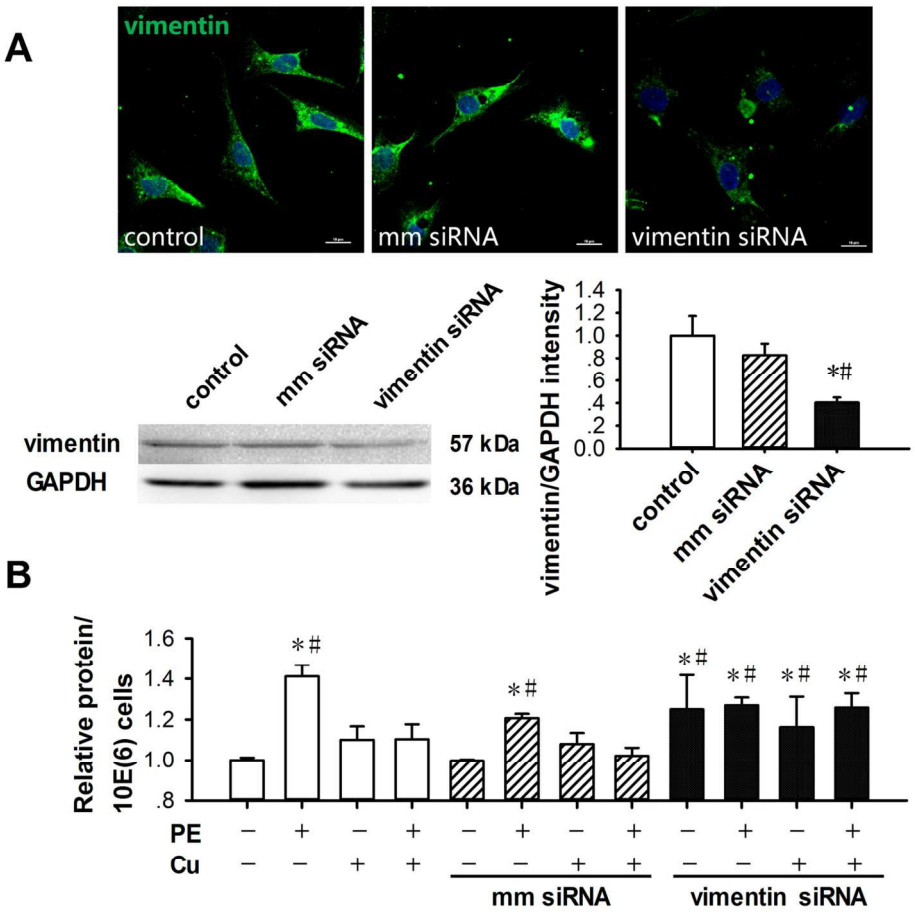

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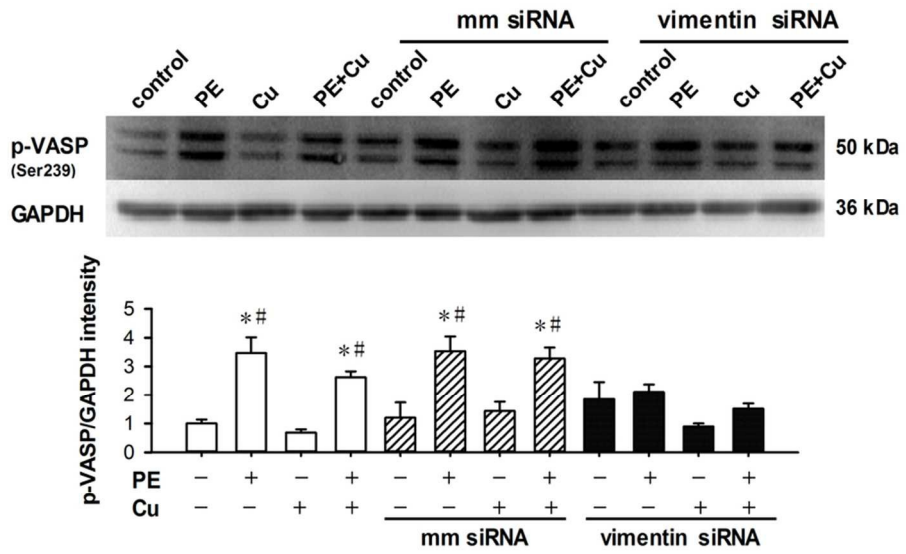

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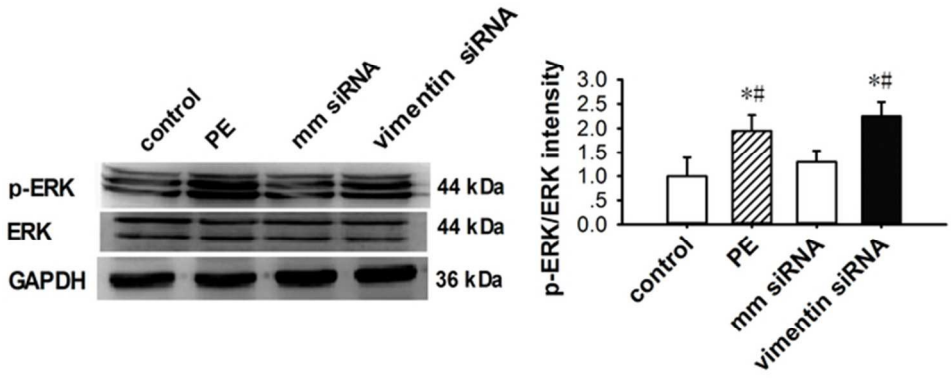

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