

# Different effects of neuropeptide Y on proliferation of vascular smooth muscle cells via regulation of Geminin

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**Abstract** The proliferation-promoting effect of neuropeptide Y (NPY) always functions in low-serum-cultured vascular smooth muscle cells (VSMCs), and the phenotypic switch of VSMCs is regulated by concentrations of serum. Whether the property of the NPY proliferative effect in VSMCs relies on phenotype of VSMCs is unclear. We aimed to explore the role of NPY on proliferation of different VSMC phenotypes in the pathogenesis of atherosclerosis. By stimulating A10 cells with 200 nM NPY in 0.5 or 10% serum, 3H-thymidine and 5-ethynyl-2'-deoxyuridine (EdU) and CCK8 measurements were used to detect VSMC proliferation. RT-PCR and Flow cytometry were performed to detect the factors involved in different properties of the NPY proliferative effect in VSMCs. Instead of facilitating proliferation, NPY had no significant effect on the growth of VSMCs when cultured in 10% serum (VSMCs stayed at synthetic states). The underlying mechanism may be involved in down-regulation of Y1 receptor ( $P < 0.05$  vs. Vehicle) and up-regulation of Geminin ( $P < 0.05$  vs. Vehicle) in 10% serum-cultured VSMCs co-incubated with 200 nM NPY. Besides, modulation of Geminin was effectively blocked by the Y1

receptor antagonist. The stimulation of NPY on proliferation of VSMCs could be a double-edged sword in the development of atherosclerosis and thus provides new knowledge for therapy of atherosclerosis.

**Keywords** NPY · Geminin · Vascular smooth muscle cells (VSMCs) · Serum · Phenotype

## Introduction

Atherosclerosis, a major form of cardiovascular disease that is one of the leading causes of mortality all over the world, involves complicated molecular and cellular responses including endothelial dysfunction, inflammation, proliferation, and matrix alteration. Notably, it has been demonstrated previously that VSMC proliferation plays critical roles in atherosclerosis and is linked to other pathological processes [1, 2]. Different cell types such as endothelial cell, platelets, and inflammatory cells release growth factors and cytokines to respond to the lesions of atherosclerosis, which will promote the changes of VSMCs from contractile type (differentiation) to synthetic type (dedifferentiation). Indeed, proliferation potential and migration rates are higher in synthetic than contractile VSMCs [3–5]. Thus, emerging strategies are required to develop therapeutics that could prevent the aberrant phenotypic switch and proliferation of VSMCs toward multiple mediators during atherosclerosis.

NPY, a 36-amino acid peptide neurotransmitter, is an endogenous vasoconstrictor and is released in response to direct stimulation of cardiac sympathetic neurons in the periphery [6, 7]. Besides, NPY has been shown to remarkably aggravate the proliferation of VSMCs by binding with Y1 receptors and promote atherosclerosis

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[8, 9]. An intriguing observation is that the proliferation-promoting effect of NPY always functions in low-serum-cultured VSMCs [10–12]. Wilson and Reusch [13, 14] found that in a serum-free media, expression of smooth muscle myosin heavy chain (SM MHC) increased in VSMCs. SM MHC is an isoform of SM contractile proteins SMC, whose stable production is associated with the contractile phenotype of VSMC and rapidly degrades in the synthetic phenotype [5, 15]. It has also been proved that factors contained in serum could be able to weaken the contractile response of rat aortic vessels [16]. In that case, we can confirm that the phenotype of VSMCs are regulated by concentrations of serum. Our previous study showed that silence of Geminin, an inhibitor of DNA replication licensing and cell cycle, could promote DNA synthesis and proliferation of VSMCs [17]. Most importantly, our group also found that over expression of Geminin stimulated phenotypic transformation of VSMCs from contractile type to synthetic type.

Given that the dynamic changes of VSMC phenotype were mediated by multiple factors in development of atherosclerosis, we aimed to determine whether the enhancing effect of NPY on the proliferation of VSMCs was affected by its aberrant phenotypic switch during atherosclerosis and investigate the potential mechanisms.

## Methods

### VSMC culture

The thoracic aortic smooth muscle cell line of rats, A10 cells (ATCC, USA), were cultured in Dulbecco's modified essential medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and a 1% penicillin/streptomycin/epidermal growth factor I in a humidified atmosphere (5% CO<sub>2</sub>, 95% air). The morphology and protein markers were examined as indicated in Zhang et al. [17]. Cells from passages 3 through 6 were used in all experiments. In experiments with NPY Y1 receptor antagonist BIBO3304, cells were incubated with indicated concentration of BIBO3304 for 1 h prior to NPY stimulation.

### CCK8 assay

A10 cells were seeded onto with 5000 cells/well and grown in 10% serum for 24 h. After serum-starved for 24 h, A10 cells were incubated in four groups for 24 h: 0.5% serum, 0.5% serum + 200 nM NPY, 10% serum, 10% serum + 200 nM NPY. Then 20  $\mu$ L CCK8 (cell count kit, Boster, Wuhan, China) was added to each group for 4 h,

followed by light absorbance measurement at a wavelength of 450 nm.

### Measurement of EdU incorporation

After plated onto 96-well plate and starved as CCK8 assay, A10 cells were stimulated with or without 200 nM NPY (Novoprotein, Shanghai, China) for 24 h in 10% serum. At the same time, different concentrations of BIBO3304 were used to treat A10 cells cultured with 10% serum and NPY for 24 h. Next, A10 cell proliferation was determined by the 5-ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Assay Kit (Ribobio, Guangzhou, China), which was performed according to the manufacturer's protocol. The cell nuclei were stained with DAPI (Beyotime), and the incorporated EdU in A10 cells was detected by fluorescence microscopy.

### RNA isolation and real-time PCR

Total RNA was extracted from the cultured cells using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. Total RNA was reverse transcribed using PrimeScript<sup>TM</sup> RT reagent Kit (Perfect Real Time) (Takara, Japan), and Real-time PCR was performed using a CFX96 Touch<sup>TM</sup> (Bio-rad, USA) with One-Step SYBR<sup>®</sup> PrimeScript<sup>TM</sup> RT-PCR Kit (Perfect Real Time) (Takara, Japan). GAPDH served as a housekeeping gene. Cycling conditions were as follows: 30 s at 95 °C, (5 s at 95 °C and 30 s at 60 °C)  $\times$  40. Melting curve analysis was performed to confirm the exclusive amplification of the expected PCR product. The values for each target gene were calculated as  $2^{\Delta\Delta C_t}$  values (Table 1).

### Cell cycle analysis

A10 cells were seeded in the 6-well plates with 500,000 cells/well and incubated and grown in 10% serum for 24 h. After starvation for 24 h, the cells were cultured in 10% serum with administration of 200 nM NPY at 0, 12, and 24 h. Then, cells were fixed in 70% ethanol and incubated overnight at  $-4$  °C. The cell cycle was analyzed by propidium iodide (PI) and examined by a flow cytometer (Beckman Coulter, USA).

### Data analysis

The statistical significance of differences between groups was assessed via Student's *t* tests of comparison, and *P* values less than 0.05 were considered to be statistically significant. Statistical analyses were conducted using the GraphPad Prism 6.0 software (GraphPad Software, Inc.).

**Table 1** Primer sequences for gene expression analyzed by qRT-PCR

Target gene	Forward (5'–3')	Reverse (5'–3')
GAPDH	TACCCACGGCAAGTTCAA	GCCAGTAGACTCCACGACAT
Y1	GTTGACGCAGGTGGAGATCA	GCTGCCCTTACCATCTTCA
Y2	GCAGCTCCAGGACTAACGTG	CCGGGGAGGAGAAGAGTGTG
Geminin	GAGCCCAAGAGAACGTGAAGAGTAG	CCTCCGTTGTTCTGCCACTTCTTTC

## Results

### Effects of different serum concentrations on NPY-mediated proliferation of VSMCs

To determine whether NPY promoted the proliferation of low-serum-cultured VSMCs, the A10 cells were incubated in 0.5% serum with additional 200 nM NPY for 24 h. Previous study has demonstrated that NPY increased proliferation of VSMCs via Y1 receptor which is the major subtype of NPY receptors [18]. We confirmed this result by NPY Y1 receptor antagonist BIBO3304 in a concentration-dependent manner. We observed that NPY increased the growth of VSMCs in an intensively low serum concentration ( $*P < 0.05$  vs. Vehicle, Fig. 1a), and this effect was blocked by Y1 receptor antagonist (Fig. 1c). In contrast, when exposed to 10% serum, there was no significant difference of VSMC proliferation between vehicle group and test group (200 nM NPY) (Fig. 1b). These data indicated that the proliferation-promoting effect of NPY on VSMCs was affected by serum concentrations of culture media.

### Expression levels of Y1 receptors are decreased in high-serum-cultured VSMCs

Considering the critical role of Y1 receptor in NPY-mediated growth of VSMCs, we explored whether NPY induced expression changes of Y1 receptor in A10 cells exposed to 0.5 or 10% serum for 24 h. Since previous study has shown that Y2 receptors were involved in stimulating angiogenesis in periphery [19], we also examined the levels of Y2 receptor in the same conditions. As shown in Fig. 2a, when A10 cells were cultured in 0.5% serum, NPY cannot induce the mRNA levels change of Y1 receptor while it stimulated down-regulation of Y1 receptors expression in 10% serum. Particularly, the mRNA levels of Y1 receptor were unchanged between 0.5% serum- and 10% serum-cultured A10 samples. Furthermore, there was no significant difference of Y2 receptor expression levels among these four groups (Fig. 2b). The results collectively revealed that down-regulation of Y1 receptor instead of Y2 receptor may limit NPY to aggravate growth of VSMCs cultured in 10% serum.

### Geminin expression modulated by NPY via Y1 receptor in 0.5% or 10% serum-cultured VSMCs

Previous study has proved that there was a close connection between Geminin expression and proliferation of VSMCs [17]. Thus, we focused on the regulation of NPY on the expression of Geminin in 0.5 and 10% serum-cultured VSMCs. Based on the role of Y1 receptor in VSMC proliferation, we also added Y1 receptor antagonist in the medium to investigate whether the regulation is involved in NPY/Y1 pathway. Although levels of Geminin were not significantly different between vehicle (0.5% serum) and 200 nM NPY (0.5% serum) groups, expression of Geminin was significantly up-regulated in 10% serum-cultured VSMCs co-incubated with 200 nM NPY compared with vehicle (10% serum) group. This modulation was effectively blocked by the Y1 receptor antagonist (Fig. 3). These results implied that Geminin could have a role in NPY-mediated proliferation effect on VSMCs.

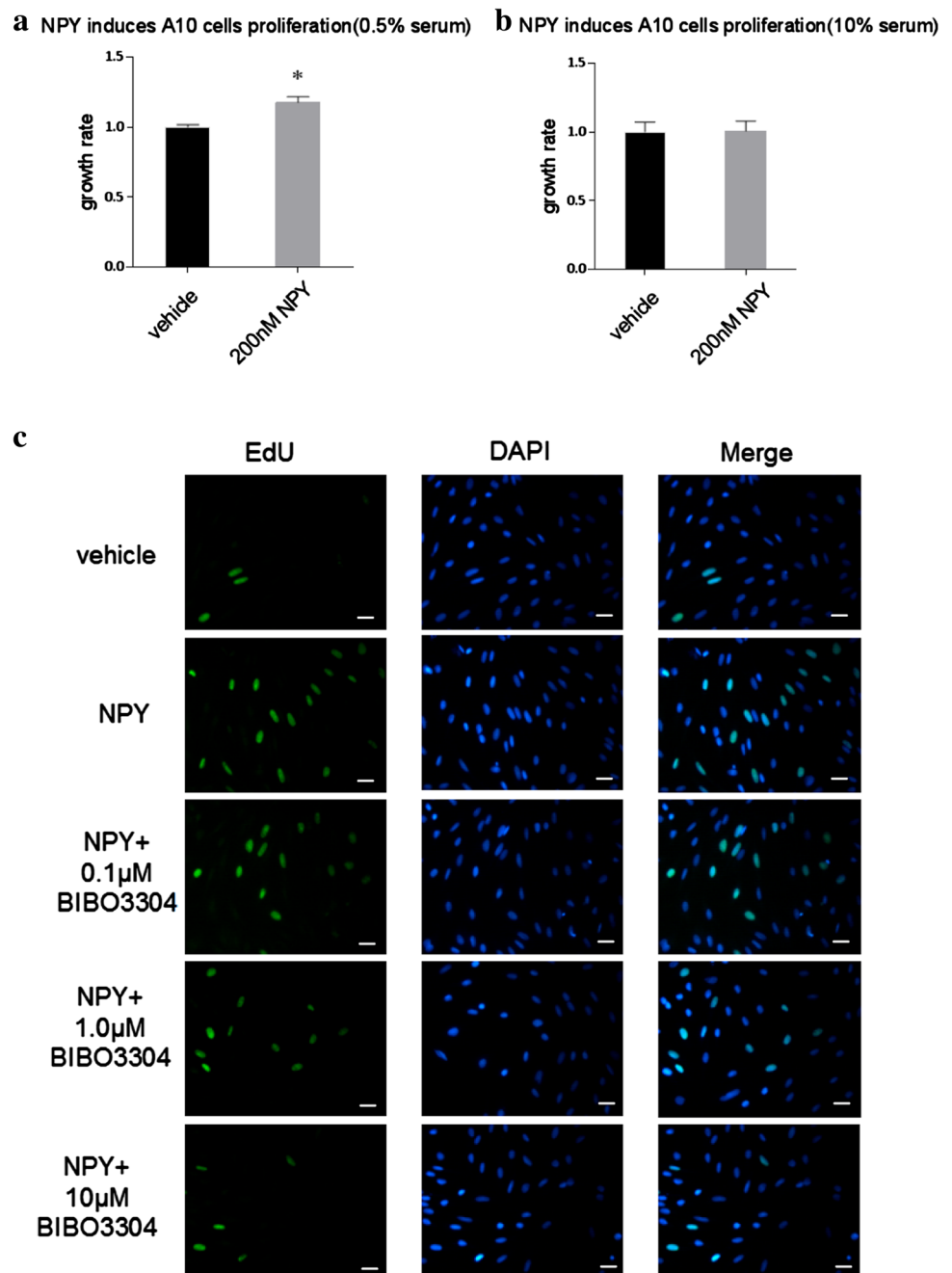
### Role of NPY on cell cycle progression of 10% serum-cultured VSMCs

Geminin is a negative regulator of cell cycle. Thus, we evaluated whether NPY-induced changes of Geminin expression play a role in cell cycle progression of 10% serum-cultured VSMCs. We performed the flow cytometry on A10 cells incubated in 10% serum by following time points: 0, 12, and 24 h after NPY stimulation. In 200 nM NPY group, more cells were in S phases compared to vehicle group at same time points (11.9 vs. 12.86% at 12 h, 9.45 vs. 13.82% at 24 h). It has been reported that Geminin prevents DNA replication at S phase and induces cell cycle arrest in S phase [20, 21]. Thus, cell cycle arrest is another reason that NPY could not facilitate proliferation of VSMCs cultured in high serum concentration (Fig. 4).

## Discussion

VSMC proliferation is commonly thought to occupy an important position in the pathogenesis of atherosclerosis, while NPY, the neurotransmitter of sympathetic postganglionic neuron, promotes VSMC proliferation. In addition,

**Fig. 1** Effects of serum concentration on NPY-induced VSMC proliferation. **a** A10 cells were stimulated with 200 nM NPY in 0.5% serum for 24 h. An index of cell proliferation was analyzed by CCK8 assay.  $*P < 0.05$  versus vehicle group. **b** A10 cells were stimulated with 200 nM NPY for 24 h. An index of cell proliferation was analyzed by CCK8 assay. There was no significant difference between NPY group and vehicle group. **c** EdU staining assay analysis. The cells with green fluorescence indicate cells undergoing proliferation, and the cells with blue fluorescence represent all the cells. A10 cells stimulated with 200 nM NPY in 0.5% serum were incubated with NPY Y1 receptor antagonist BIBO3304 in a concentration-dependent manner for 24 h. *Scale bar* 20  $\mu\text{m}$  in all images

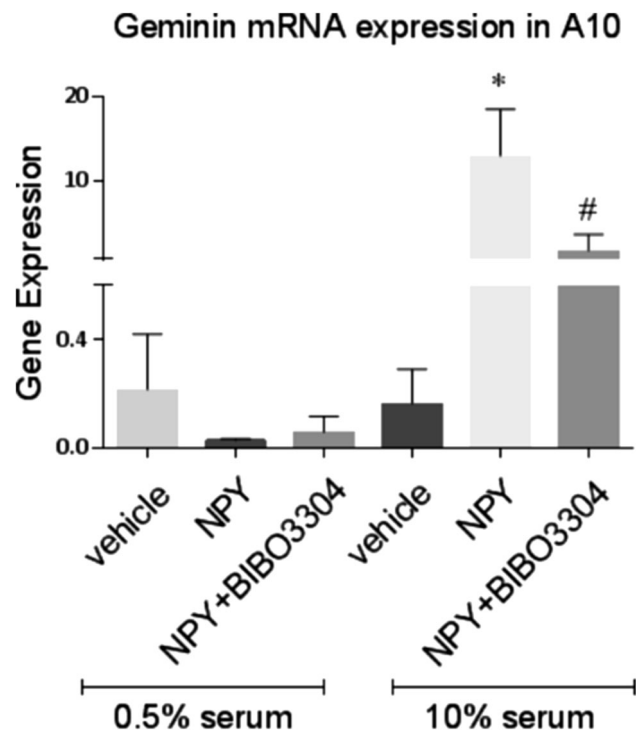
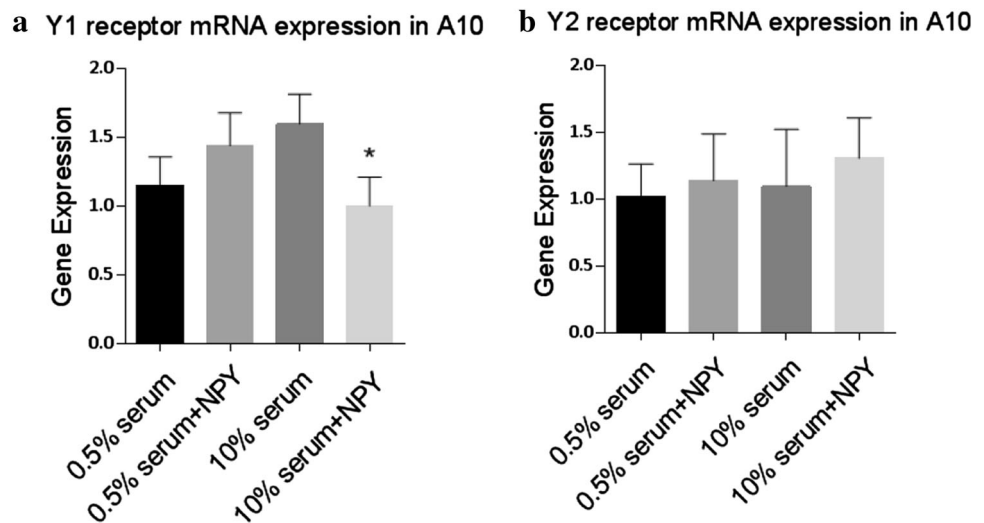


recent studies have suggested that overexpression of perivascular NPY makes contribution to atherosclerosis development [12, 22]. However, numerous studies suggested that NPY always increased growth of VSMCs that cultured in low or free serum. On the one hand, there were evidences that the makers of VSMC phenotype were regulated by serum concentrations in vitro and the phenotype switch of VSMCs was modulated by growth factors and cytokines secreted during process of atherosclerosis in vivo [23]. On the other hand, the switch of VSMCs from contractile type (differentiation) to synthetic type

(dedifferentiation) plays a critical role in atherosclerosis, such as dedifferentiated VSMCs promote plaque growth and stability [24]. Thus, it is worth exploring whether the response of contractile type or synthetic type is different when toward stimulation of NPY.

Our preliminary data suggested that expression of Osteopontin, a key marker of synthetic phenotype, is up-regulated when VSMCs were exposed to 10% serum and the condition also stimulated their growth [11]. However, we demonstrated that NPY only promoted proliferation of VSMCs incubated with 0.5% serum rather than 10%

**Fig. 2** Expression of NPY Y1 and Y2 receptors in VSMCs. **a** mRNA expression levels of Y1 in 0.5% serum, 0.5% serum + 200 nM NPY, 10% serum, and 10% serum + 200 nM NPY groups. \* $P < 0.05$  versus 10% serum group. **b** mRNA expression levels of Y2 in 0.5% serum, 0.5% serum + 200 nM NPY, 10% serum, and 10% serum + 200 nM NPY groups. There was no significant difference of Y2 expression among these four groups



**Fig. 3** mRNA expression of Geminin in VSMCs. A10 cells stimulated with 200 nM NPY in 0.5% serum or 10% serum were incubated with NPY Y1 receptor antagonist 10  $\mu$ M BIBO3304 for 24 h. NPY induced significant up-regulation of Geminin in 10% serum-cultured VSMCs compared with control group (10% serum). This regulation was effectively blocked by the Y1 receptor antagonist. \* $P < 0.05$  versus 10% serum group. # $P < 0.05$  versus NPY (10% serum)

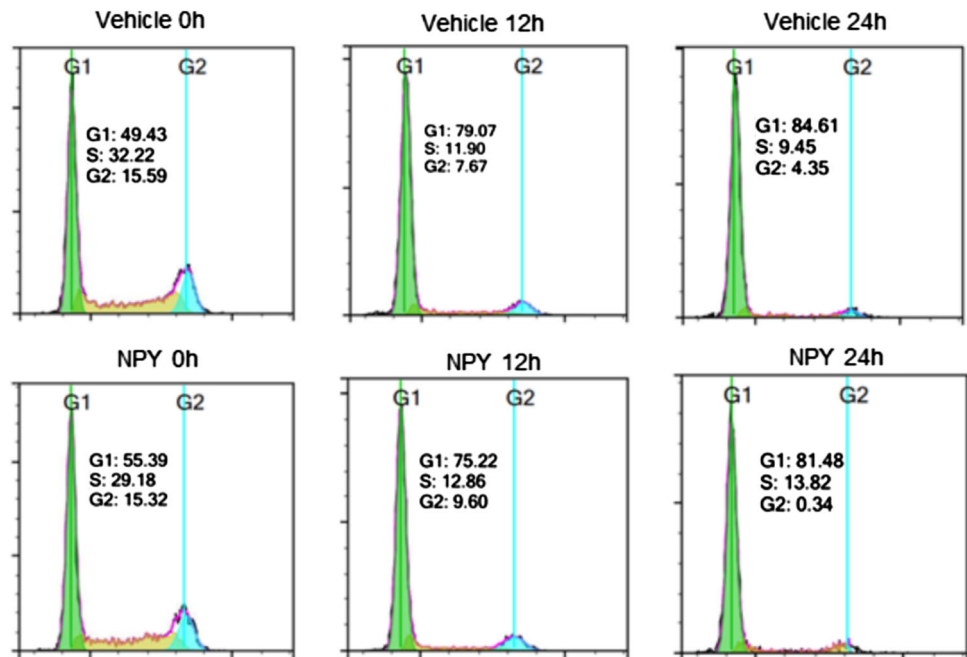
serum where VSMCs remained in differentiated and dedifferentiated state, respectively. It is well known that the proliferation-promoting effect of NPY in VSMC is mediated by Y1 receptors [8, 10, 25]. The results of Y1 receptor expression in VSMCs supported that down-regulation of Y1 receptor levels in VSMC synthetic type may

limit NPY to aggravate VSMC growth; it is likely because extra stimulating factor is not a necessity for synthetic type of VSMCs with cell proliferation activity and thus reduces Y1 receptor levels via feedback loop.

NPY induces mitogenesis signals in VSMCs via Y1 receptors [8]. We hypothesized that non-growth-promoting effect of NPY on VSMC synthetic type might be induced by alteration of cell cycle. Our previous study found that knockdown of Geminin, a cell cycle inhibitor, facilitated DNA synthesis and proliferation of VSMCs. Therefore, our present study determined whether NPY stimulation caused changes of Geminin expression in VSMC synthetic type (cultured in 10% serum). In line with our speculation, we observed an intensive increase of Geminin in VSMC synthetic type with the presence of 200 nM NPY, which was attenuated by antagonism of Y1 receptors. Furthermore, Geminin prevents DNA replication during S phase of cell cycle and up-regulation of Geminin may induce cell cycle arrest in S phase. In our study, flow cytometry analysis indicated that more cells remained in S phase after administration of NPY in VSMC synthetic type, which may be mediated by increased expression of Geminin.

We cannot exclude the possibility that some other factors might also be involved in the cell cycle arrest in S phase of VSMCs; however, we can confirm that the property of the NPY proliferative effect in VSMCs is influenced by phenotype of VSMCs. As for contractile type (differentiation), NPY promotes growth of VSMCs via interactions of multiple signaling pathways including calcium/calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC), and mitogen-activated protein kinase, MEK1/2 [25]. Instead of facilitating proliferation, NPY might negatively control VSMC growth when they transform into synthetic type (dedifferentiation).

**Fig. 4** NPY promotes cell cycle arrest in S phase of 10% serum-cultured VSMCs. A10 cells were stimulated with 200 nM NPY in 10% serum and harvested at 0, 12, and 24 h. The cells were labeled with propidium iodide (PI). Cell samples were analyzed by using a 488-nm excitation wavelength and a 610-nm emission wavelength



The molecular pathogenesis of VSMC proliferation in atherosclerosis is complicated and still far from fully illuminated. Our results suggest that NPY plays different roles in the VSMC proliferation during atherosclerosis, but there are still many questions that need further investigations. For instance, Serum contains many biologically active factors, whether other concentration gradients of serum and different time points of NPY administration are associated with the effect of NPY on VSMCs [26, 27]; abnormal phenotype switch induces VSMCs to secrete multiple cell cytokines, there is a possibility that these factors are also involved NPY-mediated changes of Geminin; calcium/calmodulin-dependent kinase II (CaMKII) pathway is the dominant element of NPY-induced growth of VSMCs, whose changes are also needed to be determined. The VSMC-specific knockdown models of Geminin and Y1 receptor are planned to use in the future to detect the interplays of NPY and Geminin in VSMCs, which will help us profoundly understand the molecular mechanisms of aberrant phenotypic switch and proliferation of VSMCs in response to NPY and other factors. In short, aberrant phenotypic switch of VSMCs is induced by multiple factors and NPY release is long-lastingly activated during the development of atherosclerosis; our findings showed that the stimulation of NPY on VSMCs could be a double-edged sword in the pathogenesis of atherosclerosis. This may provide surprising knowledge into treatments for atherosclerosis with the purpose of alleviating side effects of agents used for atherosclerosis and leads to generating a

more rational strategy to prevent diseases related to VSMC abnormal transformation.

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#### Compliance with ethical standards

**Conflict of interest** We declare there was no commercial, proprietary, or financial interest conflict in the products or companies described in this article.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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