

## Antisense oligodeoxynucleotides downregulated *c-sis* mRNA expression to inhibit proliferation of vascular smooth muscle cells<sup>1</sup>

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**KEY WORDS** proto-oncogenes; antisense oligonucleotides; vascular smooth muscle; genetic transcription; polymerase chain reaction; cultured cells; messenger RNA; cell division; thymidine; down-regulation (physiology)

### ABSTRACT

**AIM:** To assess the inhibitory effect of *c-sis* antisense oligodeoxynucleotides (ODN) on vascular smooth muscle cells (VSMC) proliferation and *c-sis* proto-oncogene mRNA expression.

**METHODS:** The VSMC were cultured with Dulbecco's modified Engle's medium (DMEM) containing synthesized *c-sis* antisense or sense oligomers. [<sup>3</sup>H]thymidine (TdR) incorporation into DNA was determined by liquid scintillation counter and the numbers of cells were counted by cell counting plate, the mRNA level was observed by using of reverse transcription-polymerase chain reaction (RT-PCR).

**RESULTS:** *C-sis* antisense ODN 2, 4, 6, 8, and 10  $\mu\text{mol} \cdot \text{L}^{-1}$  inhibited VSMC proliferation (10.3%  $\pm$  0.7%, 22.6%  $\pm$  0.9%, 31.0%  $\pm$  1.1%, 35.4%  $\pm$  0.9%, and 43.3%  $\pm$  1.2%) and [<sup>3</sup>H]TdR incorporation (6.8%  $\pm$  0.3%, 9.7%  $\pm$  0.7%, 29.0%  $\pm$  0.6%, 32.0%  $\pm$  0.7%, and 50.6%  $\pm$  1.3%) in a concentration-dependent manner. When VSMC were treated with antisense ODN 10  $\mu\text{mol} \cdot \text{L}^{-1}$  for 4 d, the highest inhibitory rate of VSMC

growth and that of [<sup>3</sup>H]TdR incorporation were 60.3%  $\pm$  1.0% and 56.3%  $\pm$  0.9%, respectively. RT-PCR showed that *c-sis* proto-oncogene antisense ODN downregulated *c-sis* mRNA expression obviously, while *c-sis* sense ODN did not inhibit VSMC proliferation and expression of *c-sis* proto-oncogene mRNA.

**CONCLUSION:** The *c-sis* antisense ODN inhibited VSMC proliferation and downregulated *c-sis* mRNA level.

### INTRODUCTION

The migration and proliferation of vascular smooth muscle cells (VSMC) were the key event in the development of atherosclerosis and restenosis after coronary angioplasty<sup>[1,2]</sup>. Advanced proliferative lesion of VSMC could occlude the artery by increasing the thickness of the intima of the artery. It was observed that platelet-derived growth factor (PDGF) was the major serum mitogen and growth promoting activator for VSMC and consisted of two polypeptide chains (A and B), PDGF-B was the product of *c-sis* proto-oncogene<sup>[3,4]</sup>. This suggested that the *c-sis* proto-oncogene played an important role in atherogenesis and restenosis after coronary angioplasty.

In recent years, using of antisense oligodeoxynucleotides (ODN) for the inhibition of VSMC proliferation has been suggested as a potential therapeutic approach in the prevention of coronary restenosis<sup>[5,6]</sup>. It is based on the findings that these antisense sequences hybridized to specific area of DNA or RNA, disrupted normal transcription and translation of the

<sup>1</sup> Project supported by Scientific Research Foundation of the Ministry of Public Health. No 94-1-346.

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Phn 86-20-8382-7812, ext 1317. Fax 86-20-8382-7872.

Received 1997-12-05

Accepted 1998-07-15

targeted gene<sup>17,8)</sup>. Thus the antisense sequences inhibit the expression of the targeted gene.

The present study was to assess the effect on VSMC proliferation by using antisense ODN directed against the expression of *c-sis* proto-oncogene, and to explore a new therapeutic approach for atherosclerosis, especially restenosis after coronary angioplasty by using antisense ODN.

## MATERIALS AND METHODS

**Cell culture** VSMC were isolated from the rabbit (New Zealand white rabbit,  $n = 6$ ,  $\hat{\circ}$ , 2.0 kg, Certificate No 26-97042, from the Experimental Animal Center of Sun Yat-Sen University of Medical Sciences, Guangzhou) aorta by an explant method<sup>9)</sup>. Briefly, the explants were placed in Dulbecco's modified Engle's medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS), benzylpenicillin sodium salt  $100 \text{ kU} \cdot \text{L}^{-1}$ , streptomycin sulfate  $100 \text{ ng} \cdot \text{L}^{-1}$ , and glutamine  $2 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  (20% FBS-DMEM). The culture was maintained at 37 °C in a humidified incubator with 5% CO<sub>2</sub> + 95% air. The cells showed a spindle-shaped appearance, and confluent VSMC in culture showed the characteristic "hill and valley" growth pattern. The identification of VSMC was confirmed by smooth muscle  $\alpha$ -actin staining.

**Synthesis of oligomers** 18-Mer antisense and sense ODN were synthesized with a gene assembler special (Pharmacia LKB). The iodine was replaced with sulfur to enhance stability to exonuclease degradation in serum while ODN synthesized<sup>10)</sup>. The ODN was lyophilized, resuspended in PBS, and quantified spectrophotometrically. ODN from the translated initial region of human mRNA of *c-sis* gene (118 - 135 bp) were used. The sequences were: antisense ODN (3'-TACTTAGCGACCGCCG-5'), and sense ODN (3'-GCCGGTCGTCGCTAAGTA-5').

**Assessment of cell proliferation and [<sup>3</sup>H]TdR incorporation** VSMC (passages 2 - 4) were suspended in 20% FBS-DMEM at a density of  $4 \times 10^7 \text{ cell} \cdot \text{L}^{-1}$ . One mL of the suspension was distributed to each well of a 24-well plate. After plating 24 h, cells were maintained in 0.5% FBS-DMEM for 24 h. Then the cell proliferation and [<sup>3</sup>H]TdR incorporation were assessed. Original medium was replaced with 20% FBS-DMEM to stimulate cell growth, nine of the wells were added [<sup>3</sup>H]TdR at final radioactivity of  $37 \text{ MBq} \cdot \text{L}^{-1}$ . At the same time of stimulation, ODN (antisense ODN or sense ODN) 2, 4, 6, 8, and  $10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  were added. In control cells no ODN was added. The cells were incubated for 24 h, then VSMC were trypsinized and counted by a blood cell counting plate, the intracellular radioactivity of [<sup>3</sup>H]TdR was determined with a liquid scintillation counter (LKB 1209, Rackbeta) after standing overnight at 23 °C. The percent inhibition was calculated: % inhibition =  $[1 - (\text{net growth of antisense or sense-treated cells} / \text{net growth of control cells})] \times 100\%$ . The inhibited percentage of [<sup>3</sup>H]TdR incorporation was calculated as that of the cell proliferation. The net growth of VSMC was obtained by subtracting the starting cell number from the cell number at indicated time points. The net [<sup>3</sup>H]TdR incorporation was obtained by subtracting the [<sup>3</sup>H]TdR incorporation from the stimulated point. ODN-treated (antisense or sense ODN) cells with concentration of  $10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$  were incubated for 1, 2, 3, and 4 d. Then the cells were trypsinized and counted, each experiment was triplicated.

***C-sis* mRNA level in cultured VSMC** VSMC were maintained in 0.5% FBS-DMEM for 24 h. The total RNA was extracted with a single step procedure<sup>11)</sup> to assess *c-sis* mRNA level of quiescent VSMC. Then 0.5% FBS-DMEM was replaced with 20% FBS-DMEM to stimulate

VSMC proliferation, *c-sis* mRNA expression in proliferating VSMC was determined at 1, 2, 3, and 4 d after stimulation respectively. ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$  (antisense or sense ODN) with 20 % FBS-DMEM was added into quiescent cultured VSMC for 24 h. *C-sis* mRNA level was determined by RT-PCR.

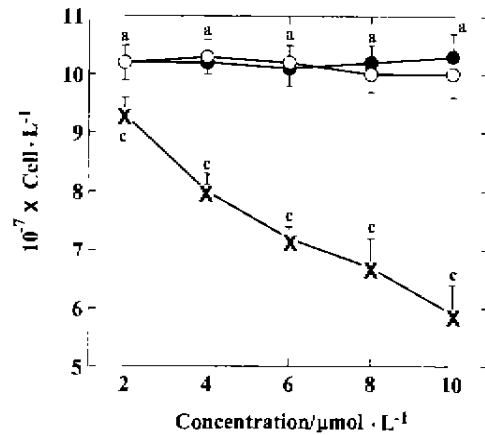
**RT-PCR** Total RNA from  $3 \times 10^6$  VSMC were used as template for first-strand cDNA synthesis with cDNA synthesis kit (Boehringer Mannheim). The primer sequences were: *c-sis* primer 5'-CCCAGCCCCCACCCTGGCC-3', 5'-GGCAATACAGCAAATACCA-3';  $\beta$ -actin primer 5'-AAGGATTCCTATGTGGGC-3', 5'-CATCTCTTGCTCGAAGTC-3'. PCR amplification of the cDNA was performed by using the Boehringer Mannheim PCR protocol. Briefly, an aliquot of cDNA was added to a reaction mixture containing primers  $20 \mu\text{mol} \cdot \text{L}^{-1}$  and Taq polymerase 5 units. Amplification was performed with a DNA thermal cycler (PTC-200, MJ Research, USA) for 20 ( $\beta$ -actin) and 30 (*c-sis*) cycles. The following thermal cycle profile was used: 1 min at 94 °C for denaturation, 1 min at 60 °C for annealing, 2 min for annealing for  $\beta$ -actin, and 2 min at 72 °C for extension. The RT-PCR products were electrophoresed in 2 % agarose gel with ethidium bromide stained. The results were analyzed with Bio-Rad 1000 Gel imagine densitometer system.

Data were expressed as  $\bar{x} \pm s$  and compared using ANOVA with unpaired *t*-test.

## RESULTS

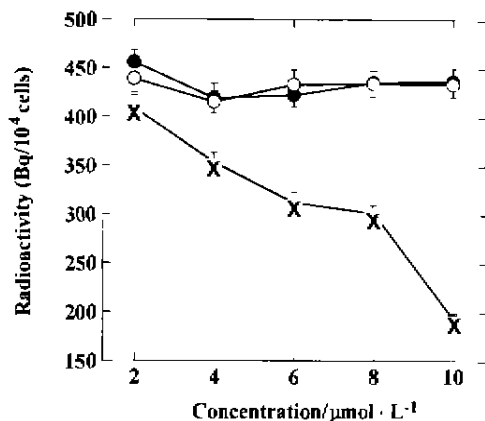
VSMC proliferation was lower in antisense ODN-treated group than that in control group ( $P < 0.01$ ). While sense ODN did not inhibit VSMC growth ( $P > 0.05$ ). This growth-inhibitory effect was raised with the increase of concentration of antisense ODN, in a concentration-dependent manner. The % of inhibition at the concentration of 2, 4, 6, 8, and  $10 \mu\text{mol} \cdot$

$\text{L}^{-1}$  was  $10.3 \% \pm 0.7 \%$ ,  $22.6 \% \pm 0.9 \%$ ,  $31.0 \% \pm 1.1 \%$ ,  $35.4 \% \pm 0.9 \%$ , and  $43.3 \% \pm 1.2 \%$ , respectively. (Fig 1)



**Fig 1.** Inhibitory effects of antisense (x) and sense ODN (●) on cultured VSMC proliferation.  $n = 9$  wells per group, experiment was repeated 3 times from 6 rabbits (2 rabbits  $\times$  3 times).  $\bar{x} \pm s$ .  $^a P > 0.05$ ,  $^c P < 0.01$  vs control (○).

The inhibitory rates of [ $^3\text{H}$ ]TdR incorporation at 2, 4, 6, 8, and  $10 \mu\text{mol} \cdot \text{L}^{-1}$  were  $6.8 \% \pm 0.3 \%$ ,  $9.7 \% \pm 0.7 \%$ ,  $29.0 \% \pm 0.6 \%$ ,  $32.0 \% \pm 0.7 \%$ , and  $50.6 \% \pm 1.3 \%$ , respectively, while VSMC growth treated with sense ODN and control medium plus [ $^3\text{H}$ ]TdR was not inhibited (Fig 2).



**Fig 2.** [ $^3\text{H}$ ]TdR incorporation into DNA was inhibited by *c-sis* antisense ODN. Experiment was repeated 3 times from 6 rabbits (2 rabbits  $\times$  3 times).  $\bar{x} \pm s$ . Control (○), sense ODN (●), antisense ODN (x).

The VSMC incubated with antisense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$  for 1, 2, 3, and 4 d produced an inhibition ( $P < 0.01$ , Fig 3). The % of inhibition were  $43.3 \% \pm 1.2 \%$ ,  $52.1 \% \pm 1.3 \%$ ,  $53.1 \% \pm 0.9 \%$ , and  $60.3 \% \pm 1.0 \%$ , respectively. When VSMC were cultured with antisense or sense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$ , and control medium plus  $[^3\text{H}]\text{TdR}$  for 1, 2, 3, and 4 d, the % of incorporation of  $[^3\text{H}]\text{TdR}$  in cells treated by antisense ODN versus the control medium were  $50.6 \% \pm 0.6 \%$ ,  $52.7 \% \pm 0.8 \%$ ,  $54.6 \% \pm 1.0 \%$ , and  $56.3 \% \pm 0.9 \%$ , respectively (Fig 4).

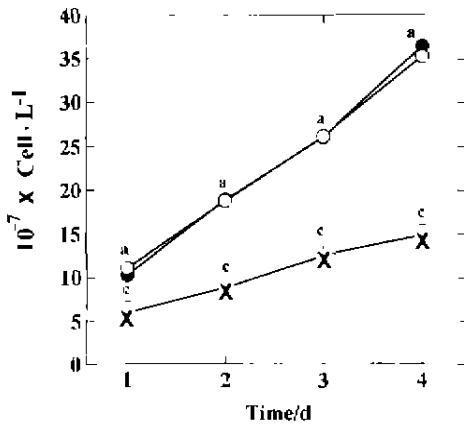


Fig 3. Proliferation of VSMC incubated with control (□), sense ODN (●) antisense ODN (×)  $10 \mu\text{mol} \cdot \text{L}^{-1}$ .  $n = 9$  wells per group, experiment was repeated 3 times from 6 rabbits (2 rabbits × 3 times).  $\bar{x} \pm s$ .  
<sup>a</sup> $P > 0.05$ , <sup>c</sup> $P < 0.01$  vs control.

VSMC growth in sense ODN and the control medium treatment were not much different ( $P > 0.05$ , Fig 3, 4). The decrease of incorporation of  $[^3\text{H}]\text{TdR}$  was essentially in good agreement with the decrease of number of VSMC after antisense, sense ODN, and control.

Amplified products showed a single band corresponding to the predicted size of 866 bp (Fig 5, 6).

In quiescent cultured VSMC, *c-sis* mRNA was not determined after agarose gel electrophore-

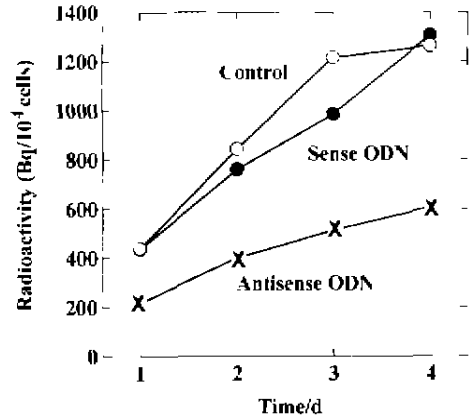


Fig 4.  $[^3\text{H}]\text{TdR}$  incorporation of VSMC incubated with *c-sis* antisense or sense  $10 \mu\text{mol} \cdot \text{L}^{-1}$  plus  $[^3\text{H}]\text{TdR}$ . Experiment was repeated 3 times from 6 rabbits (2 rabbits × 3 times).

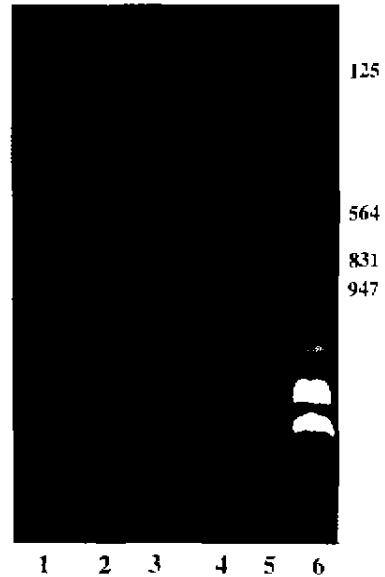


Fig 5. *C-sis* mRNA (866 bp) level in VSMC incubated with 20 % FBS-DMEM without ODN at 24, 48, 72, 96 h, and quiescent period. Lane 1) quiescent VSMC; 2) *c-sis* mRNA level in 96 h after 20 % FBS-DMEM stimulation; 3) *c-sis* mRNA level in 72 h after 20 % FBS-DMEM stimulation; 4) *c-sis* mRNA level in 48 h after 20 % FBS-DMEM stimulation; 5) *c-sis* mRNA level in 24 h after 20 % FBS-DMEM stimulation; 6) marker.

sis, after VSMC was stimulated with 20 % FBS-DMEM, expression of *c-sis* mRNA was increased

at 24 h and decreased at 72 h, the highest level was shown at 48 h. (Fig 5)

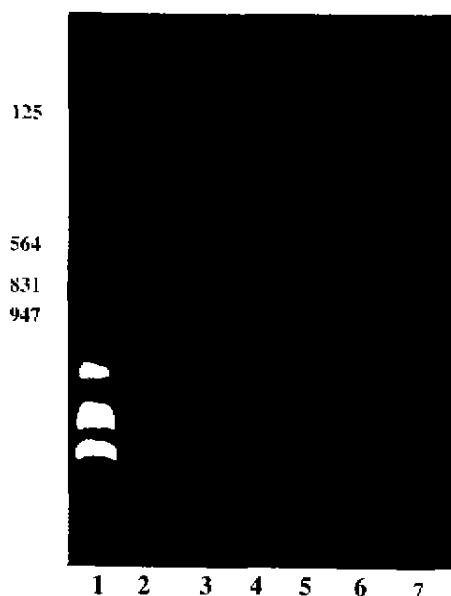


Fig 6. Effect of antisense ODN on *c-sis* mRNA (866 bp) expression in VSMC. Lane 1) marker; 2) VSMC was treated with sense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$ ; 3) VSMC was incubated without ODN; 4) VSMC was incubated with 20 % FBS-DMEM plus antisense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$ ; 5)  $\beta$ -actin mRNA (532 bp) in VSMC treated with no ODN; 6)  $\beta$ -actin mRNA (532 bp) in VSMC treated with sense ODN; 7)  $\beta$ -actin mRNA (532 bp) in VSMC treated with antisense ODN.

*C-sis* antisense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$  reduced mRNA level markedly, while sense ODN did not affect *c-sis* expression.  $\beta$ -Actin mRNA in the antisense-, sense-treated, and control VSMC presented no major differences. (Fig 6)

## DISCUSSION

Recently, antisense ODN were widely researched in vascular proliferating disease. Our study showed that VSMC proliferation was associated with increased expression of *c-sis* proto-oncogene, and supported the potential applicability of the antisense as a therapeutic approach in the restenosis after coronary

angioplasty.

In this study, *c-sis* antisense ODN were used, because VSMC derived from injured artery produced a 5-fold increase of PDGF-B mRNA as compared with those from normal vessel<sup>[11]</sup>, this revealed that increased expression of *c-sis* mRNA was very important in VSMC proliferation. We hypothesized that upregulation of *c-sis* expression would be able to stimulate proliferation of VSMC and downregulation of *c-sis* expression would be able to inhibit proliferation of VSMC. The result demonstrated that *c-sis* transcripts were elevated in proliferative, but not quiescent VSMC (Fig 5). Whether enhanced *c-sis* expression was the only reason to cause VSMC proliferation was still unknown, because the previous study showed that PDGF could increase significantly *c-myc* proto-oncogene expression<sup>[12]</sup>. Now *c-myc* proto-oncogene is believed to regulate gene expression on transcriptional and posttranscriptional levels, which result in cell proliferation and differentiation<sup>[6,13]</sup>. Thus, there may be a regulatory linkage between *c-sis* and *c-myc* oncogene. This part of study is being investigated in our study.

Antisense ODN exerts inhibitory effect, there are several possible mechanisms: 1) antisense ODN binds to double-stranded DNA, then inhibits the regeneration and transcription of gene<sup>[7]</sup>; 2) antisense ODN abolishes mRNA processing, maturation and translation<sup>[8]</sup>; 3) antisense ODN activates RNAase H which shears RNA strand without binding with antisense ODN<sup>[14]</sup>; 4) antisense ODN antiproliferation activity is not due to a hybridization, rather, a stretch of four contiguous guanosine residues<sup>[15]</sup>; but the view on the research was not supported further<sup>[16]</sup>. According to our results, it was certain that *c-sis* antisense ODN inhibited transcription of *c-sis* proto-oncogene and down-regulated the expression of *c-sis* mRNA. Therefore, we postulated that downregulation of

*c-sis* expression resulted in decreased VSMC production of *c-sis* protein — PDGF-B, a powerful stimulant for proliferation and migration of VSMC. Whether *c-sis* antisense ODN inhibited reproduction, posttranscriptional mRNA processing of *c-sis* proto-oncogene or *c-myc* expression needed to be researched in the future.

Our result showed that the cause-and-effect relation between *c-sis* expression and VSMC proliferation was obvious. These observations suggested strongly that the inhibition of VSMC proliferation was probably caused by a sequence-specific effect of *c-sis* antisense ODN down-regulating *c-sis* expression.

The advantage of antisense ODN may be high specific inhibition of the target genome without influencing other genes, and selective suppression of proliferating VSMC without inhibiting quiescent VSMC, because quiescent VSMC have not an enhanced mRNA level (Fig 5). In our study, the proliferation of VSMC was inhibited in a concentration-dependent manner by *c-sis* antisense ODN (Fig 1, 3), the inhibitory rate of VSMC growth was  $60.3\% \pm 1.0\%$  with *c-sis* antisense ODN  $10 \mu\text{mol} \cdot \text{L}^{-1}$ , and the inhibitory effect of *c-sis* antisense ODN was observed in a low concentration range ( $2 - 10 \mu\text{mol} \cdot \text{L}^{-1}$ ) in this study. Previous report showed that high concentration of *c-myc* antisense ODN could inhibit the proliferation of VSMC<sup>[6,15]</sup>, it suggests that *c-sis* antisense ODN may be more effective than *c-myc* antisense ODN in inhibiting VSMC proliferation. In addition, the difference of the used proto-oncogene antisense ODN and cell lines may cause different antiproliferative effect, thus, the postulation that *c-sis* ODN was more effective than *c-myc* ODN in inhibiting VSMC growth needed to be further investigated.

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### 反义寡脱氧核苷酸下调 *c-sis* 基因表达抑制血管平滑肌细胞增殖<sup>1</sup>

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**关键词** 原癌基因类; 反义寡核苷酸类; 血管平滑肌; 遗传的转录; 聚合酶链反应; 培养的细胞; 信使 RNA; 细胞分裂; 胸苷; 下调(生理学)

**目的:** 研究 *c-sis* 反义寡脱氧核苷酸(ODN)对 *c-sis* 表达及血管平滑肌细胞(VSMC)增殖抑制效应。  
**方法:** 用合成 *c-sis* 正、反义 ODN 培养 VSMC; 用液闪测定 [<sup>3</sup>H]TdR 掺入并细胞计数, 观察细胞增殖; 用逆转录 PCR, 评价 *c-sis* 表达。  
**结果:** *c-sis* 反义 ODN 2, 4, 6, 8, 10  $\mu\text{mol}\cdot\text{L}^{-1}$  抑制 VSMC (10.3%  $\pm$  0.7%, 22.6%  $\pm$  0.9%, 31.0%  $\pm$  1.1%, 35.4%  $\pm$  0.9%, 43.3%  $\pm$  1.2%) 和降低 [<sup>3</sup>H]TdR 掺入 (6.8%  $\pm$  0.3%, 9.7%  $\pm$  0.7%, 29.0%  $\pm$  0.6%, 32.0%  $\pm$  0.7%, 50.6%  $\pm$  1.3%) 呈有剂量依赖性。反义 ODN 10  $\mu\text{mol}\cdot\text{L}^{-1}$  培养细胞 4 d, 最大抑制率达 60.3%  $\pm$  1.0%, [<sup>3</sup>H]TdR 掺入降低 56.3%  $\pm$  0.9%, *c-sis* mRNA 表达明显降低; 而正义 *c-sis* ODN 对 VSMC 无抑制, 细胞数和 [<sup>3</sup>H]TdR 掺入及 *c-sis* mRNA 水平与对照无差异。  
**结论:** *c-sis* 反义 ODN 明显下调 *c-sis* mRNA 表达, 显著抑制 VSMC 增殖。

(责任编辑 杨如华)

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