Influence of homeobox B2 antisense oligodeoxynucleotides on the biological characteristics of *in vitro* cultured primary human umbilical vein endothelial cells

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ABSTRACT

This study aims to explore the influence of homeobox B2 (HOXB2) antisense oligodeoxynucleotides (asodn) on the biological characteristics of *in vitro* cultured primary human umbilical vein endothelial cells (HUVECs). The distribution of HOXB2 asodn in the HUVECs was observed by fluorescent labelling, and the influence of different concentrations of HOXB2 asodn on the DNA synthesis of HUVECs was assessed. Flow cytometry and a reverse transcriptase-polymerase chain reaction (RT- PCR) method were employed to observe the influence of HOXB2 asodn on HOXB2 expression and the HUVEC cell cycle. After the induction of liposome, the nuclear fluorescent staining of HOXB2 asodn was weaker 15 min after transfection and the staining reached the strongest level at 4–8 h but then weakened and disappeared by 16 h after transfection. This indicated that endothelial DNA synthesis could be inhibited by HOXB2 asodn in a dose-dependent manner. Furthermore, the HUVECs could be delayed in their passage from G1 to S. Simultaneously, expression of HOXB2 mRNA had decreased significantly by 24–48 h after transfection. Clearly, HOXB2 plays important roles in the proliferation of endothelial cells and also affects the cell cycle.

KEY WORDS: Antisense oligodeoxynucleotides.
HOXB2 gene.
Human umbilical vein endothelial cells.

Introduction

The homeobox gene was first described by McGinnis *et al*.[1] in drosophilae. The proteins encoded by this kind of gene are transcription factors that contain a helix-transition-helix (HTH) structure and a characterised homologous region that contains a DNA conjunctive region that encodes 61 amino acids – a highly conservative feature during evolution. As a key gene in embryonic development,[2] it plays important roles in cell growth, differentiation and migration and the expression of specific genes in some tissues.[3]

Previous studies have shown that the homeobox gene, especially HOXB, is closely related to the proliferation of haematopoietic progenitor cells.[4] However, there is no report of the relationship between the homeobox gene and the proliferation of endothelial cells.[5]

Recent studies have demonstrated gene expression of HOXA,[6] HOXB[7] and HOXD,[8] and this is controlled by factors that modulate endothelial growth. For example, vascular endothelial growth factor (VEGF) can increase endothelial cell numbers and increase the expression of HOXB. However, TNP-470 prevents endothelial cells entering the G1 phase of the cell cycle without an evident change in the level of HOXB gene expression.

These results suggest that HOXB genes play important roles in the changing vascular endothelial morphology during angiogenesis. To date, the most convincing evidence for HOX gene participation in angiogenesis is the fact that suppression of homeobox D10 expression can inhibit angiogenesis.[9] HOXD3 was highly expressed in endothelial cells during proliferation and might induce the cells to form lumen on the basement membrane.

The expressions of αvβ3 and uPA induced by fibroblast growth factor (bFGF) were retarded and this blocked HOXD3. However, over-expression of HOXD3 induced an increase in these two proteins and altered endothelial morphology, resulting in the development of an endothelioma on the chicken allantoic membrane. These results suggest that HOXD3 participates in early angiogenesis.[8]

Recently, Myers *et al*.[10] found that the formation of capillaries by dermal microvascular endothelia was hampered by blocking HOXB3. This effect might be related to a decrease in EphA2 receptor phosphorylation. An increase in capillary density might be induced by constant or

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over-expression of HOXB3 on the chicken allantoic membrane, but no endothelioma formation was observed in this case.

This suggests that HOXB3 and HOXD3 play different roles in angiogenesis, with HOXD3 accelerating endothelial migration and HOXB3 affecting capillary development. One of the characteristics of the HOX genes is that expression of downstream genes is regulated by upstream genes. This implies that HOXB2 might control angiogenesis, probably by modulating endothelial proliferation, and thus play an important role in wound healing.

As a key cell in wound repair, endothelial cell differentiation and proliferation play important roles in angiogenesis and wound healing,[11] and the vascularisation of a wound could provide oxygen and nutrients[12,13] for the acceleration of wound healing. Thus, the degree of vascularisation is one indicator of wound healing, but there has been little study of the relationship between HOX genes and endothelial biological features.

This study explores the role of HOXB2 gene expression in endothelial cells. It uses an antisense technique employing antisense oligodeoxynucleotides (asodn) to probe the role of HOXB2 and its effects on cellular proliferation and the cell cycle. In addition, the influence of HOXB2 asodn on HOXB2 expression is assessed.

Materials and methods

Reagents used in this study included M199 nutrient (Gibco), fetal calf serum (FCS, Hyclone), trypsin (Gibco), tripure nucleic acid extraction reagent, AMV reverse transcriptase reagent, liposome (Boehringer Mannheim, Germany) and tritiated thymidine (^{3}H-TdR, Chinese Nuclear Energy Institute, Beijing).

The isolation, culture and identification of primary human umbilical vein endothelial cells (HUVECs) were performed using the methods described by An Jing *et al*.[14] The endothelial cells used were within four generations of culture.

To prepare the asodn, 18 bases were designed complementary to the starting codon of HOXB2 mRNA (5′-CATGGCTTTCAATGGTGG-3′ [81–64nt]). For the random oligodeoxynucleotide (Odn) both base number and ratio were the same as for asodn, but arranged randomly as 5′-AGCTTGCTACATGTGTGG-3′. Both HOXB2 asodn and Odn genes were not homologous to the genes in Genbank and were synthesised by the Shanghai Biological Co., thio-modified and partially labelled with a fluorescent dye.

Briefly, a solution containing the 3′-5′-phosphorylated oligonucleotide (0.1–0.2 μmol/L) in 700 μL water was supplemented with 240 mg ethylenediamine dihydrochloride (1.8 mmol/L) and 120 mg 1-ethyl-3(3′-dimethylaminopropyl) carbodiimide (0.6 mmol/L). The mixture was vortex-mixed and incubated at room temperature for 3 h.

The oligonucleotide ethylenediamine derivative was then separated from excess reagents by size exclusion on a NAP-10 column (Pharmacia LKB Biotechnology), followed by precipitation using a 10-fold excess of 2% $LiClO_4$ in acetone. Subsequently, this derivative was dissolved in 60 μL water and the solution was supplement with 20 μL of 1 mol/L carbonate buffer (pH 11) and 80 μL dimethylacetamide. Finally, 1 mg fluorescein isothiocyanate (FITC, 2.5 μmol/L) was added.

The mixture was vortex-mixed and incubated at room temperature in the dark for 2–4 h. The FITC-Odn conjugate was separated from salts and free FITC by size exclusion on a NAP-10 column and purified by 20% polyacrylamide gel electrophoresis (PAGE) in the presence of 7 mol/L urea. The presence of an FITC group at the ends of the Odn was confirmed by spectrophotometer measurement. The ratio between the extinction of FITC at 490 nm and total extinction of FITC-Odn at 260 nm corresponded to the presence of approximately two fluorescein residues to one Odn molecule.

For the design and synthesis of the primer, HOXB2 was constructed according to previous methods and β-actin was designed in-house. HOXB2: P15′–AGA AAT CCG CCA AGA AAC CCA GCC–3′ (379–403 bp), P25′–GAG AAG ACG TCT TCT GGC AAT GGC–3′ (942–965 bp); β-actin: P15′–TGG AAT CCT GTG GCA TCC ATG AAA C–3′, P25′–TAA AAA CGC AGC TCA GTA ACA GTC CG–3′. The amplification segment was 348 bp.

The above primers were all synthesised and purified by the Shanghai Biological Co. and they were made to be 10 μmol/L in sterile deionised water before use.

To prepare liposome-oligodeoxynucleotide compounds, liposomes and oligodeoxynucleotides (8:1) were dissolved in volumes of M199 nutrient without serum and antibiotics. The solutions were left at room temperature for 45 min and then mixed. The mixture was used to prepare solutions with oligodeoxynucleotide concentrations of 0.25 mg/L, 0.5 mg/L, 1.0 mg/L and 2.5 mg/L. The solutions were stored at 4°C.

The HUVECs were cultured in 24-well culture plates beneath glass. The culture medium was discarded when 60% confluence had been achieved. HOXB2 labelled by fluorescence (1.25 mg/L) and coated by liposome was added to the cells and the mixture was cultured for 6 h before the culture medium was replaced. The cells were harvested at 15 min, 30 min, 1 h and then hourly thereafter up to 72 h.

Briefly, the cell harvesting method was as follows. The culture medium was discarded and the cells were washed (x3) with cold Dulbecco's phosphate-buffered saline (D-PBS) and then fixed for 15 min in 10% formalin prepared in D-PBS. The cells were washed again (x3) in cold D-PBS and mounted in a glycerine mounting fluid The cells were observed under a fluorescence microscope (U-ND6-2, Olympus, Japan).

The effects of HOXB2 asodn on cell proliferation were determined by a ^{3}H-TdR incorporation test. Endothelial cells in their third to fifth logarithmic growth periods were digested by 0.5% trypsin and 0.04% EDTA. The mixture was centrifuged at 1000 rpm for 10 min and the pellet was resuspended in M199 containing 10% FCS. Cell viability was identified by trypan blue to be greater than 95%, and then 2500 cells were inoculated in each well of a 96-well culture plate.

When 60–70% of the cells were fused they were treated with different concentrations of liposome-HOXB2 asodn, liposome-Odn and liposome alone. The supernatant was replaced after 6 h. Then, 1 mL of ^{3}H-TdR was added to each well after 36 h and this was repeated (x3) before allowing culture to continue for a further 12 h. The reaction was stopped and the culture medium was removed.

The cells were digested by trypsin then filtered using a fibrous filter paper. The papers were dried and then placed in scintillation bottles into which 2.5-diphenyloxazole

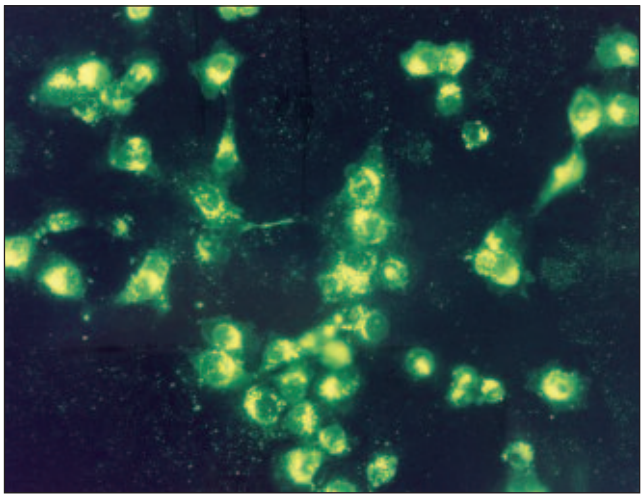

Fig.1. The distribution of HOXB2 asodn in HUVECs eight hours after transfection, showing fluorescence in the nucleus and as spots in the cytoplasm.

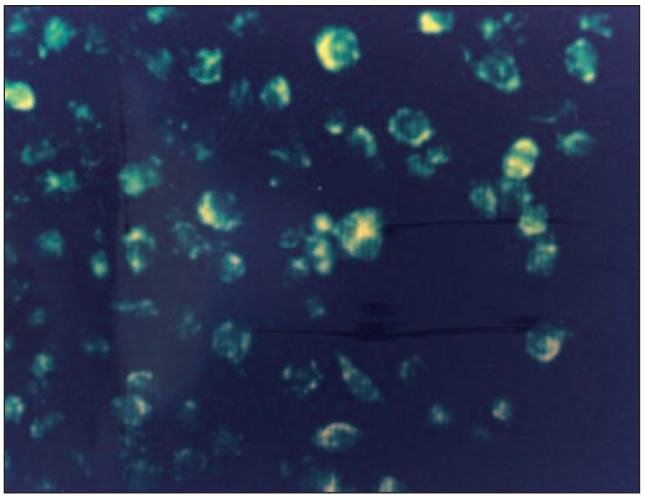

Fig. 2. Distribution of HOXB2 asodn in HUVECs 16 hours after transfection. Fluorescence is beginning to disappear.

(POP)/para-phenylene-phenyloxazole (POPOP) xylene scintillation fluid was added. Activity was measured (counts per minute [cpm]) using a scintillometer. The incorporating ratio was calculated as follows: cpm in test group/cpm in control group x 100%.

To determine the effect on the cell cycle, cells were treated as above until 60% of the endothelial cells had fused. Then, prepared liposome-HOXB2 asodn, liposome-Odn (Odn: 1.25 mg/L) and liposome. Six hours later the culture medium was replaced by M199 nutrient solution containing 10% FCS and the cells were cultured for another 24 h.

Thereafter, the culture was digested by pancreatin and the cells were harvested and washed in cold PBS (x3). Cell concentration was adjusted to 1x10⁶/L and then 1.5 mL propidium iodide was added (for 15 min) to stain the dead cells. The cells were removed by filtration through a 350 sieve and the cell cycle was analysed by flow cytometry.

To obtain cell cycle measurements, cells were resuspended in PBS containing 10 μg/mL propidium iodide and 20 μg/mL Hoechst 33342, incubated in the dark for 30 min at 37°C and analysed using a dual-laser flow cytometer. The cell cycles were obtained using the advanced Multicycle AV program (Phoenix Flow Systems).

In order to determine the change in HOXB2 mRNA expression in the target gene in HUVECs after being acted on by HOXB2 asodn, the following procedure was followed. The HUVECs were transferred to a 100 mL culture bottle. When 60% of the cells were confluent, the culture medium was discarded. Equal amounts (2 mL) of liposome-coated Odn (2.5 mg/L) and HOXB2 asodn were added to the cells and cultured for 6 h. The culture medium was replaced and culture was allowed to continue for a further 24, 48 and 72 h.

Tripure nucleic acid extraction reagents were employed to extract the total RNA from the cells in each group. The RNA purity (A260/A280) was determined by ultraviolet spectrophotometry. Then, 3 μg RNA was obtained and the downstream primer was added. A reverse transcriptase (RT) method was carried out using AMV reverse transcriptase (reaction volume: 20 μL). The reaction conditions were 37°C for 60 min and 90°C for 5 min, then storage at –20°C. The reverse transcription product was harvested (5 μL) and used in a polymerase chain reaction (PCR) method using 1xPCR buffer solution, 0.2 mmol/L dNTPs, 0.5 μmol/L primer and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase. Total volume was 50 μL.

A 5 μL sample of the PCR product was harvested at 28, 30, 32 and 35 recycling time points and analysed by electrophoresis to determine the optimum PCR recycling times. It was found that the HOXB2 PCR products showed exponential amplification at 28–32 recyclings. The reaction conditions for HOXB2 were set at 95°C for 1 min, 52°C for 1 min and 72°C for 2 min, repeated for 30 cycles, with an extension at 72°C for 10 min. For β-actin, annealing was carried out at 60°C for 1 min, with an extension at 72°C for 1 min, repeated for 30 cycles.

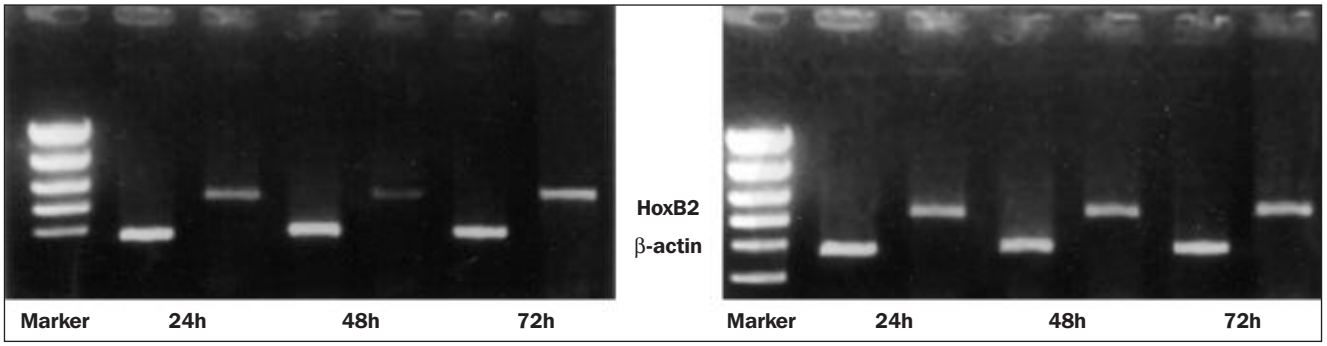

Fig 3. Changes of HOXB2 RT-PCR amplified products in HUVECs treated with Odn (right) and HOXB2 asodn (left).

The PCR products were electrophoresed through 2% sepharose and observed under ultraviolet (UV) light. The ratio of HOXB2 to β-actin was used as the parameter of HOXB2 expression level. Analysis of the DNA electrophoretic bands was undertaken using a gel scanner in order to quantify the HOXB products.

All the data were processed using the SPSS 10.0 statistical software for Windows. Data were processed by Student's *t*-test and χ^2 test. $P<0.05$ and $P<0.01$ were regarded as different and significantly different, respectively.

Results

Weak nuclear staining was observed in 10–15% of HUVECs 15 min after addition of HOXB2 asodn mediated by liposome. Nuclear fluorescence was identified in 60–70% of the cells 4–8 h after addition of HOXB2 asodn (Fig. 1). At the same time, speckled intracytoplasmic fluorescence was seen. The fluorescence began to disappear at 16 h after the addition of HOXB2 asodn (Fig. 2).

The effects of liposome-conjugated concentrations of oligonucleotides and the effects of oligonucleotides on the ratio of ^{3}H-TdR incorporation in HUVECs are shown in Tables 1 and 2, respectively. The asodn results indicate an obvious dose-dependent effect, while the Odn group exhibited no evident inhibitory effects, even when Odn concentration was 2.5 mg/L.

The effects of liposome-conjugated oligonucleotides on the cell cycle of HUVECs are shown in Table 3. The influence of HOXB2 asodn on the cell cycle was seen as an increase of cells in the G1/0 phase, while the number of cells in S phase decreased significantly. The G2/M phase showed no obvious change. However, HOXB2 exerted no obvious influence on the cell cycle in the liposome and Odn groups.

When endothelial cells were treated by liposome and liposome-coated Odn for 24, 48 and 72 h, the expression of endothelial HOXB2 mRNA exhibited no obvious change when determined by RT-PCR. However, when the cells were treated with liposome-transfected HOXB2 asodn for 24, 48 and 72 h, the mRNA expression level decreased after 24 h and reached the lowest level at 48 h and recovered to near normal level at 72 h (Fig. 3, Table 4).

Discussion

Irrespective of their size, homeobox gene fragments all possess a 183 bp region (the homology structure region) that encodes a polypeptide of 61 amino acids. The HOX genes are major controlling genes for embryonic development and cell differentiation, and play important roles in many aspects of cellular function (Table 5).

HOXB2 is a member of the class I HOX genes. It was studied by Wu *et al*.[15] who showed that the growth of mature T cells and natural killer (NK) cells could be inhibited by HOXB2 asodn. This indicates that HOXB2 might promote the cellular proliferation of T cells and NK cells. However, despite the fact that endothelial cells are derived from the same haemopoietic progenitors, the effect of HOXB2 on the endothelium remains unclear.

In the current study, synthetic and thio-modified HOXB2 asodn coated by liposome was employed to observe its

Table 1. Effects of liposome conjugated various concentrations of oligonucleotides on the value of CPM of HUVECs (±SD).

Odn concentration	Counts per minute		
	Asodn	Odn	DOTAP
0.25 mg/L	9197±723	9324±499	9569±263
0.50 mg/L	8569±361*	9528±377	9680±256
1.25 mg/L	6532±569†	9630±222	9635±194
2.50 mg/L	4922±444‡	9539±3144	9643±555

*$P<0.05$, †$P<0.01$, ‡$P<0.01$ *vs.* DOTAP group.

Table 2. Effects of oligonucleotides on the ratio of ^{3}H-TdR incorporation in HUVECs (±SD)

Odn concentration	Incorporation rate (%)	
	Asodn	Odn
0.25 mg/L	96.10±7.56	97.44±5.21
0.50 mg/L	88.52±3.72*	98.42±3.89
1.25 mg/L	67.80±5.90†	99.0±2.8
2.50 mg/L	49.63±4.15‡	96.19±3.16

*$P<0.01$, †$P<0.01$, ‡$P<0.01$ *vs.* DOTAP group.

Table 3. Effects of liposome-conjugated oligonucleotides on the cell cycle of HUVECs (±SD)

	G1/0	S	G2/M
Control	36.41±7.31	61.17±4.44	2.42±1.09
DOTAP	38.21±6.25	59.18±5.50	2.61±0.76
Odn	38.04±5.32	58.76±4.98	3.20±2.21
Asodn	52.52±3.07*	43.74±1.93†	3.74±0.27

*$P<0.01$, †$P<0.01$ *vs.* control group.

Table 4. Changes of the relative expression of HOXB2 mRNA in HUVECs treated by Odn and HOXB2 asodn (±SD).

	24 hours	48 hours	72 hours
Odn	0.789±0.029	0.800±0.045	0.783±0.014
HOXB2 asodn	0.612±0.029*	0.423±0.024†	0.759±0.010

*$P<0.05$, †$P<0.01$ *vs.* Odn group.

Table 5. The role of HOX genes.

Hox gene	Role	References
HoxB3, HoxD3	Participates in angiogenesis	8, 10
PRX-2, HoxB13	Modulates scar-free healing	16
Hox11, HSI1	Modulates the cell cycle	23, 24, 25, 26
HoxB cluster	Proliferation and differentiation of haematopoietic stem cells	28, 29
Hox gene	Participates in carcinogenesis	30, 31

influence on the biological characteristics of endothelial cells. The results indicate that liposome can coat Odn successfully. In addition. predesigned HOXB2 asodn was shown to block the expression of the target HOXB2 gene with relatively strong specificity.

Results of the RT-PCR method indicated that the expression of HOXB2 could be inhibited by HOXB2 asodn, but that expression of HOXB2 mRNA could not be inhibited by Odn. In the ^{3}H-TdR incorporation experiment, the liposome group served as a control to avoid the possible influence of pure liposome on cellular ^{3}H-TdR incorporation. HOXB2 asodn significantly inhibited endothelial DNA synthesis in a dose-dependent manner, but Odn in both similar and also higher concentrations exhibited no inhibitory effect. This suggests that HOXB2 can accelerate endothelial proliferation.

With regard to the cell cycle, it has long been recognised that a growth control point exists between the G1 and S phases,[17] which is the most important modulating point in the cell cycle. At this point, cells decide whether or not to divide, differentiate, undergo apoptosis or enter the G0 phase to repair damaged DNA. Acting as an initiating and governing factor during the G1 phase, cyclin D1 may play an important role in integrating the ectogenesis proliferating signals.[18]

Many proteins take part in the modulation of cellular mitosis at this point, and homologous protein can function as a transcription factor. Lawrence *et al*.[19] suggested that the targeting genes of mammal homologous proteins were the genes encoding extracellular proteins, adhesion molecules, growth factors and gene families affecting cellular growth, tumour development and metastasis. Hence, HOXB2 might modulate the cell cycle by some mitogens.

In the present study, there were evident changes in the endothelial cell cycle after HUVECs were co-cultured with 1.0 mg/L HOXB2 asodn for 24 h. This indicates that HOXB2 asodn delayed the transition of endothelial cells from G1 to S. At the same time, the results indicate that Odn and liposomes have no effect on the cell cycle.

London *et al*.[20] demonstrated similar results with phosphorodiamidate morpholino oligomer (PMO), an antisense inhibitor of MMP-9, which could attenuate the angiogenesis and human prostate cancer cell invasion and tumorigenicity. Brooks *et al*.[21] also found that the HOXD3 and integrin alpha v beta 3 genes were highly expressed in newly formed vascular endothelia in tumours, and that an integrin alpha v beta 3 antibody could inhibit angiogenesis. Furthermore, they showed that increased expression of HOXD3 could stimulate the production of new blood vessels.

However, the molecular mechanism of HOX proteins and their participation in the modulation of cellular proliferation and the change of phenotype remains unclear. Krosl *et al*.[22] found that the cellular proliferation induced by HOXB4 up-regulated the protein levels of Jun-B and Fra-1, which led to an increase in AP-1 activity. These changes lead ultimately to an increase in cyclin D1 level. Therefore, it is believed that HOX proteins are key factors in the control of the cell cycle. Nevertheless, the fact that HOXB2 asodn inhibits endothelial cells transferring from G1 to S implies that HOXB2 proteins have a role in modulating cell proliferation and the cell cycle.

The mechanism of asodn action remains unclear, although some hypotheses have been put forward.[27] It is suggested that it may 1) inhibit the initiation process of mRNA translation; 2) form a local asodn-mRNA double chain structure with mRNA and activate RNase H, which leads to a decrease in mRNA; 3) block the specific transportation of mRNA across the nuclear membrane; or, 4) inhibit the initiation and extension of gene duplication.

The HOXB2 asodn employed in the present study was designed according to the starting site of transcription, which might be realised by the specific combination of HOXB2 asodn and HOXB2 mRNA. This activates RNase H to cut and degrade combined mRNA, leading to the decreased expression of HOXB2 protein products.

Clearly, endothelial proliferation was inhibited by HOXB2 asodn in a dose-dependent manner. Furthermore, the transition of endothelial cells into S phase of the cell cycle was delayed by HOXB2 asodn, and the expression of target genes was inhibited over 24–48 h of co-culture.

Endothelial cells have an important role in wound repair, thus HOXB2 might also have a role in wound healing. However, the full mechanism of action of these genes requires further study. □

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