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ORIGINAL RESEARCH PAPER

Enhancement of bone formation by genetically-engineered bone marrow stromal cells expressing BMP-2, VEGF and angiopoietin-1

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Abstract To explore the potential of combined delivery of osteogenic and angiogenic factors to bone marrow stromal cells (BMSCs) for repair of critical-size bone defects, we followed the formation of bone and vessels in tissue-engineered constructs in nude mice and rabbit bone defects upon introducing different combinations of BMP-2, vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) to BMSCs with adenoviral vectors. Better osteogenesis and angiogenesis were found in co-delivery group of BMP-2, VEGF and angiopoietin-1 than any other combination of these factors in both animal models, indicating combined gene delivery of angiopoietin-1 and VEGF165 into a tissue-engineered construct produces an additive effect on BMP-2-induced osteogenesis.

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Introduction

Tissue-engineered bone is playing an increasingly more important role in critical-size bone defect repair. Bone healing can be distilled into two major synergistic and interactive processes: osteogenesis and angiogenesis. Thus, integration of these involved aspects may be more effective in achieving better bone defect repair (Murphy and Mooney 1999). Bone morphogenetic proteins (BMPs) and vascular endothelial growth factors (VEGFs) are widely used as osteogenic and angiogenic factors (Levy et al. 1998; Gerber et al. 1999). However, VEGF induced new vessels are immature and leaky due to increased permeability (Schwartz et al. 2000). On-the-other-hand, angiopoietin-1 (Ang-1) is required in the latter stage of angiogenesis for the stabilization and maturation of blood vessels and can counteract VEGF-induced inflammation in endothelial cells (Thurston et al. 1999). Therefore, the combined application of Ang-1 and VEGF may be more effective than application of either factor alone for enhancing genesis of functional vessels in tissue engineering (Chae et al. 2000). Despite this, until now there have been no reported studies on the combined delivery of BMP, VEGF, and angiopoietin in bone-tissue

engineering. In the current study we investigated the potential, feasibility and possible risks of introducing these three factors to bone marrow stromal cells (BMSCs) with adenoviral vectors for repair of critical-size bone defects.

Materials and methods

Two animal models were adopted in this study. One was for in vivo angiogenesis and osteogenesis observation using tissue-engineered grafts subcutaneously implanted on the back of nude mice. The grafts were constructed with gene-modified rabbit BMSCs seeded on cubic β-tricalcium phosphate (β-TCP) scaffolds (Bio-Lu, China). The other model was for observation of ex vivo critical-size bone defect repair in rabbits using grafts constructed with gene modified autologous BMSCs seeded on cylindric β-TCP scaffolds (Fig. 1). We conducted our study according to our institution's animal care guidelines, and the experimental protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine.

Adenoviral vectors adopted

Four adenoviral vectors were used in this study, all of which were plasmid-based systems with different gene inserts. The vectors were Ad-BMP-2, Ad-VEGF165, Ad-Null, and a bicistronic vector carrying hVEGF165 and Ang-1 genes (Ad-Bic) separated by an internal ribosome entry site region (kindly provided by Ruowen Ge). The vectors were E1-deficient recombinant adenovirus propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation.

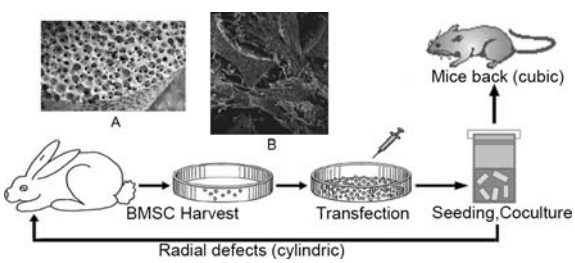

Fig. 1 Schematic of study **a** SEM surface view of blank scaffold; **b** SEM view of cells growing inside scaffold

Preparation of the cells and scaffold constructs

Bone marrow was harvested from rabbit under general anaesthesia. BMSCs were then isolated and amplified to the third passage when transfection was carried out. Twenty-four hours later, the equivalent number of cells were trypsinized and seeded in a hypobaric environment onto the β-TCP scaffolds, which had a volume porosity of 70%. We used cubic scaffolds with an edge length of 5 mm in nude mice and 4 mm diameter, 15 mm long cylindric scaffolds in rabbits. The cells and scaffold constructs were subsequently cultured for 6 days in vitro before implantation. RT-PCR, western blotting and enzyme-linked immunosorbent assay (ELISA) of target genes were performed after transfection.

Reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was extracted using the TRIzol method (Invitrogen Life Technologies) from samples of Ad-BMP, Ad-Bic and Ad-VEGF transduced BMSCs at 0, 7, 14, 21 and 28 days after transfection for RT-PCR analysis. Sense and antisense primers for BMP2, VEGF165 and angiopoietin-1 were designed on the basis of nucleotide sequences deposited in GenBank and are listed in Table 1. The RT-PCR was performed by using the QIAGEN One-Step RT-PCR Kit (QIAGEN). Briefly, a 500 ng template total RNA of each sample was added into each master mixture. Reverse transcription was carried out at 50°C for 30 min, followed by the initial PCR activation step for 15 min at 95°C, denaturation at 94°C, annealing at 64°C for 0.5 min, and extension at 72°C for 1 min. Thirty cycles were performed, followed by a final extension at 72°C for 10 min.

Western blot analysis

Bone marrow stromal cells were grown in 6-well tissue culture plates and transfected with vectors as previously described. Cell samples from each group were obtained at 7 days post-transfection. Western blotting was then performed according to conventional protocol. Antibodies adopted were: goat anti-hBMP2 monoclonal antibody (R&D system), mouse

Table 1 Primer sequences for RT-PCR

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)
BMP-2	TTGGAGGAGAAACAAGGTG	AACAATGGCATGATTAGTGG
VEGF165	CCTTGCTGCTCTACCTCCA	CAAATGCTTTCTCCGCTCT
Angiopoietin-1	AACCGAGCCTATTCACAG	GCCACTTTATCCCATTCA

anti-hVEGF monoclonal antibody (R&D system) and rabbit anti-hAngiopoietin-1 monoclonal antibody (Chemicon).

ELISA for hBMP2 and hVEGF165 expression

The detection of hBMP2 and hVEGF165 secreted from a transfected BMSCs was done with a hBMP2 ELISA kit (R&D system) and hVEGF165 ELISA kits (Chemicon). The transfected BMSCs were grown in 6-well tissue culture plates at 2×10^5 cells/well. Ad-LacZ transfected and non-transfected BMSCs were used as control. The supernatant from each well was collected at 7, 14, 21 and 28 days and the samples were kept frozen at −20°C until they were used for assay. The assay was performed according to the supplier's instructions.

In vivo osteogenesis and angiogenesis

The engineered constructs were implanted subcutaneously on the backs of 42 (84 sides) 8-week-old male nude mice. Each side was randomly assigned to one of 7 groups, with 12 sides per group. The treatments were as follows:

(1) Co-administration of Ad-BMP-2 and Ad-Bic: During seeding of the transfected cells to the scaffolds, the suspensions of Ad-BMP-2-transfected cells and of Adv-Bic-transfected cells were combined in a ratio of 4:1.
(2) Co-administration of Ad-BMP-2 and Ad-VEGF165: During seeding of the transfected cells to the scaffolds, the suspensions of Ad-BMP-2-transfected cells and of Adv-VEGF165-transfected cells were combined in a ratio of 4:1.
(3) Administration of Ad-BMP-2 alone: All seeded cells were Ad-BMP-2-transfected.
(4) Administration of Ad-Bic alone: All seeded cells were Ad-Bic-transfected.
(5) Administration of Ad-VEGF165 alone: All seeded cells were Ad-VEGF165-transfected.
(6) Administration of Ad-Null alone: All seeded cells were Ad-Null-transfected.
(7) Administration of rat bone marrow stromal cells (rBMSCs) alone: Only non-transfected cells were seeded.

At 4, 8, and 12 weeks after surgery, four implants were retrieved in each group and fixed in 10% (v/v) neutral formalin for histologic analysis [hematoxylin and eosin (H&E) staining] and immunohistochemical analysis.

Ex vivo critical-size bone defect repair

The engineered constructs were implanted in 15 mm of bone defects in the middle third of the radii in 20 (40 sides) male New Zealand White rabbits that weighed 3000 ± 250 g each. Each defect was randomly assigned to one of four groups, with 10 defects per group. The type, ratio, and concentration of cells in each group were the same as the corresponding groups in the nude mice experiment. The treatments were as follows:

(1) Co-administration of Ad-BMP-2 and Ad-Bic
(2) Administration of Ad-BMP-2 alone
(3) Administration of rBMSCs alone
(4) No implantation of construct into defects

At 2, 6, and 12 weeks after surgery, laterolateral X-rays of the forearms were obtained for all rabbits while they were under general anesthesia. After the animals were sacrificed, the forearms were harvested for biomechanical tests (maximal loading) and histologic analysis of in vivo bone formation.

Histology and histomorphometry

All samples were processed for histologic examination. Tissue sections of 6 μm thick were obtained for

H&E staining. The bone-tissue area for each section was determined by dividing the total number of bone pixels by the number of pixels for the total implant in nude mice or the trabecula area percentage in rabbits using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Three sections per side were analyzed in a blinded fashion, and the mean area of these three sections was used for statistical analysis.

To determine the extent of blood vessel in-growth, 4-, 8-, and 12-week tissue sections from nude mice were immunostained for CD31. Blood vessels, indicated by CD31 staining, were counted manually at 100× magnification. Only circular CD31 staining was interpreted as indicating a blood vessel.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Statistically significant differences in histomorphometric analysis were determined using one-way ANOVA.

Results and discussion

To our knowledge, this is the first report exploring the orchestration of BMP, VEGF and angiopoietin in tissue engineering. A new bicistronic vector carrying the genes of VEGF165 and Ang-1 was developed and used in this study. The ratio (4:1) of cells when they were combined after transfection was chosen on the basis of other reports (Peng et al. 2002) and our earlier work (data not shown). We also used two animal models rather than one because they are not interchangeable for our purposes. Angiogenesis and osteogenesis cannot be properly studied in New Zealand rabbits because of the genotypic diversity among individual rabbits, and it is difficult to make a critical-size bone defect in nude mice.

RT-PCR, western blotting and ELISA of target genes were performed after transfection during this study as outlined in the materials and method section and indicated stable gene expression and protein secretion for 3–4 weeks. Importantly, the concentration of VEGF and angiopoietin-1 was not compromised in Ad-Bic transfection compared with Ad-VEGF transfection (Fig. 2).

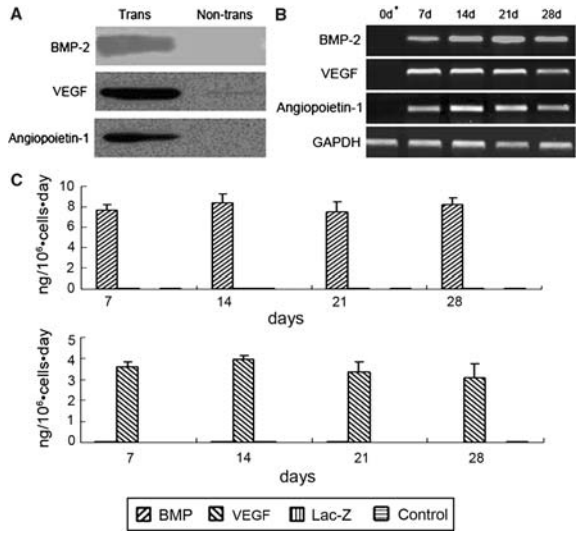

Fig. 2 Western-blotting, RT-PCR and ELISA for transfected genes **a** Western-blotting; **b** RT-PCR; **c** ELISA, All indicating stable expression of genes ($n = 4$ for each group)

Compared with amplification autologous endothelial cells or in situ administration of angiogenic factors, gene transduction to autologous stromal cells is simpler and more cost-effective for delivery of angiogenic factors in vivo. Angiogenic factors are crucial for angiogenesis especially in the early period after the engineered construct is implanted, and several reports have indicated that prolonged administration of VEGF, no matter what vector is used for transduction, will lead to an increased risk of formation of endothelial cell-derived vascular tumors (Lee et al. 2000; Masaki et al. 2002). The transient release of angiogenic factors caused by adenovirus transfection (erased within 2–4 weeks) is therefore potentially safer than the prolonged release by retrovirus or lentivirus. It is for this reason that we used replication-deficient adenoviral vectors in this study.

Figure 3 illustrates the main outcomes of the in vivo osteogenesis and angiogenesis assay in nude mice. Although the highest vessel density was found in the VEGF-Ang-1 group and VEGF-only group at 4 weeks after surgery, it remained steady only in the VEGF-Ang-1 group at 12 weeks after operation. Accordingly, at 8 and 12 weeks after surgery, vessel density in BMP-2-VEGF165-Ang-1 group was higher than BMP-2-VEGF165 group, confirming that addition of VEGF and Ang-1 to BMP-2 improves the

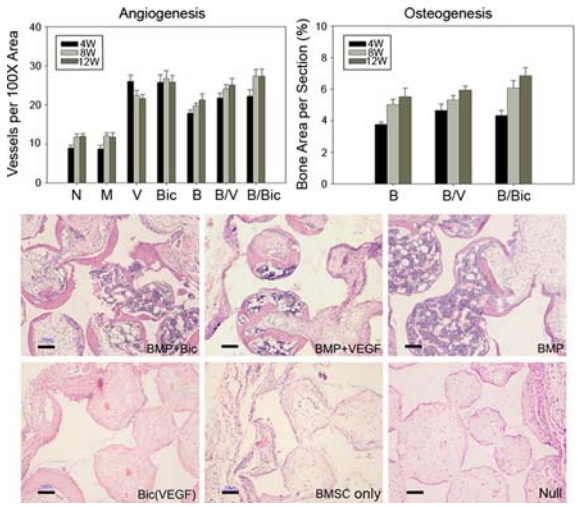

Fig. 3 Osteogenesis and angiogenesis in nude mice. Angiogenesis: At 4 weeks after surgery, angiogenesis in VEGF-Ang-1 and VEGF-only groups was significantly better than co-administration groups or BMP-2-only group ($P < 0.05$). In samples taken 8 weeks after surgery, vessel density in BMP-2-VEGF-Ang-1 and VEGF-Ang-1 groups was higher than BMP-2-VEGF group ($P < 0.05$). After 12 weeks, vessel density in the BMP-2-VEGF-Ang-1 group was significantly higher than all other groups ($P < 0.05$). Osteogenesis: Bone formation was mainly located on the wall of pores contacting the back. In the two co-administration groups, bone formation was significantly more than BMP-2-only group ($P < 0.05$), whereas without significant difference between each other ($P > 0.05$). At 8 and 12 weeks post-operation, BMP-2-VEGF-Ang-1 group showed significantly more bone formation than BMP-2-VEGF group ($P < 0.05$) or BMP-2-only group ($P < 0.01$). In the other groups, pores were filled with fibrous tissue. No osteogenesis was observed in the groups without BMP-2 administration. N = Null; Bic = bicistronic vector carrying VEGF and angiopoietin-1 genes; B = BMP = bone morphogenic protein; V = VEGF = vascular endothelial growth factor 165; M = BMSC = bone marrow stromal cell ($n = 12$ for each group) (bar = 100 μm)

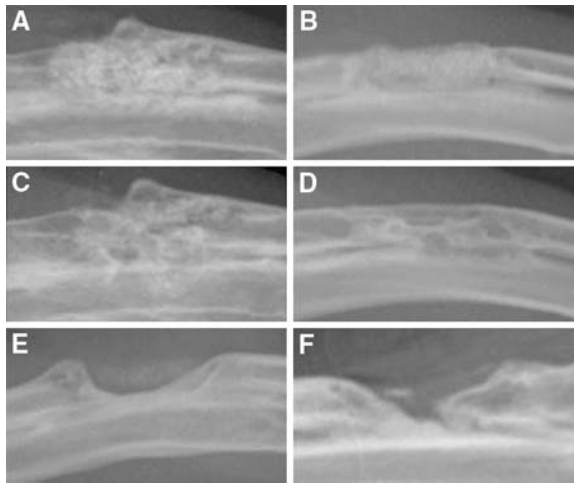

Fig. 4 Radiographs of treated defects taken at different time-points after surgery At 6 weeks after surgery, more bony callus can be observed in the co-administration group than in the Ad-BMP-2-only group, whereas at 12 weeks after surgery, bony union had been achieved in both groups. In the non-transfected rBMSC groups, the contour of implanted constructs could still be seen until 12 weeks after surgery. **a** Co-administration of Ad-BMP-2 and Ad-Bic, 6 weeks; **b** administration of Ad-BMP-2 only, 6 weeks; **c** co-administration of Ad-BMP-2 and Ad-Bic, 12 weeks; **d** administration of Ad-BMP-2 only, 12 weeks; **e** non-transfected bone marrow stromal cells, 12 weeks; **f** blank graft, 12 weeks

angiogenic effect observed when there was the addition of VEGF alone to BMP-2. Since osteogenesis proceeds in parallel with angiogenesis in the presence of BMP-2, the addition of Ang-1 to the BMP-2-VEGF system should contribute to better bone formation as well as vessel formation. This is supported by the higher osteogenesis in BMP-2-VEGF165-Ang-1 group than other groups.

The feasibility and effect of this combination on critical-size bone defect repair was explored in New Zealand white rabbits. The experimental groups were designed on the basis of the results in nude mice. Radiological and morphometric evaluation (Figs. 4, 5 and 6a) were both consistent with those in nude mice, indicating significant improvement on bone formation. Additionally, in the co-administration group, 5 of the 10 bone marrow cavities had recanalized in the co-administration group, but only 1 of the 10 cavities in the Ad-BMP-2-only group had recanalized. To our surprise, the biomechanical characteristics (Fig. 6b, c) of the samples in the BMP-2-VEGF165-Ang-1 group were not significantly different to those of the BMP-2-only group. This is possibly because the blood supply in the bone-defect model was confluent to allow both groups to achieve bone union. If this is the reason, bone volume would be the only indicative parameter of angiogenesis-induced improvement on bone formation.

We note that there are some concerns about the safety of adenoviral vectors. We carried out the gene therapy method ex vivo, which is simpler and more controllable than in vivo because viral vector particles would hardly be conveyed into the body and tests for safety could be run before seeding or implantation if necessary. To date we have detected no evidence of oncogenesis in our studies.

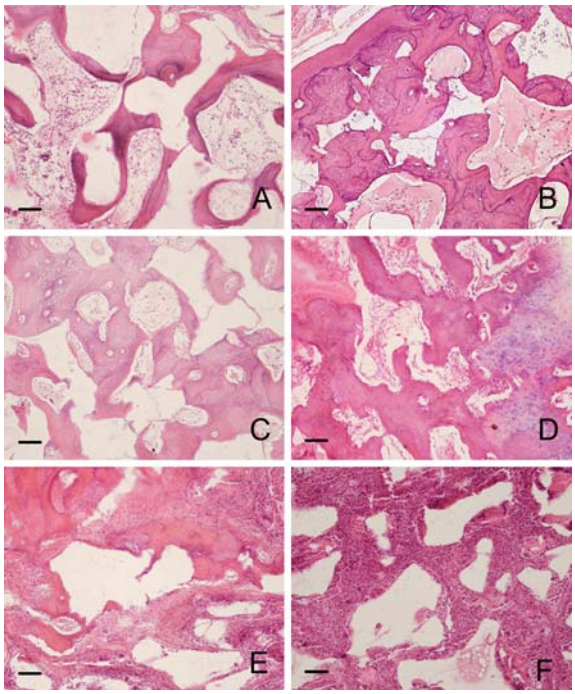

Fig. 5 Rabbit samples stained with hematoxylin and eosin. New woven bone and cortex bone formation with irregular osteon formation were found in the co-administration and the Ad-BMP-2-only groups. More new bone can be observed in the co-administration group than in the Ad-BMP-2-only group. The control groups turned out to be non-union, in which only fibrous tissue could be seen in the defect area. **a** administration of Ad-BMP-2 only, 6 weeks; **b** administration of Ad-BMP-2 only, 12 weeks; **c** co-administration of Ad-BMP-2 and Ad-Bic, 6 weeks; **d** co-administration of Ad-BMP-2 and Ad-Bic, 12 weeks; **e** non-transfected bone marrow stromal cells, 12 weeks; **f** blank scaffold, 12 weeks ($n = 10$ for each group) (bar = 100 μm)

In conclusion, this study confirmed the importance of combining multiple factors in bone regeneration. Combined factors can also be used in engineering a variety of other tissue types because regeneration of all tissues is dependent on the interplay of various growth factors and cell types. Our results show that combined gene delivery of Angiopoietin-1 and VEGF165 into a tissue-engineered construct produces an additive effect on BMP-2-induced osteogenesis through enhanced angiogenesis compared with either therapy alone, even better than that produced by the coadministration of VEGF and BMP-2. To our knowledge, this is the first study showing the efficacy of treatment combining Ang-1, VEGF, and BMP-2 in the field of bone-tissue

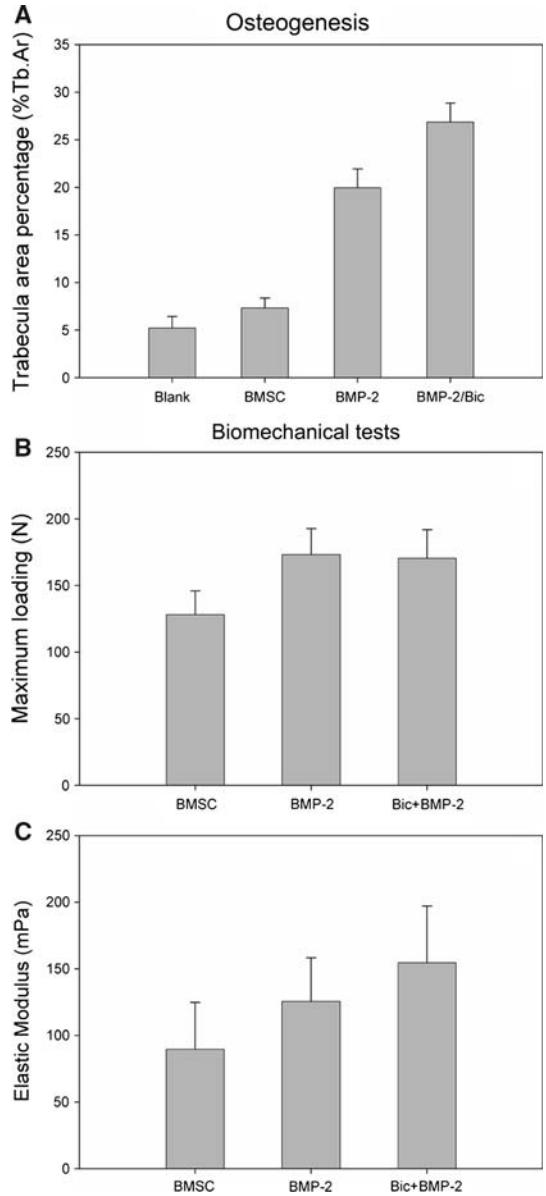

Fig. 6 Osteogenesis and biomechanical tests in rabbits: The trabecula area percentage in the co-administration group (26.85% ± 2.01%) was significantly higher than BMP-2-only group (19.95% ± 1.92%; $P < 0.05$), which was also significantly higher than the non-transfected groups (7.332% ± 1.03%; $P < 0.05$). The difference in maximal loading between the co-administration group and the Ad-BMP-2-only group was not statistically significant ($P > 0.05$), although both were higher than the non-transfected group. Similar results were found for the elastic modulus. **a** Osteogenesis; **b** maximum loading; **c** elastic modulus. BMP = bone morphogenic protein; BMSC = bone marrow stromal cell. BMP = bone morphogenic protein; BMSC = bone marrow stromal cell ($n = 10$ for each group)

engineering. We expect that combined growth-factor gene therapy will become an accepted curative modality in critical-size bone defects.

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