JUN siRNA regulates matrix metalloproteinase-2 expression, microvascular endothelial growth and retinal neovascularisation

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Summary

Transcription factors link changes in the extracellular environment with alterations in gene expression. As such, these molecules serve as attractive targets for intervention in pathological settings. Since JUN has been linked with microvascular disease in humans, we hypothesised that small interfering RNA (siRNA) targeting this immediate-early gene may be useful agents that suppress endothelial growth and neovascularisation. Here we show that *Jun* siRNA inhibits *Jun* mRNA and protein expression in murine microvascular endothelial cells, blocks cell proliferation and suppresses migration in a scratch-wound assay. It also inhibits three-dimensional tubular formation on basement membrane extracts and reduces angiogenesis in mice bearing Matrigel plugs as subcutaneous implants. Single intravitreal administration of *Jun* siRNA reduces neovascularisation in a murine model of proliferative retinopathy, and suppresses endothelial JUN and matrix metalloproteinase-2 (MMP-2) immunoreactivity in retinal vessels, data supported by its repression of MMP-2 expression and gelatinolytic activity in vitro. Co-administration of TGFβ with the siRNA reverses this neovascular inhibitory effect, which is in turn abrogated by cis-9-octadecenoyl-N-hydroxylamide, consistent with the involvement of a metalloproteinase such as MMP-2. Thus, JUN siRNA can serve as a specific inhibitor of aberrant endothelial and neovascular growth.

Key words: JUN, siRNA, Gene targeting, Endothelial cells, Retinal neovascularisation

Introduction

The angiogenic process involves a complex series of cellular events including microvascular sprouting, endothelial proliferation, migration and tubule formation. Although it is known that some of these events are controlled at the level of transcription by nuclear regulatory factors such as Ets-1 (Iwasaka et al., 1996), hypoxia-inducible factor-1 (Boudreau et al., 1997), FOS (Marconcini et al., 1999) and early growth response-1 (Fahmy et al., 2003), more work is needed to provide greater understanding of the transcription factors involved. This information would help facilitate the design of factor-specific, anti-angiogenic strategies.

Recent studies by our group using gene-specific catalytic DNA (DNAzymes) demonstrated a role for the basic region leucine zipper protein and immediate-early gene product, JUN in tumour angiogenesis (Zhang et al., 2004). JUN, a member of the AP-1 superfamily, is activated by multiple angiogenic stimuli including vascular endothelial growth factor (Zhang et al., 2004), (basic) fibroblast growth factor-2 (Viard et al., 1993) and serum (Zhang et al., 2004). JUN has been linked with neovascular disorders in humans. For example, JUN is expressed in the proliferative membranes of patients with proliferative diabetic retinopathy but not in patients with quiescent retinopathy (Ren et al., 2000). JUN might also be one of the factors influenced by endostatin in its inhibition of angiogenesis (Abdollahi et al., 2004).

Small interfering RNAs (siRNAs) provide a new approach to gain insight into the function(s) of the targeted gene in biological systems. siRNAs are RNA duplexes of 21-23 nucleotides (nt) in length that facilitate mRNA destruction in a RISC (RNA-induced silencing complex)-dependent manner (Elbashir et al., 2001; Hamilton and Baulcombe, 1999). In the present study, we evaluated the capacity of novel siRNA molecules targeting *Jun* mRNA to serve as inhibitors of JUN and JUN-dependent gene expression, microvascular endothelial cell proliferation, migration, tubule formation and retinal neovascularisation.

Results

Jun siRNA inhibits JUN and MMP-2 protein expression, and proliferation of murine microvascular endothelial cells

Synthetic siRNA targeting nts 2465-2485 of murine *Jun* mRNA (GenBank Acc. No. J04115) was introduced into growth-quiescent bEND-3 cells using the commercial transfection agent, RNAiFect, before incubation in medium containing fetal bovine serum (10%) for 2 hours. Murine, rather than human, microvascular endothelial cells were used for in vitro experiments because the siRNA would be evaluated in murine models. Serum induced JUN protein expression (Fig. 1A), which was inhibited by *Jun* siRNA but not by its scrambled counterpart, *Jun* siRNAscr at 0.1 and 0.4 μM, relative to levels of β-actin (Fig. 1A).

To complement these data, and demonstrate the functional relevance of siRNA inhibition of JUN protein, we used luciferase activity as a surrogate measure of JUN-AP-1 DNA-binding activity. Two tandem JUN-AP-1 DNA binding sites were subcloned into the *Sma*I site of pGL3-prom 5′ to the SV40 promoter upstream of firefly luciferase cDNA. Inducible JUN-AP-1-dependent reporter gene expression in transfected bEND-3 cells was inhibited by the *Jun* siRNA in a sequence-dependent manner (Fig. 1B). RT-PCR analysis also revealed that *Jun* mRNA expression was suppressed by the siRNA (Fig. 1C). An siRNA molecule (Egr-1 siRNAscr) of irrelevant sequence but identical size as *Jun* siRNA, like *Jun* siRNAscr, failed to influence inducible *Jun* mRNA (Fig. 1C) or JUN-AP-1-dependent reporter activity (Fig. 1B). *Jun* siRNA also suppressed TGFβ-inducible mRNA expression of matrix metalloproteinase-2 (MMP-2), a JUN-dependent gelatinase (Zhang et al., 2004) implicated in extracellular proteolysis during angiogenesis (Pepper, 2001) at 0.4 μM (Fig. 1D). The siRNA inhibited TGFβ-inducible-gelatinolytic activity in the culture supernatant corresponding to 72 kDa, the size of pro-MMP-2 (Fig. 1E).

We next examined the effect of siRNA on bEND-3 proliferation by quantifying cell numbers 2 days following serum stimulation of growth-quiescent cells. bEND-3 proliferation was strongly induced by serum (Fig. 2) and inhibited by the *Jun* siRNA, whereas siRNAscr did not inhibit at any concentration tested (Fig. 2). Cell counts in the scrambled siRNA cohort were not different from those in the mock-transfected group (Fig. 2). A second siRNA targeting *Jun* (siRNA2) also inhibited endothelial cell proliferation, whereas its own scrambled counterpart (siRNA2scr) failed to affect cell growth (Fig. 2).

Jun siRNA inhibits bEND-3 regrowth after injury

The capacity of *Jun* siRNA to block bEND-3 cell regrowth after wounding was next assessed by quantifying cell numbers in the denuded zone. In this system, cells regrow into the denuded zone virtually to completion within 2 days in the absence of siRNA but in the presence of vehicle (Fig. 3A,B). This reparative process, which involves both migration and proliferation, was suppressed by the *Jun* siRNA. siRNA inhibition was sequence specific because cell counts in the scrambled siRNA cohort did not differ from those in the mock-transfected group (Fig. 3A,B).

Jun siRNA blocks bEND-3 tubular formation on Matrigel in vitro

Seeding an endothelial cell suspension onto Matrigel (basement membrane extract) facilitates the relatively rapid formation of a three-dimensional tubular capillary-like network. The *Jun* siRNA inhibited tubule formation within 6 hours (Fig. 4), whereas network formation in the presence of *Jun* siRNAscr was indistinguishable from the vehicle control (Fig. 4).

Jun siRNA inhibits angiogenesis in murine Matrigel implants

Subcutaneous Matrigel implantation in C57BL/6 mice triggers an angiogenic response from the host vasculature and the eventual colonisation of the plugs within 14 days (Fahmy et al., 2003). *Jun* siRNA inhibited angiogenesis in these plugs by ~50% (Fig. 5). By contrast, *Jun* siRNAscr possessed no inhibitory effect on new blood vessel formation within the plug (Fig. 5) indicating again, sequence-specific inhibition by *Jun* siRNA.

Jun siRNA inhibits JUN protein expression and retinal neovascularisation in mice following hyperoxia-induced proliferative retinopathy

We reasoned that because *Jun* siRNA blocks matrix angiogenesis in mice (Fig. 5), the molecule may be useful in a pathophysiologically relevant angiogenic setting such as ocular angiogenesis. Exposure of neonatal mice to hyperoxic conditions followed by normoxia results in retinal neovascularisation (Smith et al., 1994) (Fig. 6A). Single intravitreal administration of the JUN siRNA inhibited retinal neovascularisation compared with mice treated with siRNAscr (Fig. 6A). Blood vessel counts in mice treated with siRNAscr did not differ from those treated with vehicle alone (Fig. 6A) indicating that siRNA inhibition was sequence specific and not merely due to a mass effect. Histological examination further indicated that the siRNA did not produce adverse morphological effects in the retina, nor did terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) immunostaining indicate apoptosis (data not shown).

Recent studies have demonstrated that retinal angiogenesis is compromised in MMP-2-deficient mice (Ohno-Matsui et al., 2003). Since JUN regulates MMP-2 expression (Fig. 1D) (Zhang et al., 2004), and MMP-2 is induced in endothelial cells upon exposure to TGFβ (Fig. 1D) (Zhang et al., 2004), we evaluated the effect of the siRNA on JUN and MMP-2 expression in retinal blood vessels by immunohistochemistry. JUN and MMP-2 are poorly expressed in vessels of normal mice but dramatically induced by hyperoxia or normoxia (Fig. 6B,C). The siRNA suppressed inducible JUN (Fig. 6B) and

Fig. 1. *Jun* siRNA suppresses JUN and MMP-2 protein expression in murine microvascular endothelial cells. (A) Growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of *Jun* siRNA, siRNAscr or mock-transfected and incubated in medium containing serum for 2 hours before western blot analysis (for JUN or β-actin) of whole cell extracts. (B) Growth-quiescent bEND-3 cells transfected with 15 μg of pGL3-prom bearing two JUN/AP-1 binding sites and 0.1 μM (thin bar) or 0.4 μM (thick bar) of *Jun* siRNA, siRNAscr, Egr-1 siRNAscr or mock transfected were incubated in medium containing serum for 24 hours before determination of luciferase activity in the cell lysates. (C) RT-PCR analysis for *Jun* using extracts of growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of *Jun* siRNA, siRNAscr, Egr-1 siRNAscr or mock-transfected and incubated in medium containing serum for 2 hours. (D) RT-PCR analysis for MMP-2 using extracts of growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of *Jun* siRNA, siRNAscr, Egr-1 siRNAscr or mock transfected and incubated in serum-free medium containing 10 ng/ml TGFβ for 24 hours. (E) Gelatin zymography demonstrating MMP-2 bioactivity in supernatants of transfected bEND-3 cells. The cells were transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of *Jun* siRNA or siRNAscr and incubated in serum-free medium containing 10 ng/ml TGFβ for 24 hours. Data are representative of at least two independent experiments performed in duplicate or triplicate and are mean values ± s.e.m. **P*<0.05 compared with no siRNA control by Student's *t*-test.

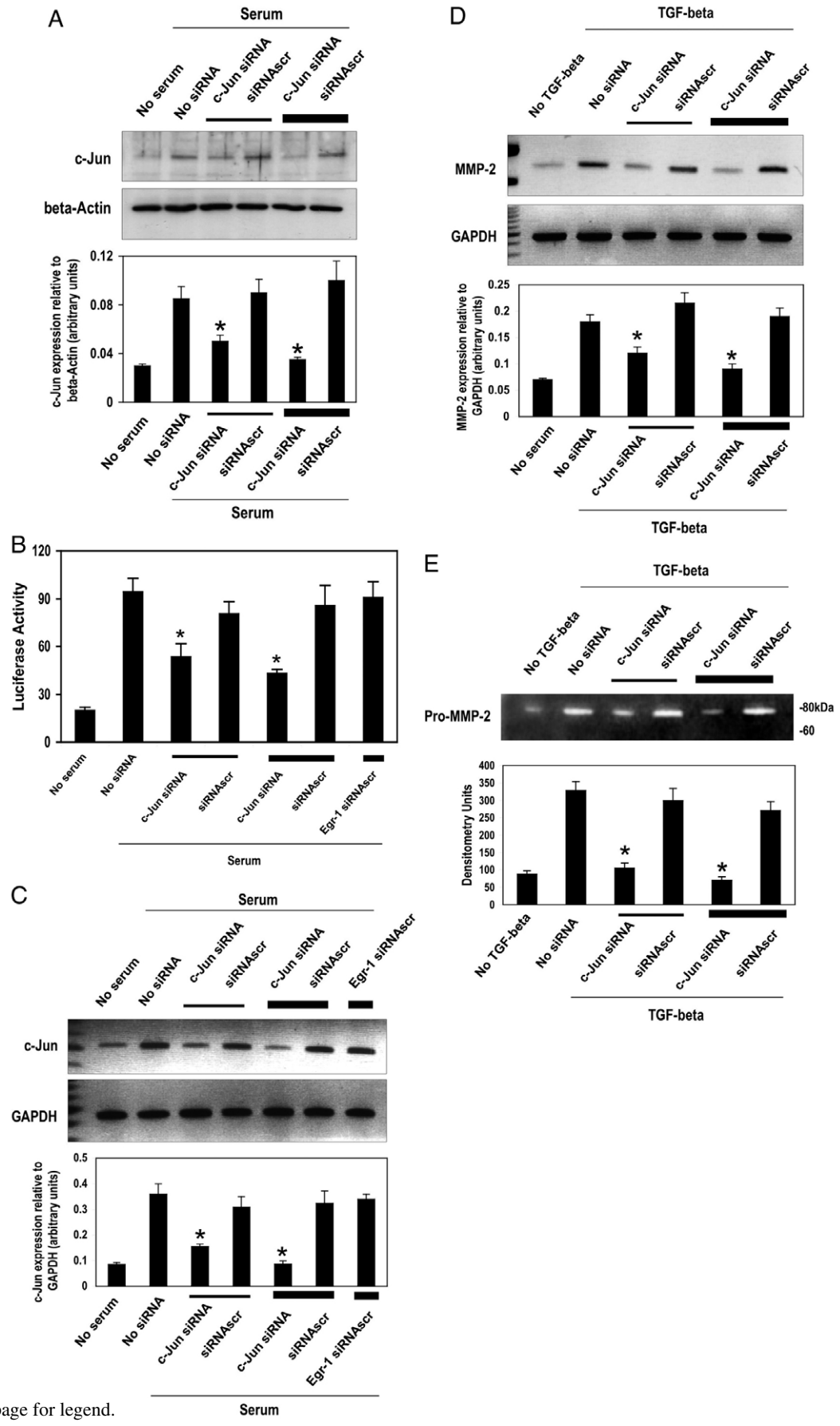


Fig. 1. See previous page for legend.

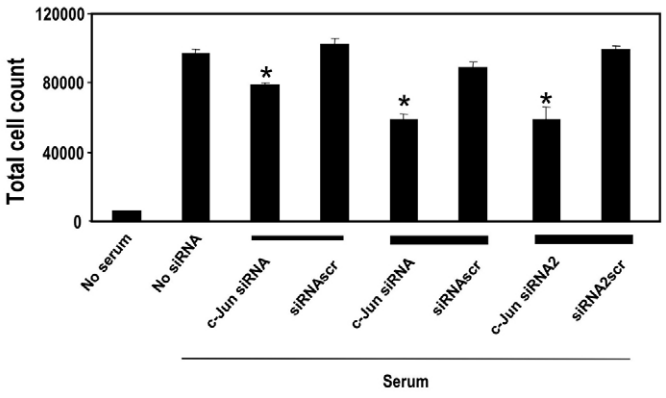
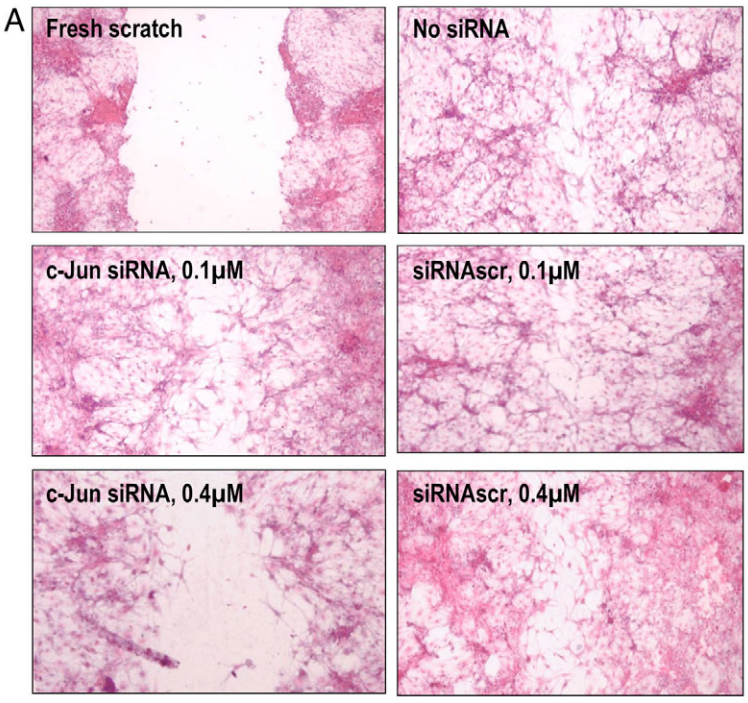
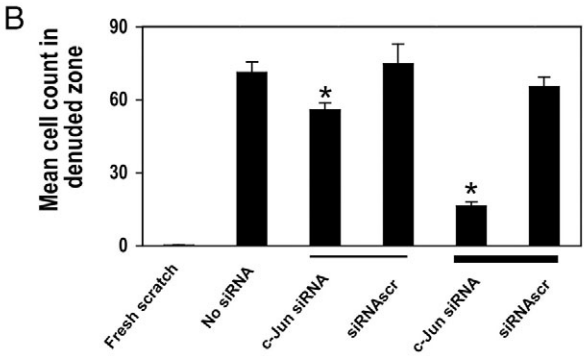


Fig. 2. *Jun* siRNA inhibits growth of murine microvascular endothelial cells. Growth-quiescent bEND-3 cells transfected with 0.02 μM (thin bar) or 0.05 μM (thick bar) of *Jun* siRNA, siRNAscr, siRNA2, siRNA2scr or mock transfected were incubated in medium containing serum for 48 hours before trypsinisation and quantification of cell numbers using a Coulter counter. The data are representative of at least two independent experiments performed in triplicate. **P*<0.05 compared with the no siRNA control by Student's *t*-test.





MMP-2 (Fig. 6C) expression in these vessels. By contrast, the siRNA had no effect on levels of the zinc-finger transcription factor Sp1 whose levels were unaltered by hyperoxia or hypoxia (Fig. 6D). We hypothesised that local co-administration of recombinant TGFβ with the JUN siRNA would override the suppressive effect of the siRNA alone, because TGFβ can rapidly stimulate JUN expression (Davidson et al., 1993; Tang et al., 1998). Retinal vascularity in the siRNA group was increased by TGFβ (Fig. 6A). Rescue by the growth factor was blocked by the presence of 9-octadecenoyl-N-hydroxylamide (Fig. 6A). These findings demonstrate that TGFβ can override siRNA inhibition of retinal vascularity in a metalloproteinase-dependent manner (Fig. 6A).

Discussion

This study demonstrates that siRNA targeting the transcription factor JUN inhibits serum-inducible microvascular endothelial cell proliferation and blocks cellular regrowth in an in vitro model of wounding. The siRNA suppressed three-dimensional tubular formation on basement membrane extracts in a time-dependent manner, and blocked angiogenesis in mice implanted with subcutaneous Matrigel plugs. Moreover, a single intravitreal administration of the siRNA suppressed retinal neovascularisation in mice subjected to the hyperoxia-induced retinopathy of prematurity model, which has been used to evaluate the anti-angiogenic properties of several compounds including VEGF antisense oligonucleotides (Robinson et al., 1996), Macugen/EYE-001 (Eyetech, 2002), combretastatin-A4 (Griggs et al., 2002) and soluble EphA2 receptor (Chen et al., 2006). TGFβ, co-administered with the siRNA, increased retinal vascularity, which was abrogated by cis-9-octadecenoyl-N-hydroxylamide, consistent with the involvement of a metalloproteinase such as MMP-2 (Aye et al., 2004; Emonard et al., 1999; Liao et al., 2003). The scrambled counterpart of the JUN siRNA had no effect in any of these systems indicating the sequence specificity of the siRNA. siRNA targeting VEGF and the VEGF receptors have previously been used to inhibit angiogenesis in animal models, and are presently under evaluation in clinical trials in patients with age-related macular degeneration (Kim et al., 2004; Reich et al., 2003; Shen et al., 2006; Tolentino et al., 2004).

JUN, like JUNB and JUND, is a member of the JUN sub-family of dimeric basic region leucine zipper

Fig. 3. *Jun* siRNA inhibits murine microvascular endothelial cell regrowth from the wound edge. bEND-3 cells were transfected with 0.1 or 0.4 μM of *Jun* siRNA, siRNAscr or mock transfected (No siRNA). Injury was performed by scraping the monolayer, leaving the cells in medium containing serum for 48 hours before fixation, Haematoxylin-Eosin staining and (A) microscopy (200×) or (B) quantification of cell numbers in the denuded zone (0.1 μM, thin bar; 0.4 μM, thick bar). The data are representative of at least two independent experiments performed in triplicate. **P*<0.05 compared with the no siRNA control by Student's *t*-test.

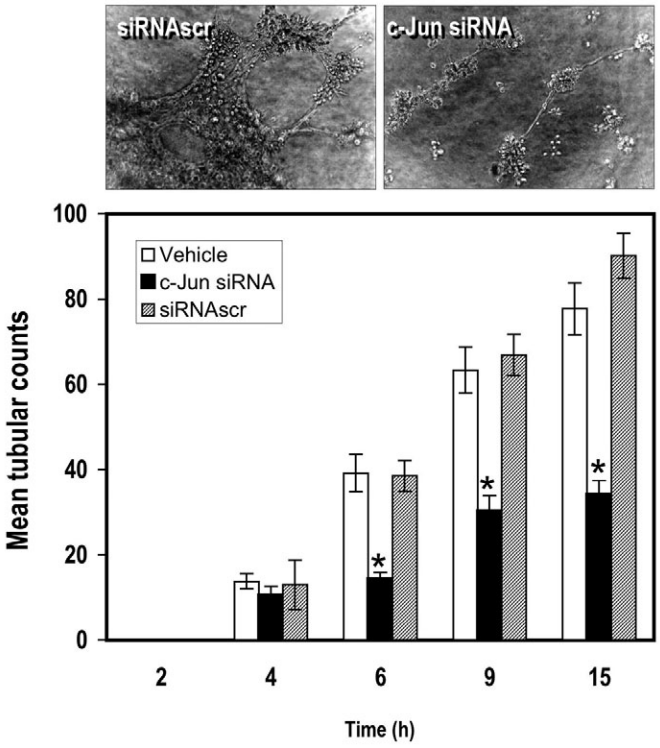


Fig. 4. *Jun* siRNA inhibits murine microvascular endothelial tubule formation. bEND-3 cells transfected with 0.1 μM of *Jun* siRNA, siRNAscr or mock transfected were seeded onto Matrigel-coated wells and spontaneous tubule formation was quantified by counting tubules using phase-contrast microscopy (under 100× magnification) at the times indicated. The data is representative of at least two independent experiments performed in triplicate. The figure shows representative photomicrographs after 6 hours at 200× magnification. **P*<0.05 compared with control by Student's *t*-test.

(bZIP) proteins in AP-1. The AP-1 family regulates a plethora of cellular processes, including proliferation, differentiation, apoptosis and survival (Shaulian and Karin, 2002). Gene transactivation by JUN is mediated by physical interactions with promoter recognition elements (5′-TGAG/CTCA-3′), which are also known as phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements (Angel et al., 1987) and/or interactions with other factors. JUN proteins are able to form dimers with bZIP proteins, such as for example, members of the CREB-ATF family of proteins and the oncogenic transcription factor Maf. JUN also dimerises with the p65 subunit of NF-κB, Sp1, CBP/p300 (CRE-binding-protein binding protein), SMAD-3 and -4, and retinoblastoma protein (Rb), among others (Chinenov and Kerppola, 2001). JUN does not necessarily have to bind DNA to influence gene transcription, as has been observed in the human 12(S)-lipoxygenase promoter where Sp1 serves as an anchor protein (Chang and Chen, 2005). The spectrum of genes influenced by JUN, directly and indirectly, is therefore impressive, and includes those regulating the cell cycle, growth, the extracellular matrix, membrane and cytoskeleton (Vogt, 2001). The JUN siRNA inhibited gelatinolytic activity corresponding to 72 kDa, the size of pro-MMP-2. Our previous studies using DNAzymes have demonstrated that MMP-2 is a JUN-dependent gene (Zhang et al., 2004). JUN proteins have been found to regulate MMP-2 transcription (Bergman et al., 2003). Our inability to detect a smaller migrating species in vitro corresponding to the active form of MMP-2 (59-65 kDa) may reflect the limit of detection of zymography. It is also possible that although these cells produce pro-MMP-2, they may express insufficient levels or lack essential co-factor(s)/enzymes that activate MMP-2 in culture. These co-factor(s) are, nevertheless likely to be present during retinal neovascularisation which is inhibited by cis-9-octadecenoyl-N-hydroxylamide (Fig. 6A) and unlike the in vitro setting, involves a variety of cell types. Indeed, studies have demonstrated that MMP-2, MMP-9 and MT1-MMP are all upregulated in the retina of mice undergoing a neovascular response (Majka et al., 2001). Previous studies have also demonstrated the appearance of a single band on a zymogram corresponding to the 72 kDa form of MMP-2 (Steinbrenner et al., 2003). A limitation of this study is that of the plethora of genes JUN controls, the influence of the siRNA on the altered expression of just one metalloproteinase was investigated. Thus, JUN siRNA can inhibit endothelial cell growth and neovascularisation and this may involve suppression of MMP-2 production.

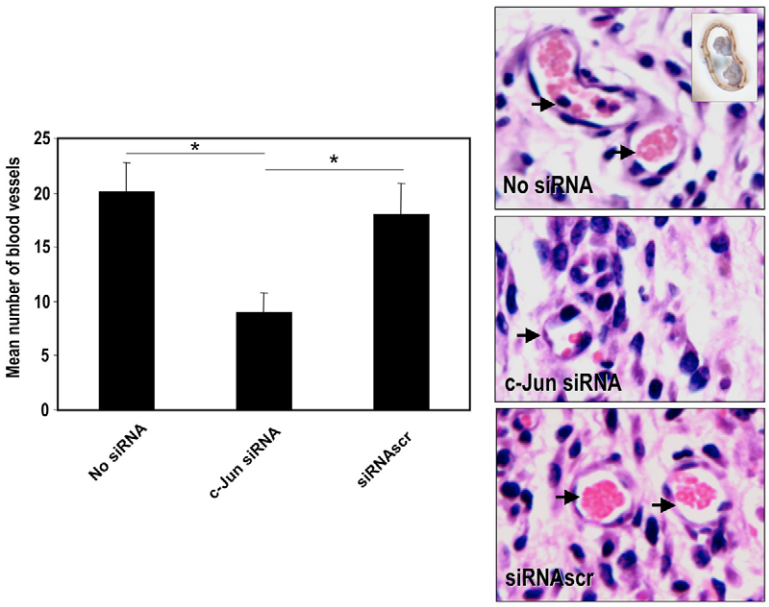


Fig. 5. *Jun* siRNA inhibits angiogenesis in mice. C57BL/6 mice were implanted subcutaneously with Matrigel plugs containing 0.5 μg FGF-2 and 100 μg *Jun* siRNA or *Jun* siRNAscr or no siRNA. Blood vessels ($CD31^+$, top panel inset) in the cross-sectioned plugs were quantified by light microscopy 14 days after implantation. The figure shows representative cross-sections of the treated plugs (magnification, 400×). **P*<0.05 by Student's *t*-test between groups as indicated.

Materials and Methods

Cell culture and siRNA transfection

Murine microvascular endothelial cells (bEND-3) (gift from the Inflammatory Research Unit, University of New South Wales, Australia) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 2 mM glutamine, 5 U/ml penicillin-strepto-

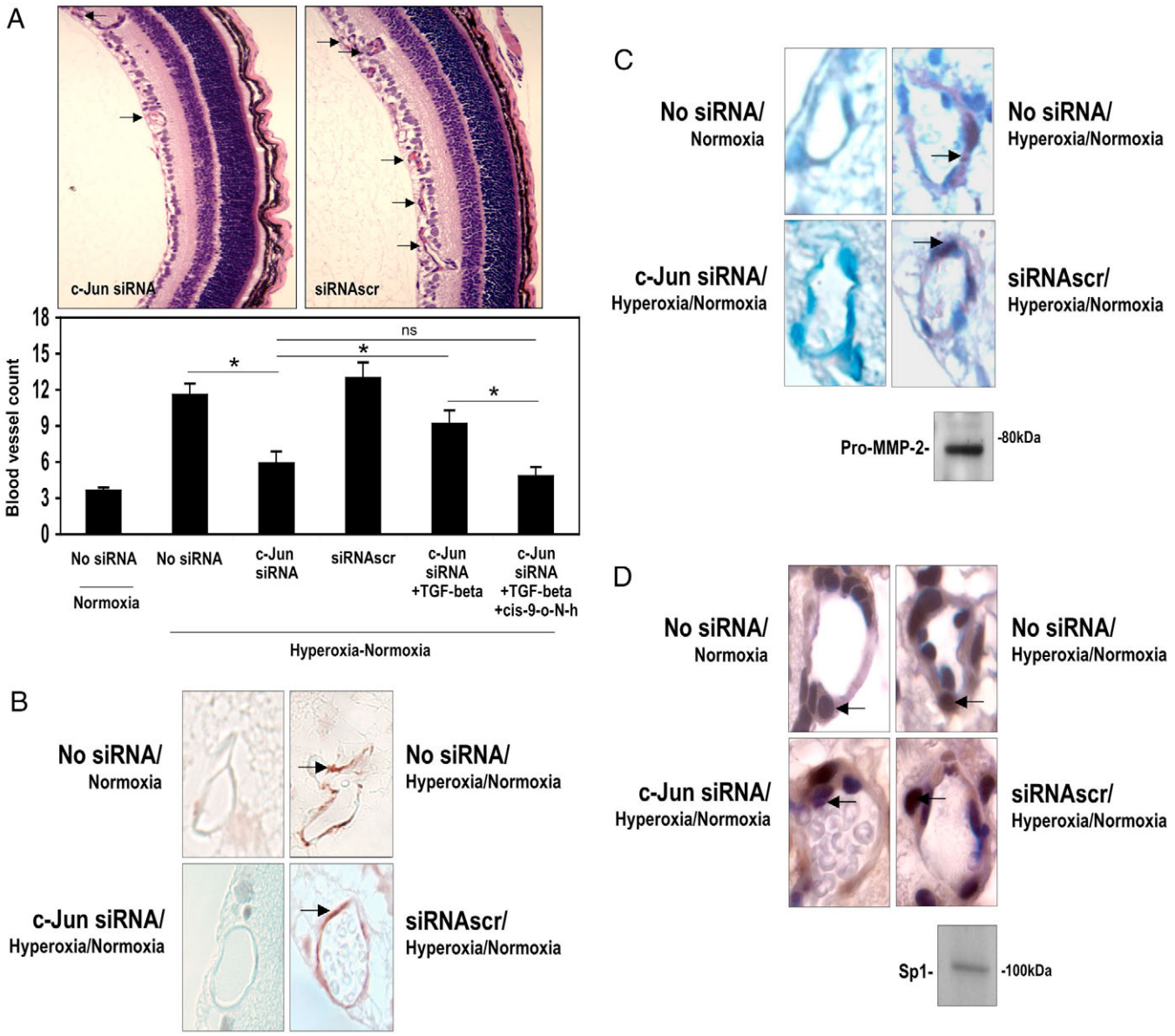


Fig. 6. siRNA targeting *Jun* inhibits neovascularisation in mice following hyperoxia-induced proliferative retinopathy. Postnatal day 6 (P6) C57BL/6 mice were exposed to hyperoxia (75% oxygen) for 4 days. P10 mice were returned to normoxia, and a bolus intravitreal injection of 20 μg of either *Jun* siRNA or siRNAscr was administered. Where indicated the siRNA was co-administered with 0.08 μg TGFβ with or without 20 μM cis-9-octadecenoyl-N-hydroxylamide (cis-9-o-N-h). Mice were left at room oxygen for a further 7 days before P17 pup eyes were enucleated and fixed in 10% formalin in PBS. (A) Serial cross-sections of the eyes were stained with Haematoxylin and Eosin and blood vessels in the retina were quantified by light microscopy under 400× magnification and expressed as the mean ± s.e.m. Representative images at 100× magnification (upper panels). *Jun* siRNA inhibits hyperoxia-induced neovascularisation (lower panel). **P*<0.05 by Student's *t*-test between groups as indicated. Immunoreactivity for (B) JUN, (C) MMP-2 or (D) Sp1 in retinal microvessels of mice treated intravitreally (20 μg) by single administration of *Jun* siRNA or siRNAscr or vehicle (no siRNA). Western blot analysis in C and D was performed with bEND-3 lysates as described in the Materials and Methods. Arrows in B-D indicate specific immunostaining (magnification, 1000×).

mycin and 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. *Jun* siRNA was synthesised with the sequence, 5′-r(CAGCUUCCUGCCUUUGUAA)dTT-3′. The scrambled counterpart, *Jun* siRNAscr, had the sequence 5′-r(GAUUACUAGCCGUCUUCCU)dTT-3′. Sequence of *Jun* siRNA2 was 5′-r(GCGCAUGAGGAACCGCAUU)dTT-3′ and its scrambled counterpart, *Jun* siRNA2scr2 was 5′-r(GCCGAACUAUAGGCCGUAG)dTT-3′. *Egr-1* siRNAscr, which also served as a negative control as indicated, had the sequence 5-r(AAGCGAGUAGCGCUAGGAAGU)dTT-3′. RNAiFect transfection reagent (Qiagen) was used to deliver the siRNA according to the manufacturer's instructions 6 hours after the change of culture medium to serum-free conditions to initiate growth arrest at subconfluence. The 'No siRNA' group was mock transfected with vehicle alone. Eighteen hours after transfection, the cells were transfected again and the medium was replaced with medium containing 10% serum.

Western blot analysis

Serum-starved bEND-3 cells transfected twice with siRNA, siRNAscr or mock transfected with vehicle alone were incubated in 10% FBS for the indicated time. The cells were washed in phosphate-buffered saline (PBS), and then harvested into RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 5 mM EDTA, 1% aprotinin, 2 mM phenylmethyl sulphonyl fluoride (PMSF), followed by two freeze-thaw cycles and centrifugation at 8000 ***g*** in a microfuge (Sigma,

Germany) for 10 minutes at 4°C to remove cell debris. 2 μg of protein was resolved on 12% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) nylon membranes (Millipore, Bedford, MA). The membranes were then probed with rabbit polyclonal antibodies to JUN (1:1000 (0.2 μg/ml), Santa Cruz Biotechnology, CA), MMP-2 (1:1000 (0.2 μg/ml), Santa Cruz Biotechnology, CA) and Sp1 (1:1000 (0.2 μg/ml), Santa Cruz Biotech) in TPBS [0.05% Tween 20 in PBS (v/v)] and incubated with horseradish-peroxidase-conjugated swine-anti rabbit IgG (1:1000, DAKO, Denmark). The protein bands were visualised using the Western Lightning chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA).

RT-PCR

Growth-quiescent bEND-3 cells in 100 mm Petri dishes were transfected with siRNA or siRNAscr and incubated in medium containing 10% serum for the times indicated. Total RNA was prepared using Trizol reagent (Invitrogen). cDNA was generated in 20 μl reactions containing 4 μg total RNA, 200 U Superscript II reverse transcriptase (Invitrogen), 500 μM of each dNTP and 0.5 μg oligo $(dT)_{15}$ (Invitrogen). PCR analysis was performed in 20 μl reactions containing reverse-transcribed cDNA template, 1 U DNA polymerase (Promega, Annandale, Sydney, Australia), 100 μM of each dNTP, 30 mM $MgCl_2$ (Invitrogen) and 0.1 μM of forward and reverse primer (for JUN: 25 cycles at 95°C 25 seconds, 61°C 30 seconds and 72°C 45 seconds; for MMP-2: 22 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 40 seconds). The volume of cDNA template required to achieve equivalent levels of GAPDH (452 bp product) between samples was to amplify JUN cDNA (514 bp amplicon), MMP-2 (445 bp amplicon) or GAPDH (452 bp amplicon). Primers were as follows: JUN (forward primer, 5′-GGAAACGAC-CTTCTACGACGATG-3′; reverse primer, 5′-GAAGTTGCTGAGGTTGGCGTA-GA-3′); MMP-2 (forward primer, 5′-GGGACAAGAACCAGATCACATAC-3′; reverse primer, 5′-CTTCTCAAAGTTGTAGGTGGTGG-3′); GAPDH (forward primer, 5′-ACCACAGTCCATGCCATCAC-3′; reverse primer, 5′-TCCACCACC-CTGTTGCTGTA-3′).

JUN AP-1-dependent reporter gene expression

The pGL3-prom vector (Promega) was digested with *Sma*I and double-stranded oligonucleotides (Oligo JUN) (Zhang et al., 2004) bearing JUN-AP-1 binding sites (GCTTGATGAGATCAGCCGGAAGCTTGATGAGATCAGCCGGAA) were subcloned upstream of the SV40 promoter driving Firefly luciferase. Transient transfections were performed at 70% confluence in 100 mm Petri dishes using FuGENE6 with 15 μg of the reporter construct and either 0.1 or 0.4 μM siRNA. Luciferase activity was determined 24 hours after transfection using the Luciferase assay system (Promega).

Gelatin zymography

Culture supernatants were collected from bEnd-3 cells transfected twice with siRNA as indicated or mock transfected with vehicle alone and resolved (equal amounts of protein per lane) at 4°C on a 10% polyacrylamide gels (with a 4% polyacrylamide stacking gel) containing 1 mg/ml bovine type B gelatin (Sigma). The gel was soaked in 2.5% Triton X-100 (Sigma) for 1 hour then incubated in substrate buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3) overnight at 37°C. The gel was stained in 0.2% Coomassie Blue R-250 (Bio-Rad) in water, methanol and glacial acetic acid at volume ratio (5:4:1) for 1 hour, then destained in H_2O:methanol:glacial acetic acid (5:4:1 vol) to reveal clear bands corresponding to protein bands with gelatinolytic activity.

Cell proliferation assay

bEND-3 cells seeded (3×10^3/well) in 96-well titre plates were rendered growth quiescent by incubation in serum-free medium for 24 hours then transfected with siRNA or siRNAscr and incubated in medium containing 10% serum for 2 days. The cells were harvested by trypsinisation and resuspended in Isoton II (Coulter Electronics, Brookvale, New South Wales, Australia). Cell suspensions were quantified using a Coulter counter (Z series; Coulter Electronics).

bEND-3 scratch-wound assay

Cells (3×10^4) were seeded into eight-well chambers (Nalgene Nunc International, Copenhagen, Denmark) and grown to 70-90% confluency. Cells were transfected (as indicated) and the monolayers were scraped using a P200 micropipette tip. Two days after wounding, the cells were washed twice in PBS, fixed in 10% paraformaldehyde (vol:vol) and stained in Haematoxylin-Eosin. Cells in the denuded zone (three random fields) were quantified by light microscopy under 100× magnification.

Endothelial tubule formation assay

Tubule formation was determined essentially as previously described (Fahmy et al., 2003; Zhang et al., 2004). Briefly, bEND-3 cells grown in 100-mm Petri dishes until 70% confluent were incubated in serum-free medium for 6 hours and transfected with siRNA or siRNAscr. 18 hours later, the cells were trypsinised and resuspended in medium containing 10% serum where 2.6×10^4 were seeded (in 200 μl) into 96-well plates each containing 100 μl Matrigel (BD Biosciences, MA). Total numbers of tubules per well were assessed at the times indicated by washing the cells with PBS, fixing in 10% paraformaldehyde and quantification under phase-contrast microscopy.

Matrigel plug assay

6- to 8-week-old female C57BL/6 mice were injected subcutaneously (right flanks) with 500 μl of Matrigel containing FGF-2 (0.5 μg) (Sigma, MI), 100 μg siRNA or siRNAscr or no siRNA, and FuGENE6 (2.5 μl) (n=5 mice per group). 14 days later, plugs were resected and fixed in 10% paraformaldehyde. 5 μm cross-sections were stained with Haematoxylin-Eosin. Erythrocyte-containing vessels in the plugs (whose identity as blood vessels was confirmed by immunostaining with CD31) was quantified by light microscopy under 100× magnification and expressed as the mean of three random fields. All animal experiments were approved by the University of New South Wales Animal Care and Ethics Committee.

Murine model of proliferative retinopathy

Postnatal day 6 (P6) C57BL/6 mice were exposed to hyperoxia (75% oxygen) for 4 days in Quantum-Air Maxi-Sealed cages (Hereford, UK) (Smith et al., 1994). Following hyperoxic exposure, P10 mice were returned to normoxia, anaesthetised (17 mg/kg ketamine and 2.5 mg/kg xylazine) and a bolus intravitreal injection of 20 μg of either *Jun* siRNA or siRNAscr or no siRNA, in 2 μl saline containing 0.2 μl FuGENE6 (n=6-12 eyes per group) was administered using a 26-gauge bevelled needle attached to a micro-volume syringe (SGE international, Melbourne, Australia). The mice were left at room oxygen for a further 7 days before P17 pup eyes were enucleated and fixed in 10% formalin in PBS. Serial 6 μm cross-sections of whole eyes were cut sagittally, parallel to the optic nerve, and stained with Haematoxylin and Eosin. In experiments using recombinant human TGFβ, the siRNA was co-administered with TGFβ (0.08 μg) with or without cis-9-octadecenoyl-N-hydroxylamide (Calbiochem) (20 μM) in one bolus delivery. Blood vessels from each group were quantified blind under light microscopy (400× magnification) and expressed as the mean ± s.e.m.

Immunohistochemical analysis

Immunostaining was performed essentially as described (Khachigian et al., 2002) with rabbit polyclonal MMP-2 and Sp1 antibodies purchased from Santa Cruz Biotechnology, CA.

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