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EXTENDED REPORT

A key role for Fut1-regulated angiogenesis and ICAM-1 expression in K/BxN arthritis

Mohammad A Amin,[1] Phillip L Campbell,[1] Jeffrey H Ruth,[1] Takeo Isozaki,[1] Bradley J Rabquer,[1] W Alex Stinson,[1] Martin O'Brien,[1] Gautam Edhayan,[1] Ray A Ohara,[1] Jonathon Vargo,[1] Steven E Domino,[2] Alisa E Koch[1,3]

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[1]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA
[2]Department of Obstetrics and Gynecology, University of Michigan Medical School, Ann Arbor, Michigan, USA
[3]Veteran's Administration, Ann Arbor, Michigan, USA

Correspondence to
Dr Mohammad A Amin, Department of Internal Medicine/Division of Rheumatology, University of Michigan Medical School, 4428 BSRB, 109 Zina Pitcher Drive, Ann Arbor, MI 48109-2200, USA; maamin@med.umich.edu

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ABSTRACT

Objectives Angiogenesis contributes to the pathogenesis of rheumatoid arthritis. Fucosyltransferases (Futs) are involved in angiogenesis and tumour growth. Here, we examined the role of Fut1 in angiogenesis and K/BxN serum transfer arthritis.

Methods We examined Fut1 expression in human dermal microvascular endothelial cells (HMVECs) by quantitative PCR. We performed a number of angiogenesis assays to determine the role of Fut1 using HMVECs, Fut1 null (*Fut1*$^{-/-}$), and wild type (wt) endothelial cells (ECs) and mice. K/BxN serum transfer arthritis was performed to determine the contribution of Fut1-mediated angiogenesis in *Fut1*$^{-/-}$ and wt mice. A static adhesion assay was implemented with RAW264.7 (mouse macrophage cell line) and mouse ECs. Quantitative PCR, immunofluorescence and flow cytometry were performed with *Fut1*$^{-/-}$ and wt ECs for adhesion molecule expression.

Results Tumour necrosis factor-α induced Fut1 mRNA and protein expression in HMVECs. HMVECs transfected with Fut1 antisense oligodeoxynucleotide and *Fut1*$^{-/-}$ ECs formed significantly fewer tubes on Matrigel. *Fut1*$^{-/-}$ mice had reduced angiogenesis in Matrigel plug and sponge granuloma angiogenesis assays compared with wt mice. *Fut1*$^{-/-}$ mice were resistant to K/BxN serum transfer arthritis and had decreased angiogenesis and leucocyte ingress into inflamed joints. Adhesion of RAW264.7 cells to wt mouse ECs was significantly reduced when Fut1 was lacking. *Fut1*$^{-/-}$ ECs had decreased intercellular adhesion molecule-1 (ICAM-1) expression at mRNA and protein levels compared with wt ECs. ICAM-1 was also decreased in *Fut1*$^{-/-}$ arthritic ankle cryosections compared with wt ankles.

Conclusions Fut1 plays an important role in regulating angiogenesis and ICAM-1 expression in inflammatory arthritis.

INTRODUCTION

Fucosyltransferases (Futs) are involved in the synthesis of glycoconjugates and blood group antigens. We and others have shown that a number of Futs contribute to increased angiogenesis, tumour growth and metastasis.[1–7] Fut1 plays an important role in angiogenesis and tumour growth. siRNA directed against Fut1/Fut4 inhibits tumour growth and decreases phosphorylation of Erk1/2 and epidermal growth factor receptor in human epidermoid carcinoma A431 cells.[5] Fut1 has been implicated in tumour necrosis factor-α (TNF-α) induced endothelial cell (EC) migration, tube formation and capillary formation in spheroid angiogenesis assays by increasing Lewis^{y} (Le^{y}) expression.[8] Silencing of Fut1 results in a decrease in the rolling and adhesion of immature dendritic cells over ECs.[8] The expression and activities of nucleolin are influenced by Fut1 and Fut2, and Fut1 induces cell adhesion and proliferation of bovine postcapillary venular ECs.[9]

There is increased expression of fucosylated glycans present on serum immunoglobulin in juvenile and adult patients with RA, suggesting the importance of Futs in these diseases.[10 11] There are also elevated levels of α(1,2)fucosylated and β-N-acetylgalactosaminylated prostate-specific antigen that serve as specific markers for prostate cancer.[12] *Helicobacter pylori*, the micro-organism involved in gastritis and gastric ulcers expresses Le^{y}/Le^{x}/H, which is also found in gastric mucin.[13 14] Antibodies directed against *H pylori* Le^{y} result in gastritis via an autoimmune reaction directed against gastric mucin Le^{y}. Missense mutation of Fut1 and deletion of Fut2 are responsible for Indian Bombay phenotype of ABO blood group system.[15] These individuals lack A and B antigens in their red blood cells and have anti-H, anti-A and anti-B antibodies in their serum. In everyday life, they do not have a deleterious phenotype, but they will develop acute haemolytic transfusion reaction when they receive blood from A or B blood group donors.[15–17]

The importance of glycoconjugates in cell adhesion and leucocyte trafficking into inflammatory sites has been described in a number of studies.[18] Maly *et al* found that mice deficient in enzyme Fut7, which generates Le^{x}, have an impairment in selectin-dependent leucocyte-endothelial adhesion and in vivo leucocyte rolling.[19] Altered expression of fucosylated oligosaccharides is found in pathological processes like cancer and atherosclerosis, and fucose deficiency results in leucocyte adhesion deficiency disorder in humans.[20] These studies suggest that Futs are involved in chronic inflammatory diseases by increasing leucocyte ingress and by generating the ligands for selectins required for leucocyte adhesion to ECs.

In this study, we found that Fut1, an α(1,2)fucosyltransferase, is inducible in human dermal microvascular endothelial cells (HMVECs) and plays a key role in angiogenesis in vitro and in vivo. We tested our hypothesis by using Fut1 null (*Fut1*$^{-/-}$) ECs and *Fut1*$^{-/-}$ mice. *Fut1*$^{-/-}$ mice developed less K/BxN serum transfer arthritis and had significantly reduced angiogenesis in arthritic ankles compared with wild type (wt) mice. RAW264.7 macrophage-

like cells had less adhesion to mouse ECs when Fut1 was lacking. Fut1 also plays an important role in EC intercellular adhesion molecule-1 (ICAM-1) expression. Our data provides a novel role for Fut1 in K/BxN arthritis, angiogenesis, and ICAM-1 expression.

RESULTS

TNF-α increases Fut1 mRNA and protein expression in HMVECs

Fut1 mRNA is inducible in HMVECs as determined by quantitative PCR. TNF-α increased Fut1 mRNA expression ~2.5-fold compared with non-stimulated HMVECs (figure 1A). We found that the maximum increase in Fut1 mRNA expression was at 1 h but not at 3 h and 6 h. TNF-α-stimulated Fut1 expression was significantly decreased in HMVECs transfected with Fut1 antisense oligodeoxynucleotide (ODN) compared with Fut1 sense ODN transfected cells (figure 1B). To further confirm Fut1 expression induced by TNF-α, we performed western blots with HMVECs stimulated for various time points, and found a marked increase in Fut1 expression in HMVECs (figure 1C).

Fut1 is involved in HMVEC tube formation on Matrigel

HMVECs transfected with Fut1 antisense ODN formed significantly fewer tubes on Matrigel compared with Fut1 sense ODN transfected HMVECs when stimulated with basic fibroblast growth factor (bFGF), suggesting a role for Fut1 in HMVEC tube formation (figure 1D,E).

Fut1 plays an essential role in EC tube formation

Fut1$^{-/-}$ ECs formed significantly less tubes on Matrigel compared with wt ECs in response to bFGF. Tubes formed by *Fut1*$^{-/-}$ ECs were almost twofold less compared with wt ECs, suggesting the contribution of Fut1 to EC tube formation in vitro (figure 1F,G). Tubes formed by bFGF-stimulated wt ECs were significantly higher compared with wt and *Fut1*$^{-/-}$ ECs treated with phosphate buffer saline (PBS), a negative control.

Fut1 regulates bFGF-mediated EC morphogenesis ex vivo

EC morphogenesis assays reflect EC migration and vascular rearrangement.[21] In this assay, sprouting is induced in mouse ECs in the cornea ex vivo. There was a marked decrease in

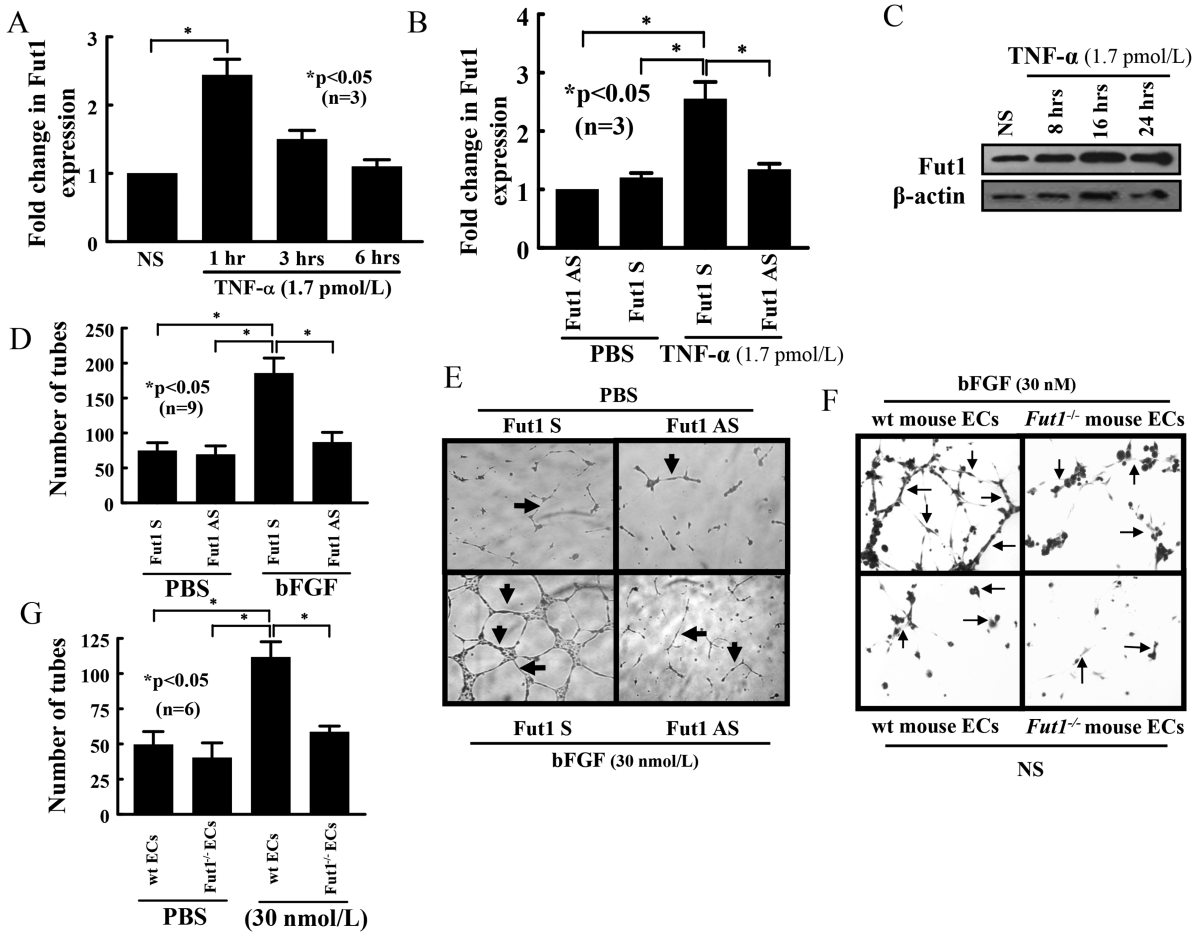

Figure 1 (A) Tumour necrosis factor-α (TNF-α) induces fucosyltransferase 1 (Fut1) mRNA expression in human dermal microvascular endothelial cells (HMVECs). TNF-α-induced Fut1 expression in HMVECs was ~2.5-fold at 1 h compared with non-stimulated HMVECs, as determined by quantitative PCR. NS=non-stimulated. (B) HMVECs transfected with Fut1 antisense (AS) ODNs had more than a twofold decrease in Fut1 expression compared with sense (S) ODN transfected HMVECs. Results are expressed as the mean±SEM and *p<0.05 was considered statistically significant. n=number of the experiments; PBS=Phosphate buffered saline. (C) TNF-α induces Fut1 expression at the protein level at various time periods. This is one of two assays. (D) HMVECs transfected with Fut1 AS ODN form significantly less tubes on growth factor reduced (GFR) Matrigel compared with S transfected HMVECs. Arrows indicate the tubes formed by HMVECs. (E) Fut1 S ODN transfected HMVECs form significantly more tubes compared with Fut1 AS ODN transfected HMVECs. n=number of replicates in each group. (F) Fut1 null (*Fut1*$^{-/-}$) mouse endothelial cells (ECs) form less tubes on GFR Matrigel in response to bFGF (30 nmol/L). (G) Tubes formed by *Fut1*$^{-/-}$ ECs on Matrigel were twofold less compared with wild type (wt) mouse ECs. Arrows indicate the number of tubes formed. n=number of replicates in each group.

bFGF-induced sprouting in *Fut1*$^{-/-}$ mouse corneas compared with wt mouse corneas (figure 2A).

Fut1$^{-/-}$ mice have less angiogenesis in the Matrigel plug in vivo

After finding that *Fut1*$^{-/-}$ is inducible in HMVECs and that *Fut1*$^{-/-}$ ECs form fewer tubes, we examined if Fut1 also regulates angiogenesis in vivo. *Fut1*$^{-/-}$ mice had significantly reduced angiogenesis compared with wt mice in the Matrigel plug angiogenesis assay in response to bFGF (30 nmol/L). There was more than a fourfold decrease in hemoglobin (Hb), a correlate of neovascularisation, in *Fut1*$^{-/-}$ compared with wt mice, suggesting the importance of Fut1 in angiogenesis in vivo (figure 2B,C). We found a significant increase in Hb in Matrigel plugs containing bFGF when compared with Matrigel plugs with PBS.

Matrigel plugs harvested from *Fut1*$^{-/-}$ mice form less blood vessels compared with plugs harvested from wt mice

To visualise the effect of Fut1 in angiogenesis in vivo, immunofluorescence was performed on Matrigel plug cryosections using rabbit antimouse von Willebrand Factor (vWF) antibody, an EC marker. Plugs obtained from *Fut1*$^{-/-}$ mice had significantly decreased blood vessels compared with wt plugs having bFGF as a stimulus (figure 2D,E). We also found that Matrigel plugs harvested from *Fut1*$^{-/-}$ mice having bFGF formed more blood vessels when compared with the plugs from *Fut1*$^{-/-}$ mice treated with PBS.

Fut1$^{-/-}$ mice have reduced angiogenesis in the inflammatory sponge granuloma model

The sponge granuloma model of angiogenesis is characterised by inflammatory leucocyte ingress and blood vessel formation into inert sponges and serves as a good model for inflammatory

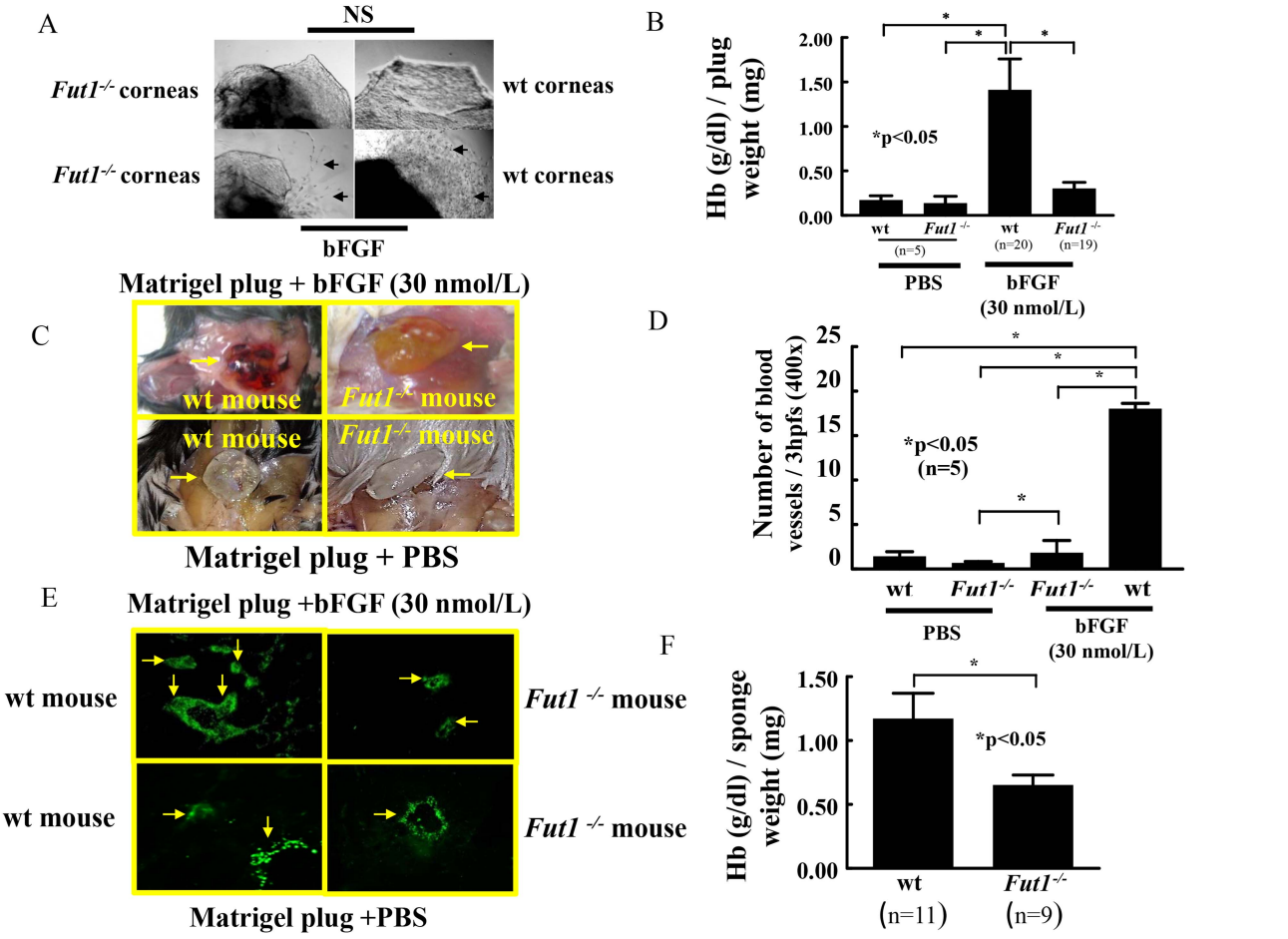

Figure 2 (A) Endothelial cell (EC) morphogenesis assays performed with fucosyltransferase 1 null (*Fut1*$^{-/-}$) and wild type (wt) mouse corneas. There was a marked decrease in EC sprouts formed by *Fut1*$^{-/-}$ mouse corneas in response to bFGF. Arrows show EC sprouts formed in wt and *Fut1*$^{-/-}$ mouse corneas. NS=non-stimulated. (B) *Fut1*$^{-/-}$ mice showed impaired angiogenesis in the Matrigel plugs containing bFGF (30 nmol/L) compared with wt mice. Hb was fourfold less compared with wt mice. (PBS served as the negative control.) Results represent the mean±SEM and *p<0.05 was considered statistically significant. n=number of mice per group. (C) On gross appearance, plugs collected from *Fut1*$^{-/-}$ mice had attenuated angiogenesis and were yellowish, while wt plugs had exuberant blood vessel formation and appeared red. (D) Immunofluorescence was performed to detect angiogenesis using anti-vWF antibody. There was a significant decrease in blood vessels formed in the plugs collected from *Fut1*$^{-/-}$ mice. Results are mean±SEM and *p<0.05 was considered statistically significant. n=number of mice per group. (E) Arrows indicate green fluorescent blood vessels stained with vWF in the Matrigel plug cryosections. *Fut1*$^{-/-}$ mice had fewer blood vessels than wt mice. (F) *Fut1*$^{-/-}$ mice had reduced bFGF-induced angiogenesis (30 nmol/L) in the mouse sponge granuloma assay in vivo. Results are shown as mean±SEM and *p<0.05 was considered statistically significant. n=number of mice.

angiogenesis. There was a significant ~twofold decrease in Hb in the sponges harvested from *Fut1*$^{-/-}$ mice compared with the sponges from wt mice ($p<0.05$, figure 2F). These results suggest that Fut1 is a key regulator of inflammatory angiogenesis.

Fut1$^{-/-}$ mice are resistant to K/BxN arthritis

Angiogenesis plays an important role in K/BxN arthritis.[22 23] We examined the contribution of Fut1-mediated angiogenesis in inflammatory arthritis by performing the K/BxN serum transfer arthritis model. *Fut1*$^{-/-}$ mice have significantly decreased ankle circumference starting from day 2 onwards compared with wt mouse ankles. The wt mice developed severe redness, swelling and inflammation of joints, while *Fut1*$^{-/-}$ mice had markedly decreased arthritis, with less joint swelling and inflammation (figure 3A,B). Figure 3C shows markedly decreased infiltrating leucocytes in the joints of *Fut1*$^{-/-}$ mice exposed to K/BxN-induced arthritis.

Fut1$^{-/-}$ mouse arthritic ankle homogenates had diminished Hb compared with wt arthritic ankles

To examine the contribution of Fut1-regulated angiogenesis to K/BxN arthritis, we determined Hb in the arthritic ankle homogenates of *Fut1*$^{-/-}$ and wt mice.[24] *Fut1*$^{-/-}$ arthritic ankle homogenates had ~twofold less Hb compared with wt arthritic ankle homogenates, suggesting that Fut1 contributes to angiogenesis in inflammatory arthritis in vivo (figure 3D).

Fut1$^{-/-}$ mouse ankle cryosections exhibit decreased blood vessel counts

Examination of cryosections obtained from *Fut1*$^{-/-}$ mice revealed significantly fewer blood vessels compared with wt mouse sections (5.2±1.14 mean±SE blood vessels/hpf in *Fut1*$^{-/-}$ mouse ankle sections vs 20.4±1.71 mean±SE in wt mouse ankle sections (figure 3E,F). These results suggest that Fut1 plays an essential role in K/BxN arthritis by reducing blood vessel formation.

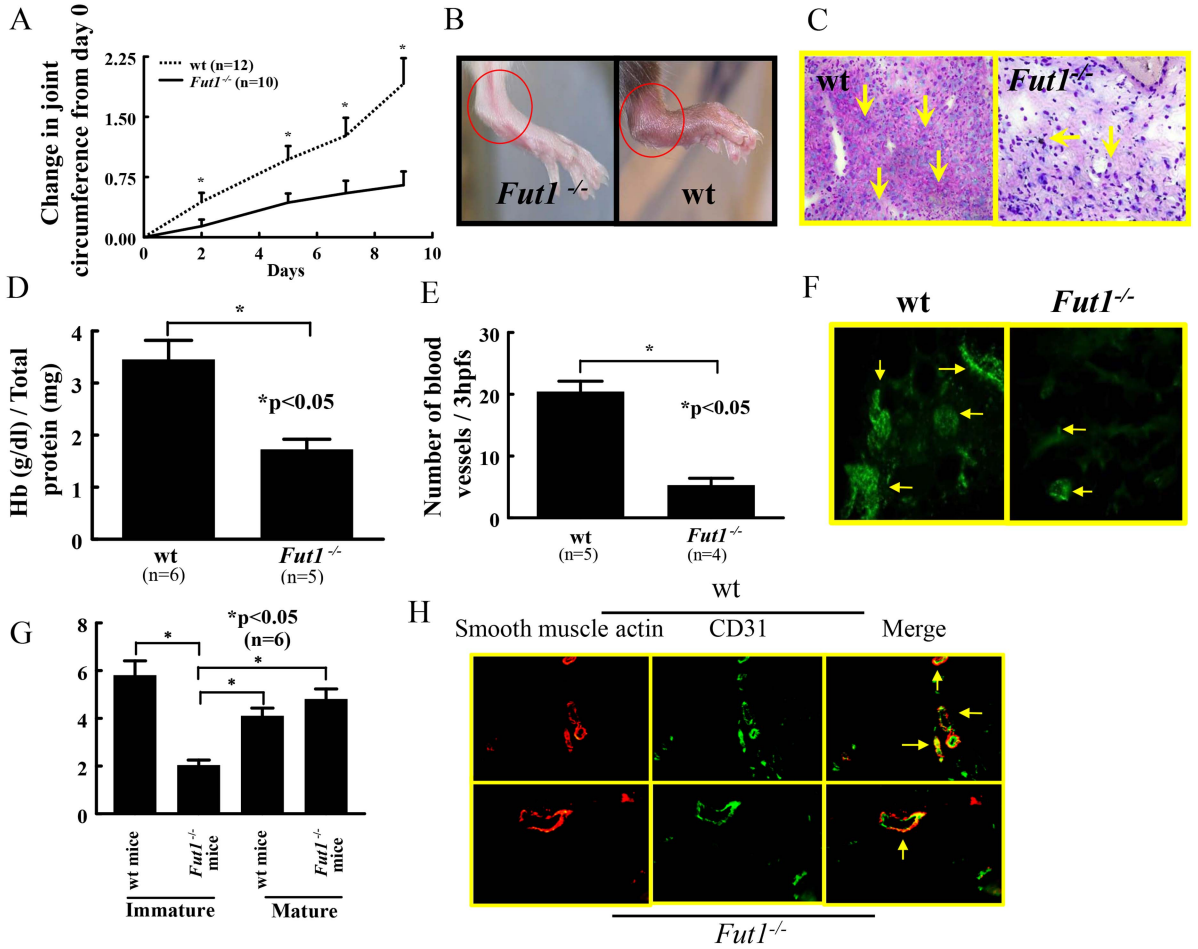

Figure 3 (A,B) K/BxN serum transfer arthritis in fucosyltransferase 1 null (*Fut1*$^{-/-}$) and wild type (wt) mice. The wt mice developed profound arthritis upon serum transfer while *Fut1*$^{-/-}$ mice had significantly decreased arthritis as measured by joint circumference. Results are expressed as mean±SEM and *p<0.05 was considered statistically significant. n=number of joints. (C) Arthritic ankles from *Fut1*$^{-/-}$ and wt mice were sectioned and H&E staining was performed. Arrows show a marked decrease in infiltrating leucocytes in the cryosections of *Fut1*$^{-/-}$ mouse ankles compared with wt mice. (D) There was a significant decrease in Hb in *Fut1*$^{-/-}$ K/BxN arthritic ankle homogenates in comparison with wt ankle homogenates. Results represent as mean±SEM and *p<0.05 was considered statistically significant. n=number of mice. (E) *Fut1*$^{-/-}$ and wt mouse cryosections were stained for vWF. Figure E shows a significant decrease in blood vessels in *Fut1*$^{-/-}$ arthritic ankle cryosections compared with wt arthritic ankle cryosections. n=number of mice. (F) Green blood vessels stained for vWF in wt and *Fut1*$^{-/-}$ arthritic mouse ankles. (G) *Fut1*$^{-/-}$ and wt mouse cryosections stained for CD31 and smooth muscle actin to detect immature blood vessels. *Fut1*$^{-/-}$ arthritic ankle cryosections have significantly less immature blood vessels compared with wt arthritic ankle cryosections. Results are expressed as mean±SEM and *p<0.05 was considered statistically significant. n=number of mice. (H) The immunofluorescence performed with arthritic ankle cryosections of *Fut1*$^{-/-}$ and wt mice using antibodies against CD31 and smooth muscle actin.

Fut1$^{-/-}$ mouse arthritic ankle cryosections displayed decreased immature blood vessels compared with wt mouse ankle cryosection

We performed dual immunofluorescence with anti-CD31 and antismooth muscle actin to determine the presence of immature blood vessels in K/BxN arthritic ankle cryosections, as immature vessels lack periendothelial cells and show active angiogenesis. Immature blood vessels are depleted in *Fut1*$^{-/-}$ mouse ankle sections when compared with wt mouse ankle cryosections. We did not find a difference in mature vessels in wt and *Fut1*$^{-/-}$ mouse ankle sections, suggesting that Fut1 regulates active angiogenesis (figure 3G,H).

Some angiogenic factors are not reduced in *Fut1*$^{-/-}$ ankle homogenates compared with wt mouse ankle homogenates

We did not find a decrease in bFGF or vascular endothelial cell growth factor (VEGF), two potent angiogenic factors, in *Fut1*$^{-/-}$ ankle homogenates compared with wt mouse ankle homogenates, indicating that these factors are not involved in the decrease in blood vessel formation or inflammation found in K/BxN arthritis (figure 4A,B).

Fut1$^{-/-}$ ECs have less leucocyte-EC adhesion compared with wt ECs in a static adhesion assay

RAW264.7 cells, a mouse macrophage cell line, adhered significantly less to *Fut1*$^{-/-}$ ECs compared with wt ECs, suggesting a basis for decreased arthritic inflammation in *Fut1*$^{-/-}$ mice (figure 4C).

Fut1 is expressed on blood vessels of RA synovial tissue

To determine if Fut1 is expressed on human RA synovial tissues, we performed immunoperoxidase staining using antihuman Fut1 antibody (Thermo Scientific) or control rabbit IgG. We found that Fut1 is present on blood vessels of RA synovial tissue cryosections, suggesting a potential role of Fut1 in human RA (figure 4D).

Fut1$^{-/-}$ ECs have reduced ICAM-1 expression when stimulated with TNF-α

We next determined the role of Fut1 in adhesion molecule expression employing *Fut1*$^{-/-}$ and wt ECs, as adhesion molecules play an important role in leucocyte ingress into inflammatory sites. *Fut1*$^{-/-}$ ECs exhibited reduced ICAM-1 expression when stimulated with TNF-α. This decrease was fourfold less in comparison with wt ECs (figure 5A). Additionally, *Fut1*$^{-/-}$ ECs displayed markedly less ICAM-1 expression compared with wt ECs by immunofluorescence (figure 5B). Another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), was not decreased in *Fut1*$^{-/-}$ ECs (data not shown).

Fut1$^{-/-}$ ECs have lower ICAM-1 expression as determined by flow cytometry

To confirm if *Fut1*$^{-/-}$ ECs have less ICAM-1 expression, flow cytometry was performed with wt and *Fut1*$^{-/-}$ ECs after overnight stimulation with TNF-α. We found a 60% decrease in ICAM-1 expression on *Fut1*$^{-/-}$ ECs compared with wt mouse ECs (figure 5C).

Fut1$^{-/-}$ mouse ankle arthritic ankle sections exhibit decreased ICAM-1 expression compared with wt ankle sections

Fut1$^{-/-}$ mouse cryosections had decreased blood vessels and ICAM-1 expression compared with wt mouse joints by dual immunofluorescence, suggesting both contribute to impaired leucocyte recruitment in K/BxN arthritis in mice lacking Fut1 (figure 5D).

DISCUSSION

Targeting new blood vessel growth, or angiogenesis, is a potential alternative to treat RA. Angiogenesis is one of the earliest pathological findings in RA and contributes to pannus development, proliferation and perpetuation of inflammation by promoting ingress of inflammatory cells.[25–27] Therefore, suppression of new blood vessels could inhibit the progression of synovial hyperplasia and mitigate joint destruction by diminishing a major source of proinflammatory cytokines.

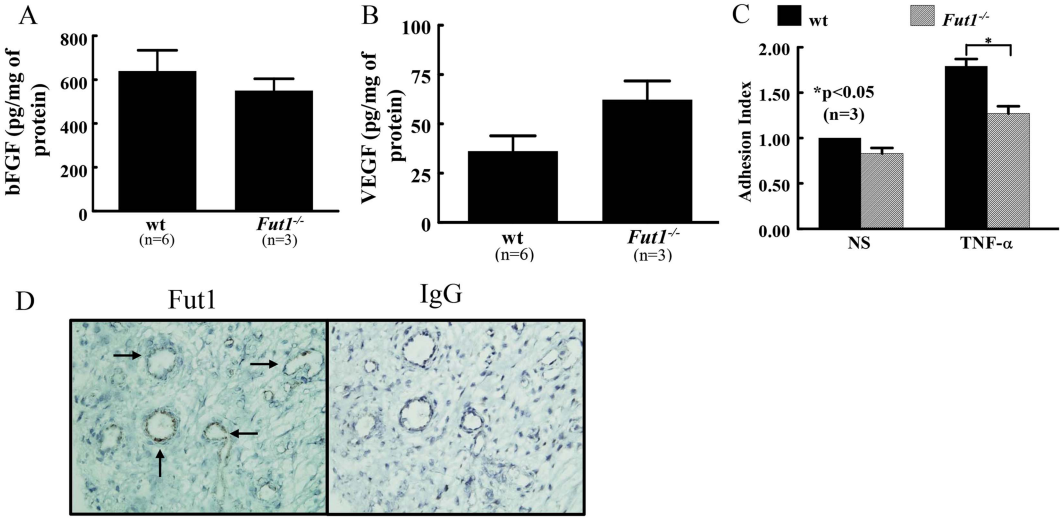

Figure 4 (A,B) Determination of angiogenic factors in fucosyltransferase 1 null (*Fut1*$^{-/-}$) and wild type (wt) ankle homogenates. Angiogenic factors VEGF and bFGF were not reduced in *Fut1*$^{-/-}$ arthritic ankle homogenates compared with wt mouse ankle homogenates. (C) Static adhesion of RAW264.7 (a mouse macrophage cell line) to endothelial cells (ECs) harvested from *Fut1*$^{-/-}$ and wt mice. There was less adhesion of RAW264.7 to ECs when Fut1 was absent. n=number of the experiments. NS=non-stimulated. (D) Expression of Fut1 on RA synovial tissue blood vessels by immunoperoxidase staining.

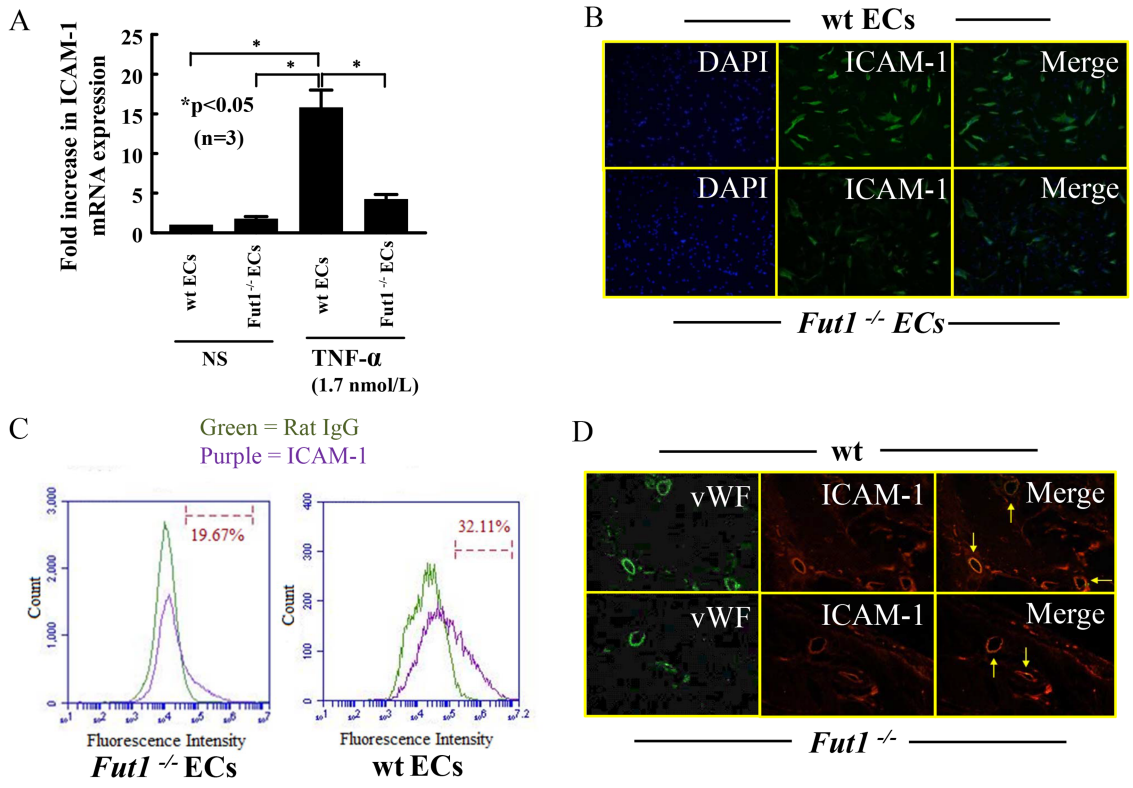

Figure 5 (A) Tumour necrosis factor-α (TNF-α) stimulated endothelial cell (EC) intercellular adhesion molecule-1 (ICAM-1) mRNA expression was ~fourfold lower in fucosyltransferase 1 null (*Fut1*$^{-/-}$) ECs compared with wild type (wt) ECs. Results represent the mean±SEM and *p<0.05 was considered statistically significant. n=number of the experiments. NS=non-stimulated. (B) TNF-α stimulated *Fut1*$^{-/-}$ ECs displayed markedly diminished cell surface expression of ICAM-1 compared with wt ECs by immunofluorescence. (C) Comparison of ICAM-1 expression on *Fut1*$^{-/-}$ and wt murine lung ECs via flow cytometry. *Fut1*$^{-/-}$ ECs (left panel) showed a 60% decrease in ICAM-1 expression compared with wt ECs when normalised to IgG control as shown by the shift in fluorescence intensity (right panel). ECs from seven wt and seven *Fut1*$^{-/-}$ mice were collected and pooled to perform flow cytometry. (D) K/BxN arthritic ankle cryosections from wt and *Fut1*$^{-/-}$ stained for blood vessels using vWF (green fluorescent) antibody while ICAM-1 was stained with red fluorescence. Arrows depict the merging of red and green. *Fut1*$^{-/-}$ mice had less ICAM-1 expression and blood vessel formation.

There is controversy regarding the role of Fut1 in angiogenesis, tumour growth and metastasis. Some authors found that introduction of Fut1 into human cancer cells inhibits vasculogenesis, tumour growth and metastasis,[28 29] while others found that Fut1 plays an active role in angiogenesis, tumour growth, and metastasis.[5 8] To resolve this issue, we performed a number of angiogenesis assays by employing *Fut1*$^{-/-}$ ECs and *Fut1*$^{-/-}$ mice.

We found that TNF-α induced Fut1 mRNA expression was ~2.5-fold higher compared with non-stimulated HMVECs by quantitative PCR. HMVECs transfected with Fut1 antisense ODN had markedly reduced Fut1 mRNA expression and formed less tubes on Matrigel compared with HMVECs transfected with Fut1 sense ODN. Our data support the notion that cytokines can induce expression of various Futs including Fut1 in cancer cells, leucocytes and ECs.[8 30 31] Moehler *et al* found that there was less than a twofold increase in Fut1 mRNA expression in human bone marrow ECs and immortalised EC lines after 24 h of incubation with TNF-α.[8] Another report found that Fut1 expression was inducible in bovine postcapillary venular ECs at 24 h when these cells were stimulated with serum.[9] We found that the maximum increase in Fut1 mRNA expression in HMVECs was at 1 h in response to TNF-α. One possible reason for this discrepancy may be because we used human primary ECs whereas the other authors used an immortalised EC line, immortalised bone marrow ECs or bovine postcapillary venular ECs. We confirmed the increased expression of Fut1 at protein level by performing western blotting.

To further confirm our results, we performed angiogenesis assays in vivo using *Fut1*$^{-/-}$ and wt mice. We found a significant decrease in Hb and in the number of blood vessels formed in response to bFGF in *Fut1*$^{-/-}$ compared with wt mice in the Matrigel plug assay. Moehler *et al* provided indirect evidence of Fut1 involvement in capillary formation in an EC spheroid angiogenesis in vitro assay. This report showed that Fut1 siRNA inhibits CD173/CD174 expression on human bone marrow ECs, which results in less tube formation in response to TNF-α.[8] In our study, we demonstrate the direct contribution of Fut1 to angiogenesis in vivo by employing *Fut1*$^{-/-}$ mice.

Angiogenesis is critical in leucocyte ingress to inflammatory sites. In an inflammatory model of angiogenesis, we found that sponges harvested from wt mice had significantly more Hb than the sponges collected from *Fut1*$^{-/-}$ mice, confirming the role of Fut1 in angiogenesis in relation to inflammation.

A number of studies have previously shown that fucosylated glycans synthesised by Futs play a critical role in angiogenesis, tumour growth and metastasis.[1 5–7] To date there is no report examining the role of Fut1 in inflammatory models of arthritis. *Fut1*$^{-/-}$ mice were resistant to K/BxN serum transfer arthritis and had markedly reduced leucocyte ingress in arthritic ankle cryosections when compared with wt mice. After finding decreased arthritis in *Fut1*$^{-/-}$ mice, we determined if this decrease in arthritis was linked to angiogenesis. The wt ankle

homogenates had significantly more Hb and blood vessels compared with *Fut1*$^{-/-}$ mouse arthritic ankles, suggesting the contribution of Fut1 in regulating angiogenesis in K/BxN serum transfer arthritis. We also examined the contribution of Fut1 in active angiogenesis in K/BxN serum transfer arthritis which is characterised by the presence of immature blood vessels. Immature blood vessels are deficient of periendothelial coverage by smooth muscle cells or pericytes. *Fut1*$^{-/-}$ mouse arthritic ankle cryosections have significant decrease in immature blood vessels when compared with wt mouse arthritic ankle cryosections. This decrease in active angiogenesis in *Fut1*$^{-/-}$ mouse may be due to the key role of Fut1 in cell proliferation, one of the aspects of angiogenesis.[32 33] RA synovial tissue contains significantly more immature blood vessels compared with normal and osteoarthritis synovial tissues which are selectively depleted by anti-TNF-α therapy, indicating the importance of immature vessels in the progression of RA.[34]

One of the limitations of using knockout mice in animal models of arthritis is that we cannot assess the therapeutic interventions in gene-deficient mice. Thus, this model cannot be used to determine the prophylactic treatment for patients with RA who present with already established disease.

We did not find a decrease in VEGF and bFGF in *Fut1*$^{-/-}$ arthritic ankle homogenates, suggesting that these potent angiogenic factors do not contribute to Fut1-mediated angiogenesis and inflammation in K/BxN serum transfer arthritis. We and others have shown that VEGF and FGF-2 are highly expressed in RA compared with osteoarthritis and normal synovial fluids and synovial tissues.[32 33] Anti-VEGF and anti-FGF-2 reduced arthritis severity and joint angiogenesis in mouse collagen induced arthritis and rat adjuvant induced arthritis, respectively.[35] We found a moderate increase, although not statistically significant, in VEGF expression in *Fut1*$^{-/-}$ arthritic ankle homogenates. We think that this increase in VEGF may be a compensatory mechanism to overcome the deficiency of an important angiogenic factor normally, Fut1.

We found that *Fut1*$^{-/-}$ mice have decreased leucocyte ingress and less severe K/BxN serum transfer arthritis in part due to decreased angiogenesis, suggesting one of the mechanisms by which Fut1 regulates inflammatory arthritis. There may be other mechanisms involved in decreased K/BxN serum transfer arthritis development in *Fut1*$^{-/-}$ mice. For instance, cytokine expression and MN ingress are two key factors which contribute to the progression of RA and inflammatory arthritis.[36–38] We have data showing that MCP-1/CCL2 expression is decreased in RA ST fibroblasts[39] and human monocytes (data not shown) when Fut1 is deficient and monocytes migrate less in the absence of Fut1, suggesting the importance and the mechanism by which Fut1 is involved in the development of K/BxN arthritis.

To further investigate the mechanism of decreased angiogenesis, leucocyte recruitment and arthritis in *Fut1*$^{-/-}$ mice, we performed a static cell adhesion assay. In contrast to our study, Kwiatowski *et al* found that increased expression of α1,2 fucosyltransferase in porcine ECs reduced the adhesion of monocytes.[40] The differences in both studies may be because we used mouse ECs and a mouse macrophage cell line while the other study used cells from two different species to examine the adhesion of human monocytes to porcine ECs.

Cell adhesion molecules mediate the inflammatory response by allowing the ingress of leucocytes in chronic inflammatory diseases such as RA. Cell adhesion molecules are shed from the cell surface, circulate in the blood and may stimulate neovascularisation. In soluble form, ICAM-1 mediates angiogenesis.[41] The expression of cell adhesion molecules such as ICAM-1 is increased by cytokines.[42] ICAM-1 is an important member of the immunoglobulin superfamily. ICAM-1 is a heavily N-glycosylated transmembrane protein, but the enzyme involved in its glycosylation has not been examined.[43 44] We studied the contribution of Fut1 to ICAM-1 and VCAM-1 expression on mouse ECs because ICAM-1 and VCAM-1 contribute to angiogenesis and leucocyte migration to RA synovial tissue.[41 45–47] In this study, we found that Fut1 is involved in regulating ICAM-1 expression post-transcriptionally, as suggested by decreased ICAM-1 protein expression on *Fut1*$^{-/-}$ ECs and mouse ankle cryosections. Fut1 also induces ICAM-1 mRNA expression, pointing to a transcriptional role of Fut1 in ICAM-1 expression.

In summary, Fut1 mediates angiogenesis in vitro and in vivo. Fut1 modulates K/BxN serum transfer arthritis by reducing angiogenesis and ICAM-1 expression leading to less leucocyte ingress, suggesting a role for Fut1 in inflammatory and immune-mediated diseases. Thus Fut1 may prove to be a potential therapeutic target for treating angiogenic, inflammatory diseases such as RA.

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Contributors All the authors have contributed substantially to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work.

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Competing interests None.

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Mohammad A Amin, Phillip L Campbell, Jeffrey H Ruth, Takeo Isozaki, Bradley J Rabquer, W Alex Stinson, Martin O'Brien, Gautam Edhayan, Ray A Ohara, Jonathon Vargo, Steven E Domino and Alisa E Koch

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