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## FGF2-induced STAT3 activation regulates pathologic neovascularization

Zhenyu Dong<sup>a</sup>, Andrea Santeford<sup>a</sup>, Norimitsu Ban<sup>a</sup>, Tae Jun Lee<sup>a</sup>, Craig Smith<sup>b</sup>, David M. Ornitz<sup>b</sup>, Rajendra S. Apte<sup>a,b,c</sup>

<sup>a</sup>Department of Ophthalmology and Visual Sciences, Washington University in St. Louis School of Medicine, 660 S. Euclid Ave, St. Louis, MO, USA 63110

<sup>b</sup>Department of Developmental Biology, Washington University in St. Louis School of Medicine, 660 S. Euclid Ave, St. Louis, MO, USA 63110

<sup>c</sup>Department of Medicine, Washington University in St. Louis School of Medicine, 660 S. Euclid Ave, St. Louis, MO, USA 63110

### Abstract

Cell-autonomous endothelial cell (EC) fibroblast growth factor receptor (FGFR) signaling through FGFR1/2 is essential for injury-induced wound vascularization and pathologic neovascularization as in blinding eye diseases such as age-related macular degeneration. Which FGF ligand(s) is critical in regulating angiogenesis is unknown. Utilizing *ex vivo* models of choroidal endothelial sprouting and *in vivo* models of choroidal neovascularization (CNV), we demonstrate here that only FGF2 is the essential ligand. Though FGF-FGFR signaling can activate multiple intracellular signaling pathways, we show that FGF2 regulates pathogenic angiogenesis via STAT3 activation. The identification of FGF2 as a critical mediator in aberrant neovascularization provides a new opportunity for developing multi-target therapies in blinding eye diseases especially given the limitations of anti-VEGF monotherapy.

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To whom correspondence may be addressed: Rajendra Apte, 660 S. Euclid Ave, Box 8096, St. Louis, MO 63110, 314-362-6690, apte@wustl.edu, David Ornitz, 660 S. Euclid Ave, Box 8103, St. Louis, MO 63110, 314-362-3908, dormitz@wustl.edu. NB's present address is: Department of Ophthalmology, Columbia University, New York, NY, USA

#### Contributions

Z.D. designed and conducted experiments, analyzed data, and prepared the manuscript; A.S. conducted experiments, analyzed data, maintained animal colonies and prepared the manuscript; N.B. conducted experiments and analyzed data; T.J.L. conducted experiments; C.S. conducted experiments and maintained animal colonies; D.M.O. devised the study and provided critical feedback on the manuscript; R.S.A. devised the study, prepared the manuscript, and supervised the overall project.

#### Conflicts of interest

The authors have declared that no conflict of interest exists.

#### Financial disclosure

The authors have no financial or property interest in the materials or methods described herein.

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## Keywords

choroidal neovascularization; endothelial cell; fibroblast growth factor; STAT3; choroid sprouting; macular degeneration; retina

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## 1. Introduction

The FGF family is composed of at least 18 signaling ligands, which interact with four cell surface tyrosine kinase receptors, FGFRs 1–4, in complex with heparan sulfate proteoglycan (HPSG) (Ornitz and Itoh, 2015). The endothelial cell response to FGF signals has been characterized using *in vitro* and *in vivo* models of angiogenesis (Chen et al., 2016; Gospodarowicz et al., 1978; Murakami et al., 2008; Oladipupo et al., 2014; Yamada et al., 2000). Using mice deficient in *Fgfr1* and *Fgfr2* in cells of both endothelial and hematopoietic lineages, we discovered that endothelial FGFR1/2 signaling is not required for embryonic development or for maintaining vascular integrity and function under homeostatic physiological conditions, despite the well-established role for FGF signaling in endothelial cells for vascular development *in vitro*. In contrast, when subjected to either skin or eye injury, or hypoxia-induced pathologic angiogenesis (House et al., 2016), mice lacking endothelial FGFR1/2 display a significant reduction in neovascular growth and tissue repair (Oladipupo et al., 2014).

Pathologic neovascularization is central to diverse disease processes including cancer, atherosclerosis and blinding eye diseases. In the eye, pathologic neovascularization can cause blindness in advanced age-related macular degeneration (AMD), the leading cause of blindness in people over 50 years of age in the industrialized world (Sene and Apte, 2014). The exudative or neovascular form of AMD is devastating as it causes the majority of acute vision loss and is characterized by the development of abnormal and leaky blood vessels underneath the retina called CNV. Although VEGF plays a significant role in the pathogenesis of CNV in AMD, it is becoming increasingly clear that VEGF-independent pathways also play a key role in the development and growth of CNV (Oladipupo et al., 2014; Sene and Apte, 2014; Sene et al., 2015; Stahl et al., 2009). In addition, resistance to anti-VEGF therapy and persistence of disease despite VEGF neutralization remains a significant clinical hurdle (Bakall et al., 2013; Gasperini et al., 2012; Spooner et al., 2018; Wagle et al., 2011; Yonekawa et al., 2013). Therefore, elucidating VEGF-independent pathways that promote CNV in diseases such as AMD is a high priority. Given the importance of the FGF-FGFR signaling pathway in promoting CNV, we conducted a series of experiments to identify the FGF ligand(s) critical to pathologic angiogenesis. Using established murine models of CNV and *ex vivo* models of vascular proliferation using explanted choroidal tissue, we identified FGF2 as the pathogenic ligand that regulates vascular endothelial proliferation and CNV via intracellular STAT3 activation. These findings have potential clinical implications given the high interest in developing multi-target therapeutic approaches that complement VEGF-based therapies in AMD.

## 2. Materials and Methods

### 2.1 Animals

All animal experiments and experimental procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the Washington University School of Medicine Animal Care and Use guidelines as well as ARVO Guidelines for Use of Animals in Research.

C57BL/6J mice (age <3 months) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice genetically deficient in *Fgf2* (*Fgf2*<sup>-/-</sup>), *Fgf8*<sup>ER-TAM Cre</sup> and *Fgf9*<sup>ER-TAM Cre</sup>, and *Fgfr1/2/3*<sup>Tie2-Cre</sup> mice in which *Fgfr1*, *Fgfr2* and *Fgfr3* genes were inactivated in Tie2-Cre-expressing endothelial cells as well as their littermates with the un-recombined *Fgfr1/2/3*<sup>fl/fl</sup> alleles, lacking the Tie2-Cre allele, were generated from published mouse lines (Colvin et al., 1996; Colvin et al., 2001; Deng et al., 1996; Meyers et al., 1998; Trokovic et al., 2003; Xu et al., 1998; Zhou et al., 1998). For the induction of ER cre in *Fgf8*<sup>ER-TAM Cre</sup> and *Fgf9*<sup>ER-TAM Cre</sup> mice, animals were fed 500mg/kg Tamoxifen-containing chow (TD150858; Envigo, Summerset, NJ) *ad libitum* for 2 weeks.

### 2.2 Antibodies

Neutralizing antibody against mouse FGF2 was purchased from Abcam (ab33103, Cambridge, MA) and its IgG isotype control from R&D Systems Inc. (MAB002, R&D Systems Inc., Minneapolis, MN). Antibodies used in western blotting were purchased from Cell Signaling Technology (Danvers MA): rabbit polyclonal antibodies against phospho-STAT1 (Tyr705, #9167), phospho-STAT3 (Tyr705, #9145), phospho-STAT5 (Tyr694, #4322), phospho-PLC $\gamma$ 1 (#2821), phospho-MEK1/2 (#9121), phospho-AKT (#9271), STAT1 (#9172), STAT3 (#12640), STAT5 (#94205), PLC $\gamma$ 1 (#2822), MEK1/2 (#9122) and AKT (#9272); and Sigma-Aldrich (St. Louis, MO): mouse monoclonal antibody against  $\beta$ -ACTIN (A2228).

### 2.3 Choroid sprouting assay

Choroid sprouting assay was performed as previously described with slight modification (Shao et al., 2013). Briefly, the RPE-choroid-sclera complex (“choroid explant”) was dissected from mouse eyes, segmented to create multiple samples of similar size and shape to either receive later treatment or serve as vehicle control for that choroid sample set, and immediately placed on the surface of growth factor-reduced Matrigel™ (Corning, NY) in 24-well tissue culture plates. Choroid explants were incubated in 500 $\mu$ L CSC complete medium (Cell Systems, Cat. 420–500) activated with growth factor boost and supplemented with 1% Penicillin/Streptomycin (GIBCO, Grand Island, NY) and 5  $\mu$ g/mL of Plasmocin™ (Invitrogen, Cat. Ant-mpt) at 37°C with 5% CO<sub>2</sub> for 48 hours before any treatment. For FGF stimulation experiment, choroid explants were treated with FGFs (FGF2: SRP4038, FGF9: SRP4054, Sigma-Aldrich; FGF8: ab205522, Abcam) in the presence of 1 $\mu$ g/ml heparan sulfate (Sigma-Aldrich). For sprouting inhibition experiment, either vehicle (DMSO, Sigma-Aldrich) or STAT3 inhibitor (Stattic, R&D Systems Inc.) was added to the medium. The final concentration of DMSO is 0.2% (v/v). Medium was changed every 48 hours. Phase contrast photos of individual explants were taken every other day for 8 days post-dissection

using an Olympus Microfire camera on an Olympus CKX41 microscope. The area of neovascular growth extending beyond the explant tissue area was measured for each sample. Each measurement of vascular area was normalized to the area of growth for vehicle-treated samples originating from the same choroid for each timepoint and the resulting data is expressed in relative units.

## 2.4 Laser-induced choroidal neovascularization

Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (100mgkg<sup>-1</sup>) and xylazine (13.4mg kg<sup>-1</sup>). Pupils were dilated with 1% tropicamide. Induction of CNV was performed with an Argon laser as previously described with a slight modification ((Nakamura et al., 2015a); (Apte et al., 2006); (Kelly et al., 2007); (Sene et al., 2013)). Briefly, after anesthesia induction and pupil dilation, four laser spots were placed around the optic disc (200mW, 0.1s, 100µm spot size) using a slit-lamp delivery system with a cover glass as a contact lens. Laser spots with vitreous, retinal, or subretinal hemorrhage were excluded from the analysis.

For measurement of CNV, RPE-choroid complex flat mounts were made as previously described (Ban et al., 2017; Nakamura et al., 2015a; Sene et al., 2013). Briefly, seven days after injury, mice were anesthetized and perfused with 2000µl of 5mg/ml FITC-labeled dextran (M.W. 2,000,000, Sigma-Aldrich) via the left ventricle. Eyes were enucleated and fixed immediately in 2% PFA for 30 minutes. Eyes were then washed with phosphate buffered saline (PBS) and the RPE-choroid complex was flat mounted onto a glass slide. Z-stack images of CNV spots were acquired using an Olympus Fluoview1000 confocal microscope and processed using ImageJ software (NIH, Bethesda, MD) to create pseudo volumetric 2D images. Pixel intensity was quantified using Metamorph software analysis (Sunnyvale, CA).

For FGF2 treatment, intravitreal injection of 1µl FGF2 or vehicle (PBS) was performed using a 30-gauge needle immediately after the laser injury.

For FGF2 neutralizing antibody treatment, intravitreal injection of either 1µl neutralizing antibody against FGF2 (ab33103, Abcam) or 1µl its IgG isotype control (MAB002, R&D Systems Inc.) was performed as described above.

## 2.5 Western blot analysis

Laser rupture of the Bruch's membrane of C57BL/6J wild type mice was conducted as described above. Three days post CNV induction, RPE-choroid complex were collected and sonicated in lysis buffer supplemented with proteinase and phosphatase inhibitors. After being centrifuged at 14,000 × g for 20 min, the supernatants were collected for western blot analysis. The electrophoresis and transfer were performed as previously described (Sene et al., 2013; Nakamura et al., 2015b; Ban et al., 2017). The membranes were then blocked for 2 hours at room temperature with 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in TBS with 0.1% Tween-20 (0.1% TBST), and incubated with the antibodies against phospho-Stat1 (1:1000), phospho-Stat3 (1: 2000), phospho-Stat5 (1:1000), phospho-PLCγ1 (1: 1000), phospho-MEK1/2 (1:1000), phospho-AKT (1:1000) (Cell Signalling Technologies, Danvers, MA; #9145, 4322, 2821, 9121, and 4058, respectively), and monoclonal β-actin (1:

5000; A5316, Sigma) overnight at 4°C. Next, the membranes were washed with 0.1% TBST and incubated with secondary antibodies conjugated to either IRDye 800CW or IRDye 680LT (1:5,000; LI-COR, Lincoln, NE) for 60 min at room temperature, respectively. Immunoreactive bands were visualized using the Odyssey Infrared Imaging System (LI-COR). Then the membranes were stripped using Re-Blot Plus Western Blot Recycling Kit (EMD Milipore, Corp. Cat No.2500), and reprobed with antibodies against Stat1 (1:1000), Stat3 (1:1000), Stat5 (1:1000), PLC $\gamma$ 1 (1:1000), MEK1/2 (1:1000) and Akt (1:1000), respectively (Cell Signalling Technologies; 9172, 12640, 9272, 2282, and 9122). The membranes were then incubated with secondary antibodies and immunoreactive bands were visualized as described above.

## 2.6 Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical evaluations were performed using GraphPad Prism Software version 6.0 (GraphPad, San Diego, CA). One-way or 2-way mixed analysis of variance (ANOVA) with Bonferroni post test, or two-tailed Student's unpaired t-test was used for comparison among groups. The accepted level of significance for all tests was  $p < 0.05$ .

## 3. Results

### 3.1 FGF2 stimulates vascular sprouting from choroid explants

In prior studies, we demonstrated that after injury to induce CNV, *Fgf2*, *Fgf8* and *Fgf9* were the only FGF ligands whose expression was significantly upregulated in addition to anticipated upregulated of *Vegfa* (Oladipupo et al., 2014). In those studies, we also demonstrated a VEGF-independent and critical role for FGFR1/2 signaling in the vascular endothelial cell response to injury. These data suggested the possibility that FGF ligands induced during tissue injury were important in the VEGF-independent vascular response.

To further identify which FGF ligand(s) is critical to ocular neovascularization, we utilized the choroidal endothelial sprouting assay, a well-established *ex vivo* model of microvascular angiogenesis (Shao et al., 2013). We compared the effect of various doses of either FGF2, FGF8 or FGF9 on the proliferation of choroidal endothelial cells. Of note, only FGF2 stimulated vascular sprouting from choroid explants of C57BL/6J mice in a dose-dependent manner (normalized sprouting area relative to vehicle control for each choroid at day 8 post-explant was  $25.5 \pm 7.1$ ,  $p < 0.05$  for 10ng FGF2 and  $38.8 \pm 7.4$ ,  $p < 0.01$  for 100ng FGF2 as compared to  $15.7 \pm 1.9$ ,  $n = 9-11$  for vehicle controls, 2-way mixed ANOVA with Bonferroni's multiple comparison test. Fig. 1, A and D top panel). In contrast, neither FGF8 ( $n = 14-16$ ) nor FGF9 ( $n = 10-14$ ), affected vascular sprouting from choroid explants as compared to controls (Fig. 1, B-D bottom panels). Furthermore, the effect of FGF2 on vascular sprouting was completely eliminated if the choroidal explants were isolated from *Fgfr1/2/3<sup>Tie2-Cre</sup>* mice where FGFR1/2/3 are selectively deficient in endothelial cells (Fig. 2, A and D top panel). As expected, neither FGF8 nor FGF9 effected vascular sprouting in *Fgfr1/2/3<sup>Tie2-Cre</sup>* explants (Fig. 2, B-D bottom panel). These results suggest that among FGF ligands upregulated after injury, FGF2 but not FGF8 or 9, regulated choroidal endothelial cell sprouting. In addition, the effect of FGF2 required endothelial FGFR

signaling as demonstrated by the failure to induce sprouting in choroidal explants isolated from mice lacking vascular endothelial FGFR signaling.

### 3.2 FGF2 promotes choroidal neovascularization in mice

Based on the above results, we next investigated whether FGF2 stimulated angiogenesis in the previously described laser injury-induced CNV mouse model. FGF2 (1ng, 10ng or 100ng) or PBS was injected into the vitreous immediately after the laser injury and evaluation of CNV was performed 7 days later by confocal microscopy. As compared to control mice that received PBS injection ( $40599.3 \pm 4508.1 \mu\text{m}^2$ ), FGF2 promoted CNV formation in a dose-dependent manner, particularly at a dose of 10ng or 100ng ( $82273.4 \pm 17833.4 \mu\text{m}^2$ ,  $p < 0.01$ , and  $93878.9 \pm 12475.6 \mu\text{m}^2$ ,  $p < 0.01$ , respectively.  $n = 13-16$ , one-way ANOVA with Bonferroni's multiple comparison test. Fig. 3A). On the other hand, intravitreal injection of anti-FGF2 neutralizing antibody significantly suppressed CNV formation as compared to the injection of control antibody ( $30369.2 \pm 5010.7 \mu\text{m}^2$  vs.  $74338.9 \pm 15250.2 \mu\text{m}^2$ ,  $p < 0.05$ , respectively.  $n = 7-9$ , Mann-Whitney u-test. Fig. 3B). To confirm the role of FGF in promoting CNV, we used *Fgf2*<sup>-/-</sup> mice that lacked expression of the FGF2 protein. In *Fgf2*<sup>-/-</sup> mice, CNV was significantly reduced as compared to littermate controls ( $93264 \pm 7690 \mu\text{m}^2$  vs.  $30613 \pm 6476 \mu\text{m}^2$ ,  $p < 0.0001$ , respectively.  $n = 6-8$ , two-tailed Student's unpaired t-test. Fig. 3C). No difference in CNV formation was noted in mice lacking FGF8 ( $30356 \pm 3989 \mu\text{m}^2$  vs.  $39511 \pm 5310$ ,  $n = 8-9$ ) (Fig. 3D) or FGF9 ( $34114 \pm 7775 \mu\text{m}^2$  vs.  $35336 \pm 9459 \mu\text{m}^2$ ) (Fig. 3F) expression compared to their respective littermate controls. Collectively, those results demonstrate that FGF2, but not FGF8 or FGF9, plays an important, pro-angiogenic role in choroidal neovascularization.

### 3.3 Activation of the STAT3 pathway occurs after induction of CNV

Next, we investigated which FGF2-mediated intracellular signaling pathway(s) were potentially critical to ocular neovascularization given the robust phenotype above. We induced laser injury in the choroid of right eye of C57BL/6J wild type mice and used the left eye as an uninjured control. Three days after the injury, eyes were enucleated and the choroid-RPE complex was harvested and processed for immunoblotting. As shown in Fig. 4A, among the four intracellular signaling pathways that are known to be activated by FGF2 (RAS-RAF-MAPK, PI3K-AKT, STAT1,3,5 and PLC $\gamma$  pathways), only STAT3 was activated following laser injury while the other signaling mediators including MEK1/2 in RAS-RAF-MAPK pathway, AKT in PI3K-AKT pathway, PLC $\gamma$ 1 in PLC $\gamma$ -regulated pathways, and STAT1 and STAT5 were not activated.

### 3.4 FGF2 stimulates choroidal neovascularization via STAT3 activation

Based on these results, we next examined whether STAT3 inhibition might suppress ocular angiogenesis in both the *ex vivo* and *in vivo* models presented above. Stattic, a small-molecule inhibitor of STAT3 activation and dimerization, significantly suppressed FGF2-induced choroidal sprouting by day 8 as compared to choroid explants treated with vehicle (relative sprouting area at day 8 is  $4.5 \pm 0.9$  vs  $9.3 \pm 0.8$ , respectively.  $n = 6$ , 2-way mixed ANOVA with Bonferroni's multiple comparison test. Fig. 4B). In the injury-induced CNV model, Stattic significantly suppressed CNV development ( $13496.7 \pm 3202 \mu\text{m}^2$  vs.  $44932.3 \pm 13152.9 \mu\text{m}^2$ ,  $p < 0.05$ , respectively.  $n = 7-8$ , two-tailed Student's unpaired t-test. Fig. 4C).



These results suggest that FGF2 may signal through the STAT3 pathway in endothelial cells to regulate ocular neovascularization.

#### 4. Discussion

Pathologic neovascularization is critical in cancers, atherosclerosis and blinding eye diseases including AMD and diabetic retinopathy. VEGF is an important driver of neovascularization in these diseases and therapies that target VEGF can reduce pathogenic vascularization in many of these diseases (Apte et al., 2019). In many advanced/metastatic cancers, anti-VEGF therapies are approved by regulatory authorities for therapy in combination with other chemotherapeutic agents (Duh et al., 2017; Miller et al., 2013; Sene and Apte, 2014; Sene et al., 2015).

As in cancer, several anti-VEGF agents have been demonstrated to prevent vision loss in eye diseases including AMD and diabetic retinopathy (Aiello, 2005; Heier et al., 2012). A key therapeutic challenge in eye diseases is the clear evidence of under-responsiveness to VEGF-targeted therapy over the long term, the increased therapeutic burden associated with the need for chronic intraocular therapy, and the resistance to therapy that develops in some patients (Apte, 2016). This may be similar to what has been known for a long time with cancer therapy where multiple therapeutic agents that target diverse molecular pathways usually lead to optimal clinical outcomes (Gerber and Ferrara, 2005). As such, there is a strong need to identify novel, VEGF-independent pathways that can be developed for eye diseases but this effort has been hampered by recent therapeutic failures.

Here, using both *in vivo* and *in vitro* models, we identify FGF2 as the critical ligand that activates *Stat3* via FGFR signaling and promotes pathologic neovascularization. Given that our previous studies demonstrate that the FGFR signaling pathway may be VEGF independent, this finding is potentially therapeutically relevant in opening a novel vista for development where either FGF2 or STAT3 may be targeted in order to reduce pathogenic neovascularization. This is especially exciting given the compounds that target both these molecules are currently being tested in clinical trials. Due to the potential binding of FGF2 to VEGFR2, it remains possible that FGF2 could contribute to VEGF signaling to some degree. Although a significant amount of work needs to be done in the translational aspects in determining whether targeting these pathways is safe and efficacious, it remains possible that should these pathways have therapeutic value. Such therapeutic agents could be combined with current anti-VEGF regimens in order to enhance efficacy and reduce the risk of vision loss in these devastating diseases.

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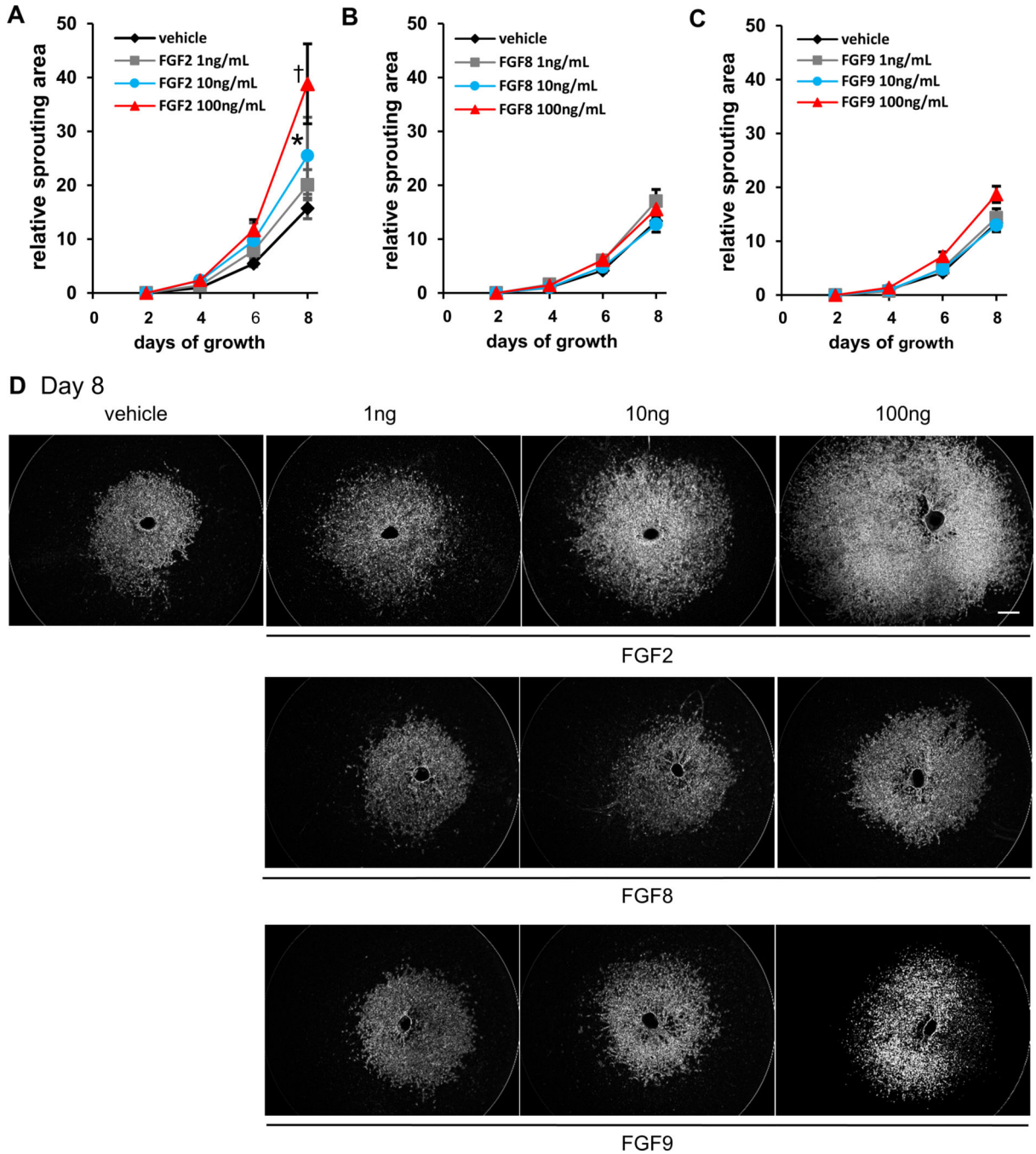
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### Highlights

- Fibroblast growth factor-2 (FGF-2) regulates choroidal neovascularization
- FGF-2 activates intracellular stat3
- The identification of the fgf-2/stat3 axis presents novel therapeutic opportunities



**Fig. 1. FGF2, but not FGF8 or FGF9, stimulates neovascular sprouting.** FGF2 stimulated neovascular sprouting in a dose-dependent manner in C57Bl/6J choroid explants by 8 days post-treatment at doses of 10 or 100 ng/ml (n=9–11; 15.7 ± 1.9 vs. 25.5 ± 7.1 relative sprouting area (RSA) (10ng/mL); \*p<0.05, and 15.7 ± 1.9 vs. 38.8 ± 7.4 RSA (100ng/mL); † p<0.01; 2-way mixed ANOVA with Bonferroni’s multiple comparison test). (A). Neither FGF8 (n=14–16; 13.4 ± 0.73 vs. 15.5 ± 1.5 RSA (100ng/mL) (B) nor FGF9 (n=10–14; 13.4 ± 0.73 vs. 18.7 ± 1.5 RSA) (C) stimulated choroidal sprouting regardless of

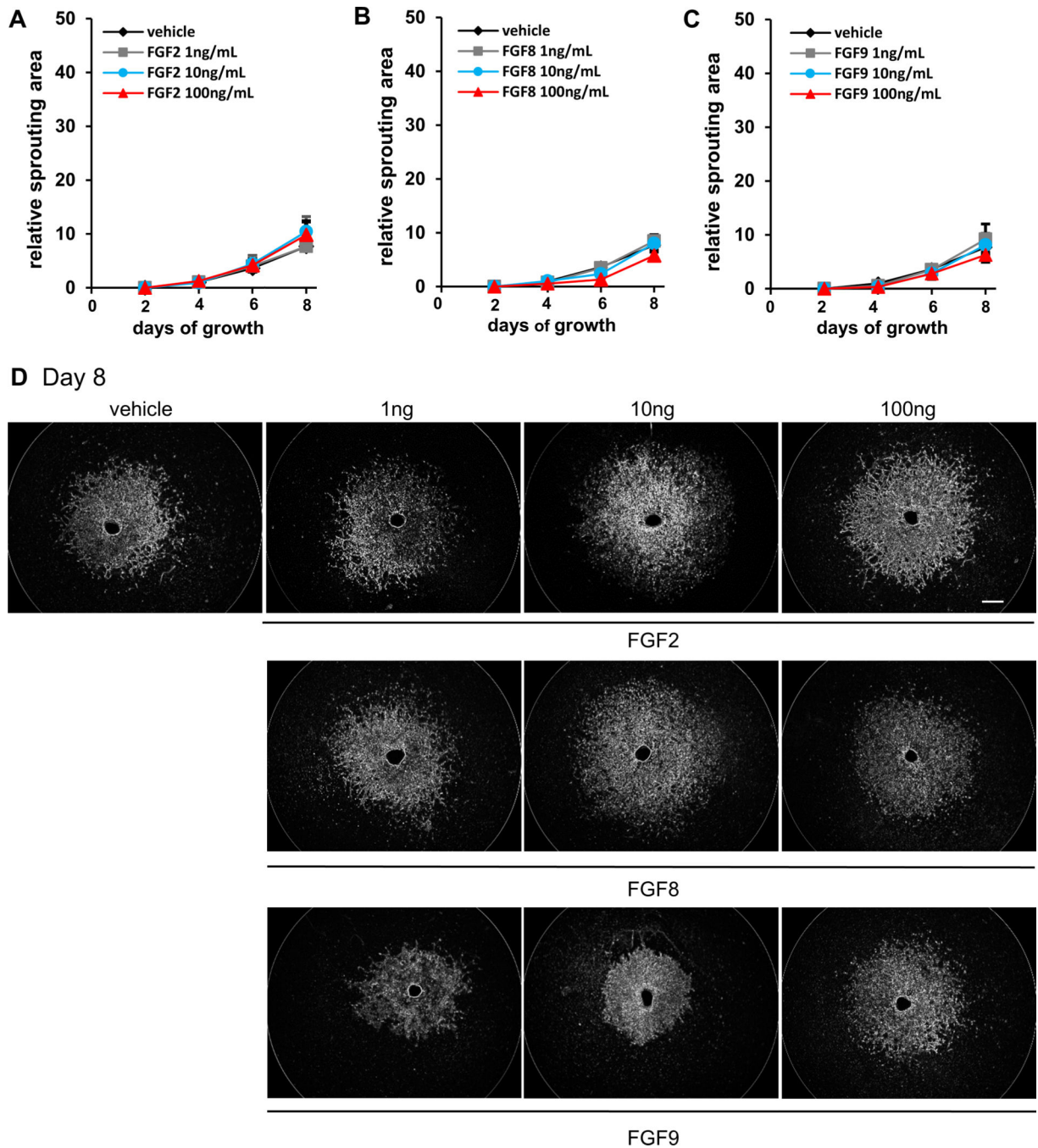
dose. Representative pictures of choroidal explants treated with FGF2, FGF8 or FGF9 (**D**).  
Scale bar: 500  $\mu$ m.

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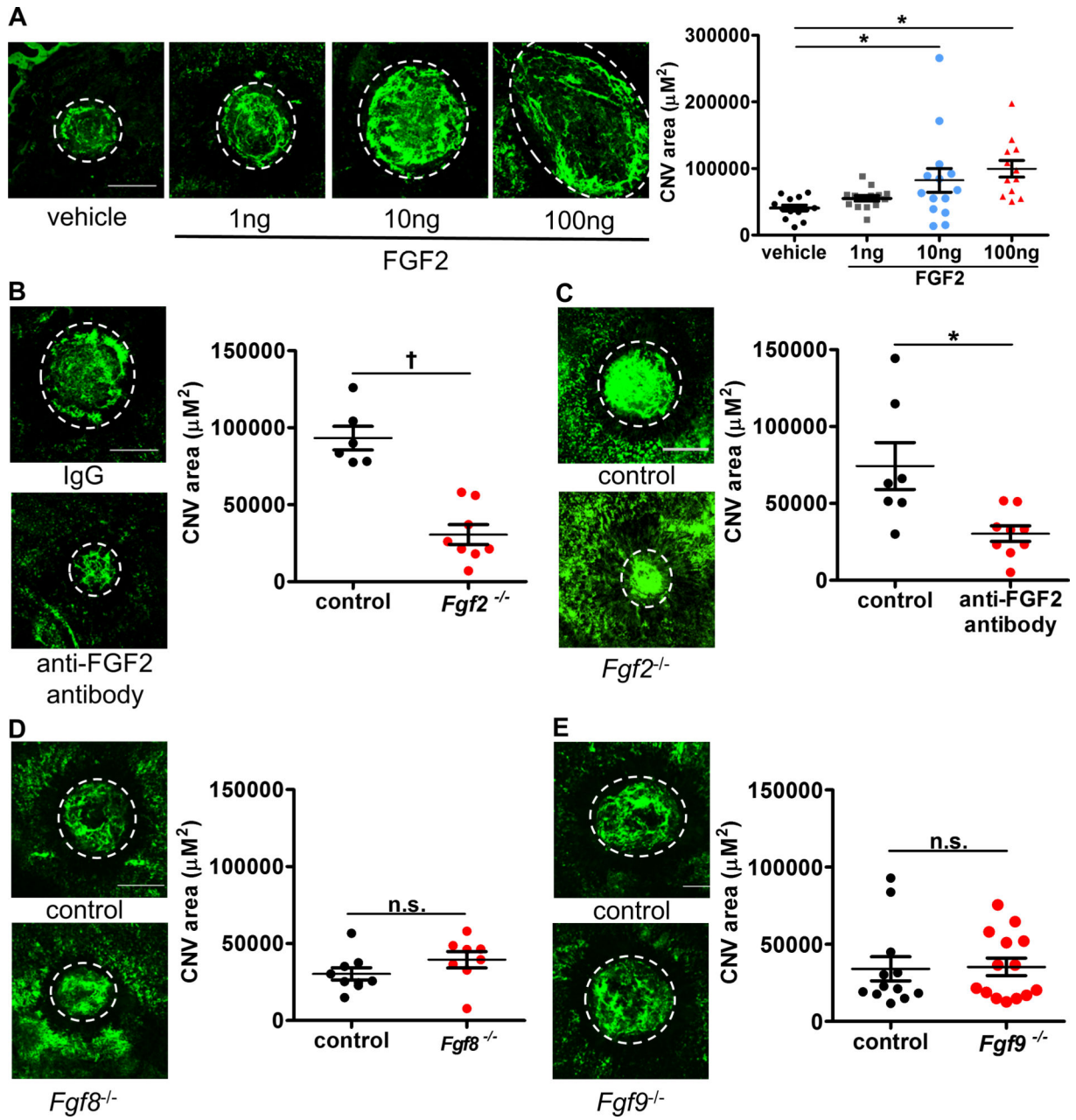
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**Fig. 2. Neovascularization stimulated by FGF2 requires endothelial FGFR.**

Neither FGF2 ( $9.8 \pm 2.4$  RSA at 100ng/mL) (A), FGF8 ( $5.7 \pm 0.88$  RSA at 100ng/mL) (B) nor FGF9 ( $6.2 \pm 1.3$  RSA at 100ng/mL) (C) ligands stimulated choroidal sprouting in *FGFR1/2/3<sup>-/-</sup>* endothelial cell triple knockout mouse choroid explants (vs. vehicle control  $7.7 \pm 0.93$  RSA at 100ng/mL; n=3-8; no significance in 2-way mixed ANOVA with Bonferroni's multiple comparison test). Representative pictures of choroidal sprouting treatments (D) Scale bar: 500  $\mu$ m.

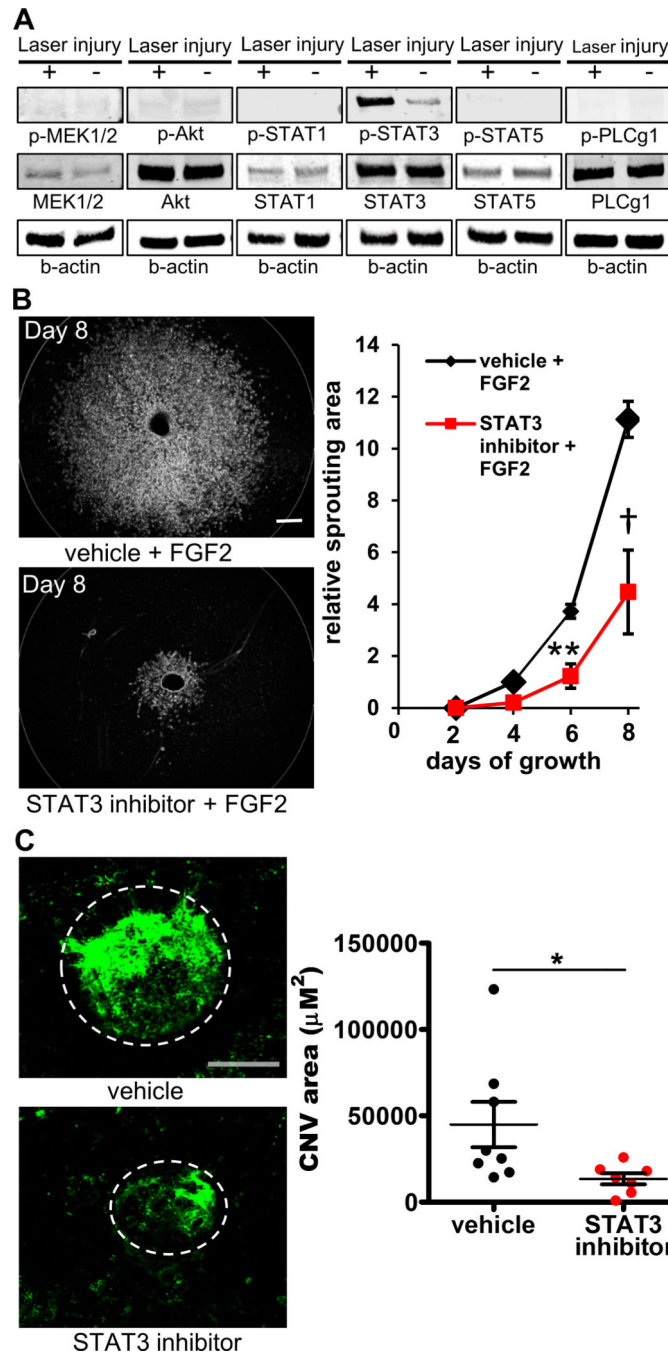




**Fig. 3. FGF2, but not FGF8 or FGF9 stimulates pathogenic CNV after laser induction.** FGF2 stimulated CNV formation following laser injury in C57Bl/6J mice in a dose-dependent manner, reaching significance at doses of 10 ng and 100 ng compared to mice receiving vehicle control (n=13–16;  $40599.3 \pm 4508.1 \mu\text{M}^2$  vs  $82273.4 \pm 17833.4 \mu\text{M}^2$ , \* $p < 0.01$  (10ng) or  $93878.9 \pm 12475.6 \mu\text{M}^2$ , \* $p < 0.01$  (100ng); one-way ANOVA with Bonferroni’s multiple comparison test (A). Neutralizing antibody against FGF2 significantly suppressed CNV formation (n=7–9;  $30369.2 \pm 5010.7 \mu\text{M}^2$  vs.  $74338.9 \pm 15250.2 \mu\text{M}^2$ , \* $p < 0.05$ ; two-tailed Student’s unpaired t-test (B). Furthermore, CNV formation was impaired in *Fgf2*<sup>-/-</sup> mice as compare to its littermates (n=6–7;  $93264 \pm 7690 \mu\text{M}^2$  vs.  $26699 \pm 5958 \mu\text{M}^2$ , † $p < 0.0001$ ; two-tailed Student’s unpaired t-test (C) while deletion of *Fgf8* (D)



(n=8; n.s.= no significance by two-tailed Student's unpaired t-test) or *Fgf9*(E) (n=13–15; n.s.= no significance by two-tailed Student's unpaired t-test) did not significantly modify CNV volumes compared to their respective littermate controls. Scale bar: 200  $\mu\text{m}$ .



**Fig. 4. The STAT3 signaling pathway is essential for CNV growth.**

Western blotting demonstrates that the STAT3 pathway in the RPE-choroid complex is activated following the induction of choroidal neovascularization, while other FGF2-induced intracellular signaling pathways remain inactivate (A). Inhibition of STAT3 by the small molecule, Stattic, significantly suppressed choroidal sprouting 8 days post-explant dissection ( $n=6$ ;  $4.5 \pm 0.9$  vs  $9.3 \pm 0.8$ ;  $*p<0.05$ ; 2-way mixed ANOVA with Bonferroni's multiple comparison test) (B). STAT3 inhibition also significantly suppressed CNV formation in vivo

(n=7–8;  $13496.7 \pm 3202 \mu\text{m}^2$  vs.  $44932.3 \pm 13152.9 \mu\text{m}^2$ , \* $p < 0.05$ ; two-tailed Student's unpaired t-test) (C) Scale bar: 200  $\mu\text{m}$ .

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