

Found in Inflammatory Zone 1 Induces Angiogenesis in Murine Models of Asthma

Yan Sun · Jinrong Wang · Huabing Li ·
Xiuzhen Han

Received: 24 February 2008 / Accepted: 12 May 2008
© Springer Science+Business Media, LLC 2008

Abstract Angiogenesis plays an important role in airway remodeling that occurs in asthma, while the mechanisms underlying this process are not fully elucidated. Found in inflammatory zone 1 (FIZZ1), a novel cysteine-rich secreted protein, is able to promote proliferation and migration of pulmonary endothelial cells, and to upregulate the expression of vascular endothelial growth factor (VEGF). However, the role of FIZZ1 in angiogenesis of asthma has not been fully investigated. Murine models of asthma were sensitized on days 1 and 14 by ovalbumin (OVA) and challenged with 2% OVA beginning from day 21. Mice were divided into four groups: asthmatic mice challenged with OVA for 7 days, 14 days, and 28 days, respectively, and healthy control mice that were sensitized and challenged with PBS. The expressions of FIZZ1, VEGF, and von Willebrand factor (vWF)-stained vascular area were measured in asthmatic mice and healthy controls ($n = 10$). Histologic examination was also performed on airway inflammation. Levels of FIZZ1 were increased significantly after allergic challenge, reached the peak by 7 days, declined by 14 days, and were reduced further by 28 days after OVA challenge. Similarly, percentages of vWF-stained vascular area (percentage of vascularity) were largely increased within 7 days and then reduced from day 14 of challenge. The expression of FIZZ1 in asthma was positively correlated with vWF-stained vascular area and

VEGF expression in a time-dependent manner. FIZZ1 expression was significantly correlated with the percentage of vascularity and VEGF expression, suggesting that FIZZ1 and VEGF have crucial roles in angiogenesis of asthma.

Keywords Angiogenesis ·
Found in inflammatory zone 1 ·
Vascular endothelial growth factor · Asthma

Introduction

Angiogenesis plays an important role in the processes of asthma. Newly formed bronchial vessels are the portal for inflammatory cells to the airway wall, and the increased vessels are the consistent pathologic components in airway remodeling. Investigation has been focused on vessel growth in asthma [1, 2], while the mechanisms underlying angiogenesis are not fully explored. The protein found in inflammatory zone 1 (FIZZ1) is also known as resistin-like molecules (RELM) alpha and hypoxia-induced mitogenic factor (HIMF). RELM beta, the most related human homolog of FIZZ1, was upregulated in hypoxia in adventitial fibroblasts and smooth muscle cells of pulmonary arteries [3]. Limited biological data have showed that FIZZ1 is a key marker of alternatively activated macrophages [4, 5]. Recently, FIZZ1 was demonstrated to stimulate proliferation of pulmonary vascular smooth muscle cells [6] and pulmonary endothelial cells, and to promote the production of vascular endothelial growth factor (VEGF) [7], suggesting that it is an angiogenic factor. In this article the expression of FIZZ1 and changes of vascularity were examined in murine models of asthma.

Y. Sun · H. Li
Department of Pediatrics, Shandong Provincial Hospital,
Shandong University, 44 Wenhua Xi Road, Jinan 250012,
People's Republic of China

J. Wang · X. Han (✉)
Department of Pediatrics, Shandong Provincial Hospital,
324 Jingwu Road, Jinan 250021, People's Republic of China
e-mail: hanxiuzhen1119@126.com

Materials and Methods

Establishment of Murine Models of Asthma

Forty female BALB/c mice (about 20 g) were habituated to experimental conditions for 1 week. Models of asthma were established as described (Fig. 1): Mice were sensitized by an injection of 200 μ l (0.5 μ g/ μ l, i.p.) of chicken OVA (Sigma, St. Louis, MO) emulsified in aluminum hydroxide (1 mg) on day 1 and day 14. From day 21, mice were challenged daily for 30 min with 2% OVA via a nebula (Bestneb Nebulizer) in the experimental chambers (30 $\times$ 18 $\times$ 13 cm) for 7 days, 14 days, or 28 days. The healthy control mice were sensitized and challenged with phosphate buffered saline (PBS) for 14 days. All animal care and protocols were in accordance with the regulations of the Care and Use of Laboratory Animals.

Twenty-four hours after the last challenge, asthmatic mice after 7 days, 14 days, or 28 days of OVA challenge and the healthy control mice were sacrificed. Specimens from lungs were subjected to histologic and immunohistochemical studies.

Histopathology

Lungs were inflated with 4% paraformaldehyde and processed in paraffin-embedded blocks. The specimens were cut at 5 μ m in thickness, and stained with hematoxylin & eosin to evaluate morphologic changes. The sections were examined under microscope (Leica DM4000B) with the IM 50 Image Manager software.

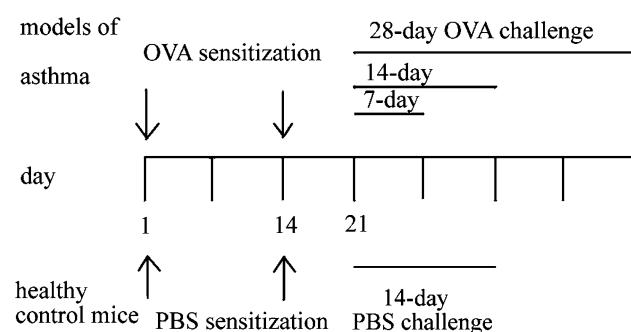


Fig. 1 Experiment design. Models of asthma were established as described. Mice were sensitized by an injection of 200 μ l (0.5 μ g/ μ l, i.p.) of chicken OVA emulsified in aluminum hydroxide (1 mg) on day 1 and day 14. From day 21, three groups of mice were challenged daily with 2% OVA via a nebula in the experimental chambers (30 min) for 7 days, 14 days, or 28 days, respectively. The healthy control mice were sensitized and challenged with PBS for 14 days. Twenty-four hours after the last challenge, mice from the asthmatic groups and the control group were sacrificed and lung specimens were removed for study

Immunohistochemistry

Sections of lung tissues were quenched of endogenous peroxides with H_2O_2 and boiled in sodium citrate (0.01 mol/L, pH 6.0) for antigen retrieval to unmask antigen epitopes. After being blocked with rabbit serum, sections were incubated in diluted primary antibodies to FIZZ1 or VEGF (1:200, Santa Cruz Biotechnology) overnight at 4°C. Then the slides were incubated with secondary antibodies and detected with SABC Elite kit reagents, followed with incubation with diaminobenzidine. Vessels were labeled with antibody of von Willebrand factor (vWF, Gene Tech, China) that binds uniformly to vascular endothelial cells.

The expression of FIZZ1 and VEGF were quantified in three fields ($\times$ 400) per respective sample, expressed as the percentages of positive bronchial epithelia [8]. Vessel area stained with vWF was quantified using Leica Owin software. The vWF-stained vascular area was divided by the total area of the image field, which indicated the percentage of vascularity [9].

Statistical Analysis

One-way ANOVA with subsequent *post hoc* test was used to examine statistical difference. The relationship was evaluated with Spearman's test. Values were expressed as mean $\pm$ SEM, and analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, IL) with $p < 0.05$ described as statistically significant.

Results

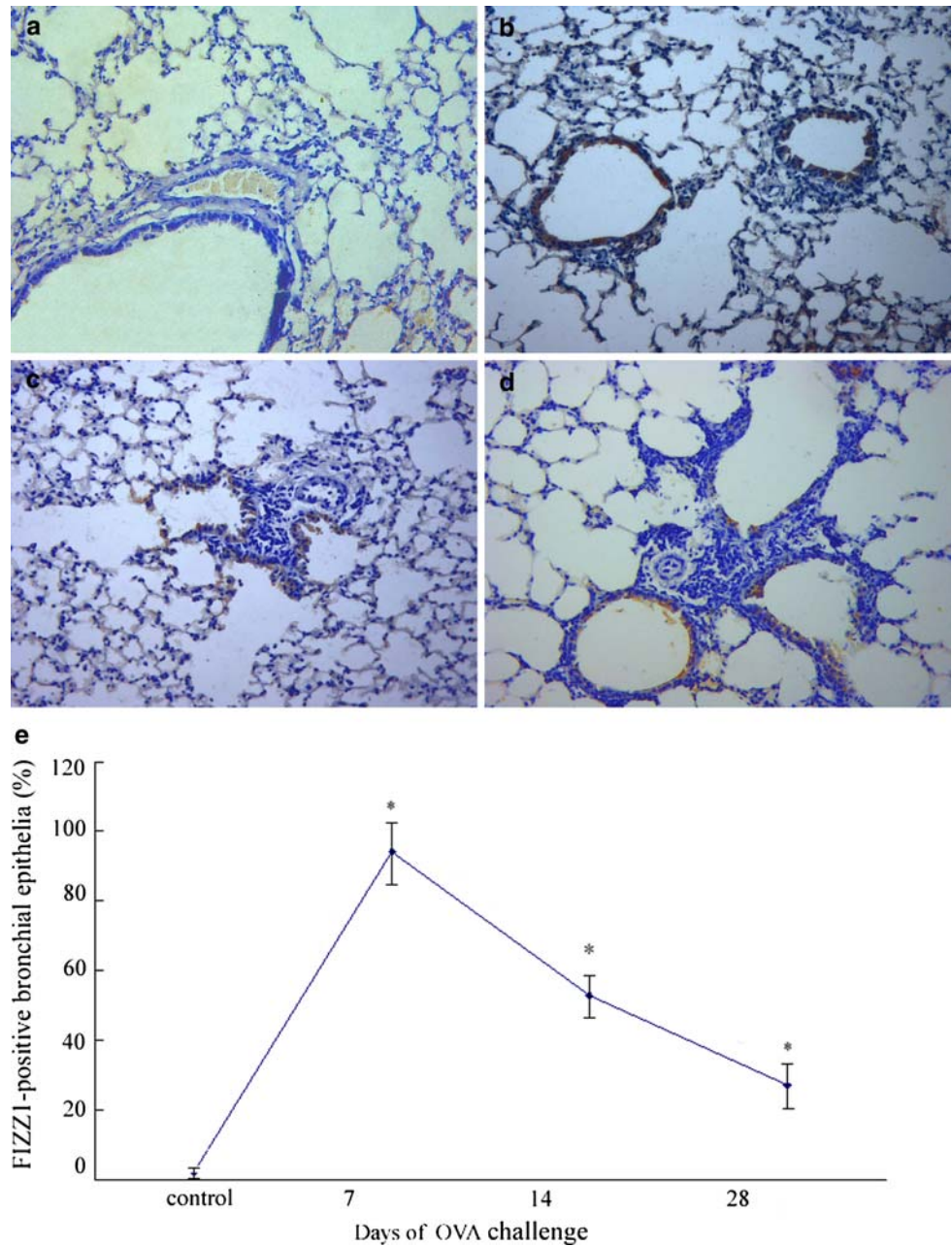
Allergic Airway Histologic Changes

The hypertrophic, hyperplastic bronchial epithelia showed structural injury, and histologic analysis indicated significant inflammation in models of asthma, a large number of infiltrated inflammatory cells in lung tissues, especially in the peribronchial and perivascular zones. The allergic inflammation was worsening with the increase of OVA-challenged time. In contrast, only a few inflammatory cells were examined in lung tissues of healthy control mice.

FIZZ1 Expression in a Time-dependent Manner

FIZZ1 was expressed at very low levels in bronchial epithelial cells in healthy control mice. In the models of asthma, FIZZ1 expression was significantly increased in bronchial epithelium and type II alveolar pneumocytes ($p < 0.001$, Fig. 2). During airway allergic inflammation, FIZZ1 levels peaked by 7 days of challenge (nearly 100% bronchial epithelia were positive), declined on 14 days of

Fig. 2 Percentages of FIZZ1-positive bronchial epithelia. FIZZ1 expression reached the peak values in 7-day OVA-challenged mice (**b**), then declined in 14-day challenged mice (**c**), and was reduced further in 28-day-challenge mice (**d**), which was still more than the expression in the healthy control group (**a**). Magnification was $\times 200$. (**e**) Percentages of FIZZ1-positive bronchial epithelia in a time-dependent manner. Values are expressed as the mean $\pm$ SE. * $p < 0.001$, groups of asthma vs. healthy control group



challenge, and was reduced further on 28 days; however, the levels of FIZZ1 were still higher than those of the controls ($p < 0.001$).

Increased Vascular Area in Models of Asthma

The percentages of vWF-stained vascular area (percentage of vascularity) were increased significantly compared to those of the controls (Fig. 3), and reached the peak values after 7 days of OVA challenge ($p < 0.001$). Endothelial sprouts arose from venules, and some grew entirely within the epithelia. Percentages of vascularity declined on 14 days of OVA challenge. Few vessels were detected after

28-day challenge, and the percentages of vascularity showed no difference between OVA-challenged asthmatic mice and the healthy controls ($p > 0.05$).

VEGF Expression in a Time-dependent Manner

VEGF is a critical regulator of angiogenesis. In healthy control mice, VEGF was expressed at low levels in bronchial epithelia, alveolar epithelia, and vascular endothelia. Similar to FIZZ1, VEGF expression was significantly increased when compared with the control levels, and also reached the peak values by 7-day challenge ($p < 0.001$, Fig. 4). VEGF levels dramatically declined on

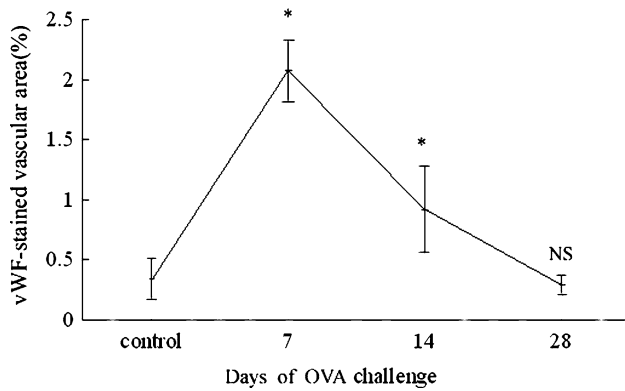


Fig. 3 Percentage of vWF-stained vascular area with respect to time. The percentages of vascularity were increased significantly to the peak levels in 7-day OVA-challenged mice, declined in 14-day-challenge mice, and were reduced to the control levels in 28-day-challenge mice. Values are expressed as the mean $\pm$ SE. * $p < 0.001$; NS, not statistically significant; groups of asthma vs. healthy control group

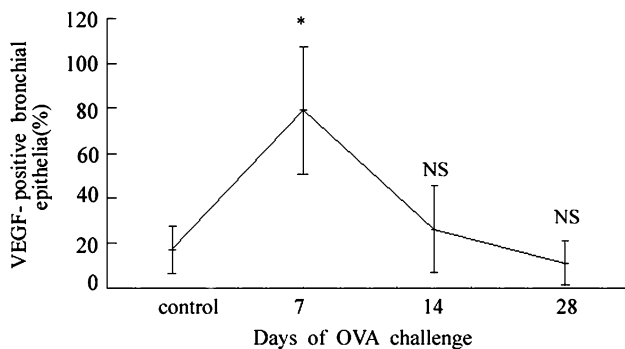


Fig. 4 Percentages of VEGF-positive bronchial epithelia reached the peak values in 7-day OVA-challenged mice, dramatically declined in 14-day-challenge mice (with no significant difference from control levels), and were reduced further in 28-day-challenge mice. Values are expressed as the mean $\pm$ SE. * $p < 0.001$; NS, not statistically significant; groups of asthma vs. healthy control group

the lung tissues from 14 days of OVA challenge, which had no significant difference from the control levels ($p > 0.05$). So did the VEGF expression of 28-day-challenge asthmatic mice.

Correlation of FIZZ1 with the Percentage of Vascularity and VEGF

There was positive correlation between FIZZ1 expression and the percentage of vascularity ($r = 0.805$, $p < 0.001$, Fig. 5), suggesting that FIZZ1 induces vessel growth as an angiogenic factor. FIZZ1 expression was also positively correlated with VEGF expression ($r = 0.604$, $p < 0.01$, Fig. 6), indicating that VEGF plays a role in the angiogenic effect of FIZZ1.

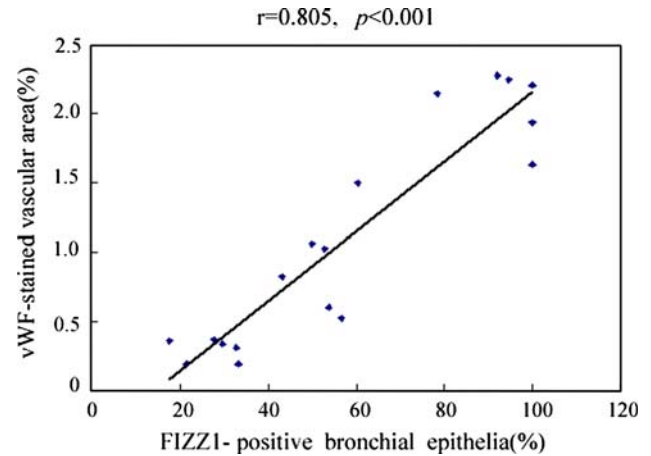


Fig. 5 Correlation between FIZZ1 expression and the percentage of vascularity. There was a positive correlation between the percentages of FIZZ1-positive bronchial epithelia and those of vWF-stained vascular area ($r = 0.805$, $p < 0.001$)

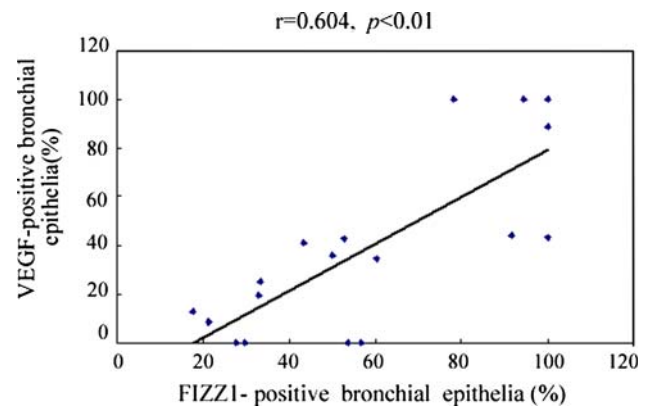


Fig. 6 Correlation between FIZZ1 and VEGF expression. There was positive correlation between the percentages of FIZZ1-positive bronchial epithelia and those of VEGF-positive bronchial epithelia ($r = 0.604$, $p < 0.01$)

Discussion

In this study we demonstrated that FIZZ1 expression induced angiogenesis in asthma. FIZZ1 levels peaked on 7 days, declined on 14 days, and was reduced further on 28 days of OVA challenge. The levels of FIZZ1 were positively correlated with the percentage of vascularity. Also, there was a positive correlation between FIZZ1 and VEGF expression.

Angiogenesis plays a key role in pathophysiologic progress of asthma. As known typical pathologic features of asthma, newly formed bronchial vessels are the portal for inflammatory cells to the airway wall and therefore contribute to chronic inflammation. Furthermore, angiogenesis is the important part of airway remodeling in asthma. The tiny mucosal vessels are inconspicuous in histologic sections of early studies, while immunohistochemical methods

with antibodies against vascular markers have made studies much easier.

In our experiment, FIZZ1 expression was significantly correlated with vWF-stained vascular area in models of asthma. This positive correlation between FIZZ1 and the percentage of vascularity has suggested that FIZZ1 is an angiogenic factor to upregulate vessel growth. Our findings are in accordance with the reports that mouse aortic rings cultured with recombinant FIZZ1 resulted in enhanced vascular sprouting. Studies have also shown that recombinant FIZZ1 stimulated the proliferation of pulmonary microvascular smooth muscle cell in rat in a dose-dependent manner [6]. Furthermore, FIZZ1 was able to promote cell proliferation and migration of pulmonary endothelial cells, which indicates that FIZZ1 contributes to pulmonary vessel growth [7].

FIZZ1 expression has been demonstrated to upgrade in hypertrophic, hyperplastic bronchial epithelium and in type II alveolar pneumocytes during allergic pulmonary inflammation [10]; however, the time-dependent changes of FIZZ1 expression are not quite clear. Our present findings show that FIZZ1 levels peaked by 7 days of OVA challenge, declined on 14 days, and were reduced further on 28 days of challenge in murine models of asthma. With the effects of angiogenic factors such as FIZZ1, new vessels form and improve hypoxia in lung tissues of asthmatic mice. At the same time, the expression of FIZZ1, as a hypoxia-induced mitogenic factor, is reduced when hypoxia has been improved by new vessel growth.

In our study a positive correlation exists between FIZZ1 and VEGF expression. It is important to mention that FIZZ1 increases VEGF expression via the PI-3K/Akt-NF-kappaB pathway [7, 11]. Also, FIZZ1-induced CD31-positive cell infiltration in Matrigel plugs *in vivo* was significantly suppressed by VEGF receptor-2 (VEGFR2) blockade, and intravenous injection of FIZZ1 also down-regulated the expression of VEGFR2 in the lung [12]. The positive correlation between FIZZ1 and VEGF expression, together with these reports, suggest that VEGF, as the downstream cytokine of FIZZ1, plays a crucial role in the angiogenic effect of FIZZ1 in asthma.

VEGF is an important growth factor in angiogenesis and microvascular remodeling [13–15]. It has been demonstrated that blood vessels respond to VEGF by sprouting angiogenesis in a few days, start to regress within 3 days when VEGF is switched off, but regress more slowly after VEGF withdrawal [16]. Similarly, we found that VEGF was overexpressed within 7 days of OVA challenge and declined to the baseline within 14 days of challenge; however, vessel growth declined slowly and returned to the control levels by 28 days after OVA challenge.

In summary, our study shows that FIZZ1 upregulates the percentage of vascularity in asthma in a time-dependent

manner, which reaches peak values by 7 days, regresses within 14 days, and is reduced further to the control levels after 28 days of OVA challenge. These results suggest that FIZZ1 induces angiogenesis in murine models of asthma. In addition, VEGF, as a downstream cytokine, plays a crucial role in FIZZ1-mediated vascularity.

Acknowledgments This work has received financial assistance from a Chinese National Research Grant.

References

- Asosingh K, Swaidani S, Aronica M, Erzurum SC (2007) Th1- and Th2-dependent endothelial progenitor cell recruitment and angiogenic switch in asthma. *J Immunol* 178:6482–6494
- Mohsenin A, Burdick MD, Molina JG, Keane MP, Blackburn MR (2007) Enhanced CXCL1 production and angiogenesis in adenine-mediated lung disease. *FASEB J* 21:1026–1036. doi: [10.1096/fj.06-7301.com](https://doi.org/10.1096/fj.06-7301.com)
- Renigunta A, Hild C, Rose F, Klepetko W, Grimminger F, Seeger W, Hanze J (2006) Human RELMbeta is a mitogenic factor in lung cells and induced in hypoxia. *FEBS Lett* 580:900–903. doi: [10.1016/j.febslet.2006.01.012](https://doi.org/10.1016/j.febslet.2006.01.012)
- Nair MG, Cochrane DW, Allen JE (2003) Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and Fizz1 that can be partly replicated *in vitro*. *Immunol Lett* 85:173–180. doi: [10.1016/S0165-2478\(02\)00225-0](https://doi.org/10.1016/S0165-2478(02)00225-0)
- Raes G, De Baetselier P, Noël W, Beschin A, Brombacher F, Hassanzadeh GG (2002) Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol* 71:597–602
- Teng X, Li D, Champion HC, Johns RA (2003) FIZZ1/RELM-alpha, a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties. *Circ Res* 92:1065–1067. doi: [10.1161/01.RES.0000073999.07698.33](https://doi.org/10.1161/01.RES.0000073999.07698.33)
- Tong Q, Zheng L, Li B, Wang D, Huang C, Matuschak GM, Li D (2006) Hypoxia-induced mitogenic factor enhances angiogenesis by promoting proliferation and migration of endothelial cells. *Exp Cell Res* 312:3559–3569. doi: [10.1016/j.yexcr.2006.07.024](https://doi.org/10.1016/j.yexcr.2006.07.024)
- Syer AK, Sullivan BT, Puder M, Arsenault D, Petrozza JC, Serikawa T, Chang S, Hasan T, Gonzalez RR, Rueda BR (2008) Ablation of leptin signaling disrupts the establishment, development and maintenance of endometriosis-like lesions in a murine model. *Endocrinology* 149:506–514. doi: [10.1210/en.2007-1225](https://doi.org/10.1210/en.2007-1225)
- Moody DM, Thore RC, Anstrom JA, Challa VR, Langefeld CD, Brown WR (2004) Quantification of afferent vessels shows reduced brain vascular density in subjects with leukoaraiosis. *Radiology* 233:883–890. doi: [10.1148/radiol.2333020981](https://doi.org/10.1148/radiol.2333020981)
- Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W, Nelson C, Lowman HB, Wright BD, Skelton NJ, Frantz GD, Tumas DB, Peale FV Jr, Shelton DL, Hébert CC (2000) FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J* 19:4046–4055. doi: [10.1093/emboj/19.15.4046](https://doi.org/10.1093/emboj/19.15.4046)
- Tang Q, Zheng L, Lin L, Li B, Wang D, Huang C, Li D (2006) VEGF is upregulated by hypoxia-induced mitogenic factor via the PI-3K/Akt-NF-kappaB signaling pathway. *Respir Res* 7:37. doi: [10.1093/emboj/19.15.4046](https://doi.org/10.1093/emboj/19.15.4046)
- Yamaji-Kegan K, Su Q, Angelini DJ, Champion HC, Johns RA (2006) Hypoxia-induced mitogenic factor has proangiogenic and proinflammatory effects in the lung via VEGF and VEGF

- receptor-2. *Am J Physiol Lung Cell Mol Physiol* 291:L1159-L1168. doi:[10.1152/ajplung.00168.2006](https://doi.org/10.1152/ajplung.00168.2006)
13. Dvorak HF, Detmar M, Claffey KP, Nagy JA, van de Water L, Senger D (1995) Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. *Int Arch Allergy Immunol* 107:233–235
 14. Hoshino M, Nakamura Y, Hamid QA (2001) Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 107:1034–1038. doi:[10.1067/mai.2001.115626](https://doi.org/10.1067/mai.2001.115626)
 15. McDonald DM (2001) Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med* 164:S39–S45
 16. Baluk P, Lee CG, Link H, Ator E, Haskell A, Elias JA, McDonald DM (2004) Regulated angiogenesis and vascular regression in mice overexpressing vascular endothelial growth factor in airways. *Am J Pathol* 165:1071–1085