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The Classical Pink-Eyed Dilution Mutation Affects Angiogenic Responsiveness

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Abstract

Angiogenesis is the process by which new blood vessels are formed from existing vessels. Mammalian populations, including humans and mice, harbor genetic variations that alter angiogenesis. Angiogenesis-regulating gene variants can result in increased susceptibility to multiple angiogenesis-dependent diseases in humans. Our efforts to dissect the complexity of the genetic diversity that regulates angiogenesis have used laboratory animals due to the availability of genome sequence for many species and the ability to perform high volume controlled breeding. Using the murine corneal micropocket assay, we have observed more than ten-fold difference in angiogenic responsiveness among various mouse strains. This degree of difference is observed with either bFGF or VEGF induced corneal neovascularization. Ongoing mapping studies have identified multiple loci that affect angiogenic responsiveness in several mouse models. In this study, we used F2 intercrosses between C57BL/6J and the 129 substrains 129P1/ReJ and 129P3/J, as well as the SJL/J strain, where we have identified new QTLs that affect angiogenic responsiveness. In the case of AngFq5, on chromosome 7, congenic animals were used to confirm the existence of this locus and subcongenic animals, combined with a haplotype-based mapping approach that identified the pink-eyed dilution mutation as a candidate polymorphism to explain AngFq5. The ability of mutations in the pink-eyed dilution gene to affect angiogenic response was demonstrated using the p-J allele at the same locus. Using this allele, we demonstrate that pink-eyed dilution mutations in Oca2 can affect both bFGF and VEGF-induced corneal angiogenesis.

Introduction

Angiogenesis is the process by which new vessels are generated from existing vasculature. In adult tissues it is rare; however it can be initiated by a number of normal and pathological processes including wounding, reproduction, cancer, macular degeneration, and cardiovascular disease. Angiogenesis is regulated by a variety of signaling molecules including VEGF, bFGF, and angiopoietins. There are a number of in vivo assays that can measure the angiogenic response to a particular stimulus. Among these is the corneal neovascularization assay. This assay takes advantage of the avascular field of the cornea as a platform in which to induce blood vessel growth. Early studies used tumor pieces to induce this growth, while later assays induced growth using slow-release pellets containing angiogenic growth factors.

Among the earliest described angiogenic proteins is bFGF [1]. bFGF induces angiogenesis largely by activating classical tyrosine kinase signaling pathways downstream of its cognate receptors FGFR1-4, with other pathways also activated [2]. Among the downstream mediators of bFGF-induced angiogenesis in the cornea is VEGF [3]. We have observed significant quantitative differences among common inbred mouse strains in their response to both bFGF [4,5] and VEGF [3,6]. Our initial efforts at mapping QTLs for these traits included several F2 intercrosses. Here we report the results of F2 mapping studies, including the identification of a new genetic modulator of angiogenic signaling.

Efforts involving recombinant-inbred strains that were initiated later, but completed earlier have been reported previously [3,4]. The pink-eyed dilution mutation in the Oca2 gene contained in the P locus is one of the oldest described in mice and, along with the albino mutation in tyrosinase, was involved in the earliest descriptions of linkage [7]. The mutation arose in the Asiatic mouse substrain Mus musculus molossinus and was introduced into common laboratory strains as a result of the 19th century mouse trade [8]. A wide variety of additional alleles are known at the P locus, all of which result in some level of hypopigmentation in mice. In humans, polymorphisms in an enhancer upstream of OCA2 play a major role in determining eye color. Individuals bearing the less active C/C genotype at rs12913832 exhibit lighter/blue eye color [9]. In addition, an R419Q polymorphism in the protein itself also results in lighter eye color [9].
Pigmentation is the result of the production of melanin in cells. The rate limiting step in this process is the oxidation of tyrosine to dopaquinone, which is catalyzed by tyrosinase (the product of the c locus) [10]. Dopaaquinone is then rearraigned and further oxidized by sequential actions of dopachrome tautomerase and tyrosinase-related protein 1 (the product of the b locus) [10]. The resulting molecules spontaneously polymerize into the black pigment eumelanin. Alternatively, in the absence of the latter two enzymes, the reaction of dopaquinone with cysteine or glutathione leads to the production of the sulfur-containing, reddish-brown pigment phaeomelanin [10]. The role of the Oca2 protein in this process is currently incompletely defined. It is homologous to several 12-transmembrane-domain transporter proteins [11]. In melanocytes lacking active p-protein, tyrosinase fails to mature and localize properly [12]. This results in protein cleavage and secretion of active tyrosinase and melanin. As a result, mice bearing the p-mutation show higher circulating levels of pigment precursors such as 5-S-cysteynyldopa (5-S-CD) and dihydroxyindolecarboxylic (DHICA) acid than fully pigmented animals [13].

Here we outline studies aimed at identifying the nature of inheritance of angiogenic responsiveness, identifying loci that control for that responsiveness, and identifying specific genetic changes within one of those loci that affect the angiogenic response.

Results

In all our crosses we used C57BL/6J as one of the parental strains. Our initial attempt at mapping differences in angiogenic responsiveness was done using 129P1/RjE mice. The angiogenic response of F2 animals was determined using the corneal neovascularization assay [14] using a dose of 10 ng bFGF. This dose was chosen because it would keep the angiogenic response of both parenta1 strains within the linear range of the assay (~0.4–2.2 mm²) [14] and thus was likely to keep most of the progeny within this range as well. We had hoped that the large phenotypic difference between this strain and C57BL/6J was the result of a few polymorphisms in major genes that would be easily identified, however this was not the case. In subsequent years, we have identified a large number of loci that affect angiogenesis [3,4,15,16] which may explain why no individual strong loci were identified in the C57BL/6J x 129P1/RjE cross. Nevertheless, by analyzing 77 F2 animals from this cross, we observed markers with uncorrected p values <0.05 for association between vessel area and genotype on chromosomes 1 (near D1Mit218), 7 (near D7Mit276 and D7Mit105), 10 (near D10Mit298), 11 (near D11Mit78), 14 (between D14Mit234 and D14Mit229), and X (See Figure 1/Table 1). To assess whether any of these loci exhibited genome-wide statistical significance, we turned to interval mapping. This demonstrated that regions on Chromosomes 1 and 7 exhibited LR scores greater than the traditional cut-off for suggestive linkage of 11.5 (equivalent to a LOD score of 2.5), exhibited LR scores greater than the traditional cut-off for significance. These demonstrated that regions on Chromosomes 1 and 7 genome-wide statistical significance, we turned to interval mapping assessed at 10 ng bFGF. Simple association tests for each of the chromosomes 1, 3, 5, 7, 11, and 17 (Table 3). Interval mapping on the full data set revealed a single region of genome-wide highly significant linkage on chromosome 7 that can explain approximately 10% of the variance in this cross (Figure 4). This peak was present when using several different measures of corneal angiogenesis, indicating that the mapping is not sensitive to the precise measure of vessel growth used (Figure S4). The use of more advanced mapping techniques, including composite interval mapping [17,18], and multiple interval mapping [19] failed to reveal additional areas of statistically significant linkage, though several regions of suggestive linkage were identified.

Based on the results from the first cross, animals congenic for the linked region on chromosome 5 and similar congenics for the chromosome 7 region were generated by repeated backcross of males heterozygous for these regions to C57BL/6J females. Because of decreasing support for linkage on chromosome 5 in the second and third F2 crosses, backcrossing for these congenics was terminated at seven generations. These animals showed no difference in angiogenic response when compared to C57BL/6J.
controls using bFGF-induced corneal neovascularization as the measure (Figure S5). We thus conclude that there are no genetic polymorphisms between SJL/J and C57BL/6J animals that alter bFGF-induced angiogenesis within this region of chromosome 5.

Several chromosome 7 congenics (Figure 5) were also generated as outlined in Materials and Methods. These strains exhibited a decrease in bFGF-induced angiogenesis when compared to C57BL/6J controls, with the B6 allele exhibiting partial dominance (Figure S6). In addition, intercrosses of 8th generation backcross animals which contained SJL/J congenic regions on both chromosome 7 and the Y chromosome were further suppressed, when compared to animals containing either region alone, indicating that the chromosome 7 region and the Y chromosome region exhibit additive effects. Together, these results indicate that there are genetic differences between SJL/J and C57BL/6J animals on chromosome 7 near the pink-eyed dilution locus that affect the angiogenic response. We have named the chromosome 7 QTL \textit{AngFq5}.

Classical mouse strains including the C57, SJL, and 129 strains are the result of inbreeding of a few small populations. As a result they share large genomic regions that are identical by descent. Using data from the mouse HapMap project, we determined that a genomic region surrounding the pink-eyed dilution locus is shared by several strains including SJL/J, 129P2/OlaHsD, and 129X1/SvJ. In this analysis, data from the closely related 129P2/OlaHsD strain was used in place of 129P3/J and 129P1/ReJ strains used in the initial crosses. This resulted in 4 regions of appropriate haplotype containing 1, 15, 7, and 2 annotated genes distributed across a total of 5.5 Mbp. Of these, only 2 exhibited SNPs predicted to alter amino-acid sequence (cSNPs). \textit{Herc2} (hect domain and RCC1-like domain 2) exhibited a K3566R polymorphism and \textit{Oca2} exhibited the L266V polymorphisms that results in the pink-eyed dilution phenotype (Figure 5).

To directly test whether a pink eyed dilution mutation can suppress angiogenesis, we used the p-J mutation. This mutation arose spontaneously at The Jackson Laboratory on a C3H background. As a result, C3H and C3H\textsuperscript{p-J} represent a pair of strains that differ only in a deletion at the pink-eyed dilution locus.

**Figure 1. C57BL/6J x 129P1/ReJ F2 cross simple interval mapping.** Whole genome simple interval map. Top: likelihood ratio statistic or likelihood of a region being linked to bFGF-induced corneal neovascularization. Dashed line indicates P = 0.05 as determined by bootstrapping. Bottom: predicted additive effect of a region on bFGF-induced corneal neovascularization.

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are albino in appearance regardless of their genotype at dilution mutation in mutation at tyrosinase interacts epistatically with pink-eyed AngFq5 epistatic interaction with the polymorphism responsible for not included in B6.SJL.M1P animals had either a compensating or (Figure 3D). This suggests that the portion of SJL chromosome 7 chromosome 7 did show a decrease in angiogenic responsiveness while B6.SJL.M1P animals, which contain a smaller region of SJL decrease in angiogenic response relative to C57BL/6J controls, Oca2 (which include animals heterozygous at one or both of the segregate in that cross. Wild-type pink-eyed dilution allele. Thus, the trait doesn’t VEGF similarly. Importantly both parents in that cross bear the angiogenesis-controlling QTLs in that cross affect bFGF and between bFGF and VEGF-responsiveness [3], indicating that most results from BXD strains in our lab showed a strong correlation in contrast to bFGF, VEGF-induced angiogenesis is potentiated by this mutation, suggesting differences in the mechanism by which these two angiogenesis signaling pathways are affected by the pink-eyed dilution mutation. This is noteworthy because previous results from BXD strains in our lab showed a strong correlation between bFGF and VEGF-responsiveness [3], indicating that most angiogenesis-controlling QTLs in that cross affect bFGF and VEGF similarly. Importantly both parents in that cross bear the wild-type pink-eyed dilution allele. Thus, the trait doesn’t segregate in that cross.

It is also noteworthy that in contrast to intercross F2 animals (which include animals heterozygous at one or both of the Tyr and Oca2, loci), fully homozygous B6.SJL.M1A animals showed no decrease in angiogenic response relative to C57BL/6J controls, while B6.SJL.M1P animals, which contain a smaller region of SJL chromosome 7 did show a decrease in angiogenic responsiveness (Figure 3D). This suggests that the portion of SJL chromosome 7 not included in B6.SJL.M1P animals had either a compensating or epistatic interaction with the polymorphism responsible for AngFq5. Importantly, in the coat color phenotype the albino mutation at tyrosinase interacts epistatically with pink-eyed dilution mutation in Oca2. Animals bearing the albino mutation are albino in appearance regardless of their genotype at Oca2. We have observed that, animals bearing an albino mutation show no difference in corneal angiogenic response to bFGF [5]. Thus, the absence of a difference in angiogenic response in B6.SJL.M1A animals indicates that similar epistatic interactions occur in the case of angiogenic responsiveness. It further suggests that soluble biochemical products of pigment production play a role in the differences in angiogenic response observed.

**Discussion**

Both previously published data, and the data presented here clearly demonstrate that angiogenic responsiveness is controlled by a large number of different polymorphic genes. It may be instructive that Oca2 is not part of a classical angiogenesis-regulatory pathway. This may indicate that a wide variety of angiogenesis-regulatory pathways remain to be discovered. In addition, it may be the consequence of the importance of angiogenesis in reproduction and development. As a result, polymorphisms in pathways that are central to angiogenesis may be incompatible with long-term strain viability. Indeed, VEGF gene knockout mice exhibit haploinsufficiency [21] and low expressing polymorphisms in the VEGF gene are associated with reproductive difficulties (reviewed in [16]).

Repeated identification of similar regions in independent crosses is a useful means of establishing the robustness of linkage between genomic regions and phenotypic traits. When the crosses are between different strains, it has the added advantage of identifying

| Table 1. Markers showing near-significant and significant association with bFGF-induced corneal neovascularization in a 77-animal C57BL/6J × 129P1/ReJ F2 cross. |
|----------------|--------|-----|--------|-----|--------|--------|--------|
| **Marker**     | **Chrom.** | **cM** | **b0** | **b1** | **F**  | **%**  | **P**  |
| D1Mit218       | 1      | 67   | 0.708  | 0.19  | 8.979  | 11%    | 0.004  **|
| D2Mit396       | 2      | 69   | 0.712  | 0.119 | 2.805  | 4%     | 0.098  |
| D7Mit76        | 7      | 3.4  | 0.713  | –0.173| 6.007  | 8%     | 0.011  * |
| D7Mit246       | 7      | 15   | 0.707  | –0.193| 10.01  | 12%    | 0.002  **|
| D7Mit229       | 7      | 23   | 0.706  | –0.189| 10.05  | 12%    | 0.002  **|
| D7Mit201       | 7      | 37   | 0.702  | –0.184| 8.595  | 10%    | 0.004  **|
| D7Mit253       | 7      | 53   | 0.709  | –0.181| 6.933  | 8%     | 0.01   * |
| D7Mit105       | 7      | 64   | 0.715  | –0.162| 5.062  | 6%     | 0.027  * |
| D10Mit298      | 10     | 3    | 0.715  | 0.151 | 5.028  | 6%     | 0.028  * |
| D11Mit78       | 11     | 2    | 0.727  | 0.128 | 4.167  | 5%     | 0.045  * |
| D12Mit150      | 12     | 59   | 0.707  | 0.123 | 3.775  | 5%     | 0.056  |
| D14Mit152      | 14     | 15   | 0.708  | 0.129 | 3.006  | 4%     | 0.087  |
| D14Mit234      | 14     | 23   | 0.692  | 0.203 | 7.634  | 9%     | 0.007  **|
| D14Mit30       | 14     | 29   | 0.699  | 0.191 | 7.504  | 9%     | 0.008  **|
| D14Mit39       | 14     | 30   | 0.699  | 0.191 | 7.504  | 9%     | 0.008  **|
| D14Mit228      | 14     | 46   | 0.697  | 0.151 | 5.072  | 6%     | 0.027  * |
| D16Mit139      | 16     | 43   | 0.706  | –0.14 | 3.512  | 4%     | 0.065  |
| D17Mit155      | 17     | 56   | 0.719  | 0.118 | 3.193  | 4%     | 0.078  |
| D19Mit128      | 19     | 11   | 0.715  | 0.144 | 3.88   | 5%     | 0.053  |
| DXMit166       | 20     | 16   | 0.708  | –0.11 | 3.984  | 5%     | 0.05   * |
| DXMit186       | 20     | 69   | 0.702  | –0.139| 6.54   | 8%     | 0.013  * |

* = P<0.05; ** = P<0.01; *** = P<0.001. doi:10.1371/journal.pone.0035237.t001
Figure 2. CS7BL/6J × 129P3/J F2 cross simple interval mapping. Whole genome simple interval map. Top: likelihood ratio statistic or likelihood of a region being linked to bFGF-induced corneal neovascularization. Dashed line indicates $P = 0.05$ as determined by bootstrapping. Bottom: predicted additive effect of a region on bFGF-induced corneal neovascularization.

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Table 2. Markers showing near-significant and significant association with bFGF-induced corneal neovascularization in a CS7BL/6J × 129P3/J F2 cross.

| Marker   | Chrom. | cM  | b0       | b1       | F     | %      | P     |
|----------|--------|-----|----------|----------|-------|--------|-------|
| D7Mit76  | 7      | 3.4 | 0.76     | -0.163   | 4.943 | 7%     | 0.03  |
| D7Mit246 | 7      | 15  | 0.757    | -0.142   | 3.636 | 5%     | 0.061 |
| D7Mit318 | 7      | 37  | 0.755    | -0.168   | 4.491 | 7%     | 0.038 |
| D7Mit62  | 7      | 43  | 0.751    | -0.213   | 7.008 | 10%    | 0.01  |
| D7Mit126 | 7      | 50  | 0.76     | -0.189   | 5.470 | 8%     | 0.023 |
| D7Mit105 | 7      | 64  | 0.76     | -0.168   | 4.482 | 7%     | 0.038 |
| D12Mit105| 12     | 6   | 0.783    | 0.258    | 11.97 | 16%    | 0.001 |
| D12Mit136| 12     | 13  | 0.788    | 0.268    | 12.33 | 16%    | 0.001 |
| D12Mit46 | 12     | 16  | 0.794    | 0.254    | 11.88 | 16%    | 0.001 |
| D12Mit34 | 12     | 29  | 0.793    | 0.143    | 3.309 | 5%     | 0.074 |
| D14Mit14 | 14     | 10  | 0.784    | -0.144   | 3.290 | 5%     | 0.074 |
| D14Mit182| 14     | 14  | 0.783    | -0.154   | 3.653 | 5%     | 0.061 |
| D14Mit228| 14     | 46  | 0.766    | -0.165   | 5.315 | 8%     | 0.024 |
| D14Mit265| 14     | 48  | 0.763    | -0.184   | 6.486 | 9%     | 0.013 |
| D14Mit198| 14     | 54  | 0.766    | -0.177   | 5.265 | 8%     | 0.025 |
| D14Mit75 | 14     | 54  | 0.758    | -0.142   | 3.482 | 5%     | 0.067 |

Marker = marker tested; Chr. = chromosome the marker is on; % = fraction of the experimental variance attributable to genotype at the marker indicated under an additive model; $P =$ likelihood that there is no relationship between the marker genotype and choroidal neovascularization area (by F test), when the data are fit to the simple linear regression model $y = b_0 + b_1 x + e$. The results give the estimates for $b_0$, $b_1$ and the $F$ statistic for each marker. $b_0$ is approximately the average area of CS7BL/6J-allele-containing strains. $b_1$ is an indication of the effect of substitution of the 129P3/J allele at that marker. Asterisks indicate uncorrected $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***)

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alleles that are shared between the differing strains at a particular locus. This information can be used to identify appropriate haplotypes bearing the alleles in question. We observed overlap between linked regions in these crosses and two previously identified QTLs. In the C57BL/6J × 129P3/J cross, we see linkage indicating that 129P3/J bears the DBA/2J allele at AngFq4 [3]. Similarly we show evidence that 129P1/ReJ bears the DBA/2J allele at AngVq2 [4]. As outlined in the results section, we also see overlap in linked regions in several of the crosses described here. 129P1/ReJ and 129P3/J share alleles at a locus on chromosome 14 that we name AngFq7. Similarly, an angiogenesis-regulating polymorphism on the Y chromosome in several 129 strains results in AngFq8. As outlined above, haplotype analysis allowed the identification of the SJL allele of the Oca2 gene as a strong candidate for AngFq5.

The mechanism by which the Oca2 gene might affect angiogenesis is currently unclear, especially since the cornea does not contain melanocytes. It implies an effect that extends beyond the local microenvironment such that iris melanocytes or skin melanocytes release factors which affect the corneal limbal vessels.
For example, circulating melanin precursors might mediate the
effect on corneal angiogenesis. Alternatively, because improperly
trafficked tyrosinase is secreted by melanocytes bearing the Oca2
allele [12] and tyrosinase readily generates reactive oxygen [22],
increased circulating tyrosinase may lead to increases in reactive
oxygen species. This reactive oxygen can, in turn, stimulate
endothelial cell production of VEGF as well as other endothelial
cell activity associated with angiogenesis [23,24]. Other endothelial
cell signaling molecules affected by ROS include phosphatases,
transcription factors and bioactive lipids [25,26].

In addition, circulating tyrosinase has the capacity to alter
circulating levels of catecholamines. Spontaneous disproportiona-
tion of dopaquinone yields DOPA as one of its products. In
addition, met-tyrosinase consumes DOPA or dopamine as it is
returned to an active redox state. Finally, in addition to tyrosine,
tyrosinase can use a variety of phenols and catechols as substrates.
Since different catecholamines can produce both pro- and antiangiogenic effects [27,28,29], circulating tyrosinase has the
potential to significantly alter the angiogenic response. Similar
differences in these known angiogenesis modulators may explain
the differential response to bFGF, due to differential effects on the
timing and nature of signaling. Alternatively these differences
might be explained by regulation of novel angiogenesis signaling
molecules by the pink-eyed dilution protein.

In humans polymorphisms in the human homolog of the Oca2
gene are associated with genetic susceptibility to melanoma [9].
Importantly, this effect may not be entirely dependent on
differences in pigmentation. OCA2 polymorphisms are associated
with differences in eye color, however, OCA2-linked differences in
melanoma susceptibility persist after controlling for eye color [30].
This indicates that differences in pigmentation and therefore UV
susceptibility may not be sufficient to explain the difference in
melanoma susceptibility. It has been suggested that subtle
differences in the quality of melanin produced, or differences in
glutathione metabolism observed in Oca2-mutant melanocytes
may account for differing disease susceptibility [30]. To this, we
add the possibility that increased VEGF angiogenic responsiveness
may affect genetic susceptibility to melanoma. An increased
angiogenic response in individuals with appropriate OCA2
genotype may allow earlier conversion of melanoma to three-
dimensional growth and thus frank disease. Extrapolating this idea
to angiogenic responsiveness in general suggests that an increased
identification and understanding of angiogenesis response alleles
may allow improved identification of individuals susceptible to
angiogenesis dependent diseases such as cancer.

Materials and Methods

Ethics Statement

All animal studies were conducted according to protocols A00-
06-046R, A03-10-075R, A06-09-078R, and A09-09-1511R
approved by the Institutional Animal Care and Use Committee of
Children’s Hospital.

Figure 4. C57BL/6J × SJL/J F2 cross simple interval mapping. Whole genome simple interval map. Top: likelihood ratio statistic or likelihood of
a region being linked to bFGF-induced corneal neovascularization. Dashed line indicates P = 0.05 as determined by bootstrapping. Bottom: predicted
additive effect of a region on bFGF-induced corneal neovascularization.
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F2 Crosses

A total of 65 F2 animals were generated by intercrossing F1 progeny of 129P1/ReJ and C57BL/6J animals obtained from Jackson Labs. The resulting animals were genotyped using 89 publicly available SSLP markers. These animals were then phenotyped using the corneal neovascularization assay [14]. For the 129P3/J F2 cross, we used B6129PF1/J animals commercially available from Jackson Labs as parents. A total of 77 F2 animals were generated, genotyped at 98 SSLP markers, and phenotyped as above. In both 129 crosses, animals with a high inter-eye variability (coefficient of variation >0.45) were excluded from further analysis.

C57BL/6J x SJL/J F2 animals were generated from grandparents obtained from Jackson labs. These animals were genotyped at 112 publicly available SSLP markers and phenotyped as above.

Chromosome 5 congenics were generated from C57BL/6J x SJL/J F1 animals by selecting males in each generation that were heterozygous at D5Mit158, D5Mit188, D5Mit213, D5Mit168, and D5Mit43. These animals were backcrossed to C57BL/6J females for seven generations. After the seventh backcross generation, the animals were intercrossed to generate homozygous congenics.

Table 3. Markers showing near-significant and significant association with bFGF-induced corneal neovascularization in a C57BL/6J x SJL/J F2 cross.

| Marker | Chrom. | cM | b0 | b1 | F   | %   | P     |
|--------|--------|----|----|----|-----|-----|-------|
| D10Mit44 | 1      | 9  | 1.404 | 0.066 | 4.314 | 1%  | 0.039 * |
| D1Mit3 | 1      | 11 | 1.403 | 0.053 | 2.955 | 1%  | 0.087 |
| D1Mit306 | 1     | 59 | 1.401 | -0.051 | 2.851 | 1%  | 0.092 |
| D1Mit94 | 1      | 64 | 1.4  | -0.069 | 5.176 | 2%  | 0.024 * |
| D1Mit36 | 1      | 92 | 1.395 | -0.107 | 13.23 | 4%  | 0.000 *** |
| D3Mit51 | 3      | 35 | 1.41  | 0.065 | 4.147 | 1%  | 0.043 * |
| D3Mit106 | 3     | 55 | 1.405 | 0.052 | 2.862 | 1%  | 0.092 |
| D5Mit205 | 5      | 45 | 1.409 | 0.077 | 6.144 | 2%  | 0.014 * |
| D5Mit338 | 5     | 59 | 1.407 | 0.061 | 4.236 | 1%  | 0.040 * |
| D5Mit158 | 5      | 62 | 1.407 | 0.073 | 5.336 | 2%  | 0.022 * |
| D5Mit188 | 5     | 64 | 1.407 | 0.077 | 5.645 | 2%  | 0.018 * |
| D5Mit168 | 5      | 78 | 1.403 | 0.061 | 4.261 | 1%  | 0.040 * |
| D5Mit119 | 5     | 80 | 1.403 | 0.06  | 3.776 | 1%  | 0.053 |
| D6Mit59 | 6      | 67 | 1.401 | -0.057 | 3.045 | 1%  | 0.082 |
| D6Mit294 | 6     | 73 | 1.401 | -0.051 | 2.845 | 1%  | 0.093 |
| D7Mit76 | 7      | 3.4 | 1.402 | -0.061 | 3.919 | 1%  | 0.049 * |
| D7Mit246 | 7     | 15 | 1.401 | -0.111 | 10.99 | 3%  | 0.001 ** |
| D7Mit270 | 7     | 18 | 1.4  | -0.123 | 14.77 | 4%  | <10^{-3} *** |
| D7Mit229 | 7     | 23 | 1.4  | -0.121 | 16.77 | 5%  | <10^{-4} **** |
| D7Mit145 | 7     | 26 | 1.4  | -0.132 | 20.46 | 6%  | <10^{-3} **** |
| Oca2 | 7     | 28 | 1.4  | -0.135 | 20.98 | 6%  | <10^{-3} **** |
| D7Mit318 | 7     | 37 | 1.404 | -0.141 | 24.18 | 7%  | <10^{-5} **** |
| Tyr | 7     | 44 | 1.407 | -0.163 | 31.23 | 9%  | <10^{-7} **** |
| D7Mit126 | 7     | 50 | 1.405 | -0.155 | 25.3  | 7%  | <10^{-6} **** |
| D7Mit105 | 7     | 64 | 1.401 | -0.11 | 13.03 | 4%  | <10^{-3} *** |
| D7Mit12 | 7     | 66 | 1.4  | -0.089 | 8.23  | 2%  | 0.004 ** |
| D10Mit180 | 10    | 64 | 1.405 | 0.061 | 3.822 | 1%  | 0.051 |
| D11Mit78 | 11    | 2  | 1.402 | -0.073 | 5.454 | 2%  | 0.020 * |
| D17Mit66 | 17    | 25 | 1.402 | -0.071 | 4.847 | 1%  | 0.028 * |
| D17Mit88 | 17    | 30 | 1.402 | -0.09 | 8.212 | 2%  | 0.004 ** |
| D17Mit93 | 17    | 45 | 1.401 | -0.066 | 4.624 | 1%  | 0.032 * |
| DXMit166 | 20    | 16 | 1.412 | 0.055 | 3.676 | 1%  | 0.056 |

Marker = marker tested; Chr. = chromosome the marker is on; % = fraction of the experimental variance attributable to genotype at the marker indicated under an additive model; P = likelihood that there is no relationship between the marker genotype and choroidal neovascularization area (by F test), when the data are fit to the simple linear regression model $y = b0 + b1x + e$. The results give the estimates for $b0$, $b1$ and the F statistic for each marker. $b0$ is approximately the average area of C57BL/6J-allele-containing strains. $b1$ is an indication of the effect of substitution of the 129P3/J allele at that marker. Asterisks indicate uncorrected $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****).

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Generation of Congenic Animals

B6.SJL chromosome 5 congenics were generated from C57BL/6J x SJL/J F1 animals by selecting males in each generation that were heterozygous at D5Mit158, D5Mit188, D5Mit213, D5Mit168, and D5Mit43. These animals were backcrossed to C57BL/6J females for seven generations. After the seventh backcross generation, the animals were intercrossed to generate homozygous congenics.

Chromosome 7 congenics were generated in a similar manner except that animals heterozygous for D7Mit229, D7Mit211,
D7Mit233, D7Mit62, and D7Mit301, but homozygous for the B6 allele at D7Mit332 were selected, and backcrossing was continued for a full ten generations before intercrossing. In addition, at the 8th and 9th backcross generations, three additional lines were generated. B6.SJLChrY animals were generated by selecting an N8 animal that was homozygous for the B6 allele at all of the chromosome 7 markers for further backcrossing. B6.SJL.7M1A animals were generated from a B6.SJL.7M1A heterozygous animal that experienced a recombination between D7Mit233 and D7Mit62, resulting in animals that retained the SJL/J pink-eyed dilution allele, but had the C57BL/6J tyrosinase allele, resulting in animals that were pink-eyed dilute in coat color.

Mapping

Windows QTL Cartographer version 2.5 [31] was used throughout for linkage analysis. Default settings were used throughout. Marker genotypes were obtained using PCR and agarose gel electrophoresis. Primers for SSLP markers and associated linkage map positions were obtained from MGI version 4.21. Simple association, interval mapping [32], and composite

Figure 5. Mapping the region responsible for AngFq5. Top: Location of the congenic regions for the congenic strains used is plotted, along with boundary-defining markers for SJL congenics (brown). Blue indicates 129 congenics, with dark blue indicating those congenics that demonstrate a statistically significant difference in angiogenic response. Genes are indicated next to which DNA strand they are coded by. Finally regions of shared haplotype are indicated colored by whether the parental strains have disparate (black) or shared (grey) alleles at AngFq5. Below: Magnification of the region of interest defined by the R82 and R06 congenics. The number of genes, and genes bearing amino-acid altering polymorphisms (cSNPs), is indicated as are the locations of the pink-eyed dilution gene (p) and the tyrosinase gene (c). Scale is in Mb on build 37.1 of mouse Chromosome 7. doi:10.1371/journal.pone.0035237.g005

Pink-Eyed Dilution Mutations Alter Angiogenesis
interval mapping [17,18] were all performed. Permutation analysis using 10,000 iterations was performed for each of the three interval mapping experiments, resulting in threshold likelihood ratios (LR) for P<0.05 of 16.7, 17.3, and 15.6 for the C57BL/6J cross with 129P2/J, 129P1/ReJ, and SJL/J respectively. In the case of the SJL cross, P<0.001 corresponded to a LR of 24.8.

Haplotype Mapping

For haplotype mapping genotypes for C57BL/6J, C57BL/10J, 129P2/OlaHsd, 129X1/SvJ, and SJL/J strains were obtained from the Broad institute hapmap website on 22 July 2009 (http://www.broadinstitute.org/mouse/hapmap/). This file contains SNP position information relative to NCBI build 37 of the mouse genome, as well as genotype information for 94 different strains. Regions of shared haplotype were determined by identifying regions where flanking polymorphic SNPs were on average >1 MB from a given SNP [15]. Uncalled SNPs were not included in this analysis.

Corneal Micropocket Assay

The corneal micropocket assay was performed as described [14,33] using pellets containing the indicated quantity of FGF2 or 160 ng carrier free recombinant VEGF 165 (R&D Systems, Minneapolis, Minnesota). The area of vascular response was assessed on the fifth (FGF2) or sixth (VEGF) postoperative day using a slit lamp. Vessel area was calculated using the formula

\[ \text{vessel area} = \frac{\text{vessel length}}{\text{average width}} \]

where VL is vessel length from the limbus in mm and CH is clock hours around the cornea. Control C57BL/6J (typically 5 mice, 10 eyes) were included in each assay to confirm consistency. All mouse strains were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed in Children’s Hospital’s animal facility on standard diet and bedding until the assay was performed.

Supporting Information

**Figure S1** Composite interval mapping of the C57BL/6J×129P1/ReJ F2 cross. Whole genome composite interval map. Top: likelihood ratio statistic or likelihood of a region being linked to bFGF-induced corneal neovascularization. Bottom: predicted additive effect of a region on bFGF-induced corneal neovascularization. The indicated likelihood ratio of 11.5 is equivalent to a LOD score of 2.3.

**Figure S2** Effect of parental gender on vessel area in a 129×B6 F1 cross. Pellets contain 10 ng bFGF. The increase in vessel area in the 129B6F1 males is statistically significant (p<0.05 by ANOVA).

**Figure S3** Simple interval mapping of the initial C57BL/6J×SJL/J F2 cross.

**Figure S4** Simple interval mapping of different measures of angiogenic response in the full C57BL/6J×SJL/J F2 cross. The use of different measures of angiogenic response as the trait to be mapped was explored. AVA is the (unadjusted) average of vessel areas of the left and right eyes. AVL is the average of the vessel length of the left and right eyes. NORMAVA is the average vessel area divided by (normalized) the vessel area measured in C57BL/6J controls assayed at the same time.

**Figure S5** Effect of SJL alleles on vessel area in B6.SJL Chromosome 5 N6F1 animals. Note the truncated vertical axis. None of these differences are statistically significant (ANOVA).

**Figure S6** Effect of SJL alleles on vessel area in B6.SJL Chromosome 7 N8F1 animals. Note the truncated vertical axis.

**Table S1** Markers showing near-significant and significant association with bFGF-induced corneal neovascularization in the initial C57BL/6J×SJL/J F2 cross.

**Author Contributions**

Conceived and designed the experiments: MSR VB WFD RJD RMR. Analyzed the data: MSR VB AEB RJD. Wrote the paper: MSR RJD.

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