Published in final edited form as:

Mamm Genome. 2013 October ; 24(0): 349–357. doi:10.1007/s00335-013-9468-9.

Genetic modification of corneal neovascularization in *Dstn*^{corn1} mice

Sharolyn V. Kawakami-Schulz, Shannon G. Sattler, Anna-Lisa Doebley, Akihiro Ikeda, and Sakae Ikeda*

Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA

Abstract

Mutations in the gene for destrin (*Dstn*), an actin depolymerizing factor, lead to corneal abnormalities in mice. A null mutation in *Dstn*, termed *Dstn*^{corn1}, isolated and maintained in the A.BY background (A.BY *Dstn*^{corn1}), results in corneal epithelial hyperproliferation, inflammation and neovascularization. We previously reported that neovascularization in the cornea of *Dstn*^{corn1} on the C57BL/6 background (B6.A.BY-*Dstn*^{corn1}) mice is significantly reduced when compared to A.BY *Dstn*^{corn1} mice, suggesting the existence of genetic modifier(s). The purpose of this study is to identify the genetic basis of difference in cornea neovascularization between A.BY *Dstn*^{corn1} and B6.A.BY-*Dstn*^{corn1} mice. We generated N₂ mice for a whole genome scan by backcrossing F₁ progeny (A.BY *Dstn*^{corn1} X B6.A.BY-*Dstn*^{corn1}) to B6.A.BY-*Dstn*^{corn1} mice. N₂ progeny were quantitatively phenotyped for the extent of corneal neovascularization and genotyped for markers across the mouse genome. We identified significant association of variability in corneal neovascularization with a locus on chromosome 3 (Chr3). The validity of identified quantitative trait locus (QTL) was tested using B6 consomic mice carrying Chr3 from A/J mice. *Dstn*^{corn1} mice from F₁ and F₂ intercrosses (B6.A.BY-*Dstn*^{corn1} x C57BL/6J-Chr 3^{A/J}/NaJ) were phenotyped for the extent of corneal neovascularization. This analysis showed that mice carrying the A/J allele at the QTL show significantly increased neovascularization. Our results indicate the existence of a modifier that genetically interacts with the *Dstn* gene. This modifier demonstrates allelic differences between C57BL6 and A.BY or A/J. The modifier is sufficient to increase neovascularization in *Dstn*^{corn1} mice.

INTRODUCTION

Blood vessels act as a source of sustenance for tissues, shuttling nutrients and oxygen that travel throughout an organism to help tissues grow and function. Vasculature forms in response to a communicated need, often due to stress, for these molecules. A multitude of proteins, such as vascular endothelial growth factor (VEGF), VEGF receptors, fibroblast growth factors (FGFs), members of the NOTCH and WNT signaling pathways, and matrix metalloproteinases (MMPs) remodel tissues and make way for a vascular network to invade a previously avascular area (Jakobsson et al. 2010; Kushner and Bautch 2013; Qazi et al. 2009; Ribatti and Crivellato 2012). The normally avascular mouse cornea is an ideal tissue to use in the study of angiogenesis and has traditionally been used in the form of the cornea micro-pocket angiogenesis assay (Nakao et al. 2010; Pakneshan et al. 2008; Rogers et al. 2007). Many studies using this assay have identified factors that play a role in factor-induced neovascularization (Rogers and Amato 2011; Rogers et al. 2004; Rogers et al. 2003). While the assay has been proven useful, it falls short of identifying factors that contribute to the spontaneous development of corneal neovascularization *in vivo*. This study uses a mouse mutant that demonstrates spontaneous neovascularization in the cornea to

*corresponding author: Phone: 608-262-5991, Fax: 608-262-2976, ikeda@wisc.edu.

identify genes that contribute to the development of a vascular network. In these mice, neovascularization occurs due to deletion of the gene that encodes for the protein destrin (DSTN) (Ikeda et al. 2003; Smith et al. 1996).

In its native state and environment, DSTN facilitates turnover of filamentous actin to globular actin units. Deletion of the gene for this actin depolymerizing factor (ADF), which is located on chromosome 2, leads to corneal abnormalities in mice. Mice homozygous for this deletion, called *Dstr^{corn1}*, display spontaneous neovascularization, excess epithelial proliferation and increased inflammation in the cornea (Ikeda et al. 2003; Verdoni et al. 2008a; Verdoni et al. 2008b). In *Dstr^{corn1}* mice, vasculature invades the corneal stroma by postnatal day 14 (P14) and continues to spread throughout the cornea, often covering up to 40% of the corneal surface by P58 (Kawakami-Schulz et al. 2012; Smith et al. 1996). The *Dstr^{corn1}* cornea also displays significant epithelial hyperproliferation and inflammation, processes that both aid and are assisted by the formation of vasculature throughout the tissue (Ikeda et al. 2003; Smith et al. 1996; Verdoni et al. 2008b). Microarray analysis of gene expression differences between *Dstr^{corn1}* and wild type (WT) mice has revealed a network of changes that occur in response to lack of DSTN (Verdoni et al. 2008a). We have identified serum response factor (SRF) as a major regulator for phenotypes of *Dstr^{corn1}* cornea (Verdoni et al. 2010). Conditional ablation of *Srf* from the corneal epithelium leads to rescue of the *Dstr^{corn1}* phenotypes. In the *Dstr^{corn1}* rescued mice, neovascularization is significantly regressed and reduced (Verdoni et al. 2010). Previous work has also identified down-regulation of the soluble form of vascular endothelial growth factor (VEGF) receptor 1 (also known as FMS-like tyrosine kinase 1, sFLT1) as a contributor to the formation of vasculature in *Dstr^{corn1}* mice (Qazi et al. 2010). Conditional *Srf* knockout *Dstr^{corn1}* mice display increased levels of sFLT1, suggesting that sFLT1 is a downstream of SRF (Verdoni et al. 2010). Thus far, we have established that *Dstn*, together with *Srf* and *Flt-1*, are important in WT corneal avascularity and that their mis-expression contributes to abnormalities in *Dstr^{corn1}* cornea. However, there are other factors in the molecular network leading from the *Dstr^{corn1}* mutation to neovascularization that have not yet been identified. Identification of other genes that lead to *Dstr^{corn1}* abnormalities will provide insight into the complex network of molecules that contribute to neovascularization.

Our previous work on *Dstr^{corn1}* mice has shown that the phenotypes representative of this mutation, including neovascularization in the cornea, are subject to genetic modification (Kawakami-Schulz et al. 2012). The phenotypes are more severe in *Dstr^{corn1}* mice on the A.BY background compared to those on the B6 background. In this study, we demonstrate that a quantitative trait locus (QTL) is responsible for genetic modification of corneal neovascularization in *Dstr^{corn1}* mice.

METHODS

Mouse husbandry

A.BY-*H2^b* H2-*T18^b*/SnJ-*Dstr^{corn1}*/J (A.BY *Dstr^{corn1}*), C57BL/6J WT (B6 WT), and C57BL/6J-Chr 3^{A/J}/NaJ (B6 Chr3^{A/J}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in an animal facility at the University of Wisconsin-Madison. All mouse procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee at the University of Wisconsin-Madison and conform to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. All mice were sampled and analyzed at P58.

Generations of congenic mouse lines

B6.A.BY- *Dstn^{corn1}* mice were generated by crossing A.BY *Dstn^{corn1}* mice to B6 WT mice, selecting for mice carrying a *Dstn^{corn1}* allele, and backcrossing to B6 WT for 8 generations (Kawakami-Schulz et al. 2012). Heterozygous mice were intercrossed and progeny genotyped as homozygous for the *Dstn^{corn1}* mutation were used for analysis.

Generation of F₁ and N₂ mice

A.BY *Dstn^{corn1}* mice were crossed to B6.A.BY- *Dstn^{corn1}* mice to obtain F₁ mice, hereafter referred to as F₁ *Dstn^{corn1}* (Kawakami-Schulz et al. 2012). F₁ *Dstn^{corn1}* mice were backcrossed to the B6.A.BY- *Dstn^{corn1}* line to obtain N₂ mice, hereafter referred to as B6 N₂ *Dstn^{corn1}*.

Generation of mice with recombinant Chromosome 3 (F₂ and F₃)

B6.A.BY- *Dstn^{corn1}* mice were crossed to B6 Chr3^{A/J} mice to obtain F₁ mice that were heterozygous for both *Dstn^{corn1}* and for Chr3 of the B6 and A/J lines. These F₁ mice were intercrossed, resulting in F₂ progeny with a recombinant Chr3. Mice genotyped to be homozygous for *Dstn^{corn1}* were used for the analysis (hereafter referred to as F₂ *Dstn^{corn1}*). F₂ *Dstn^{corn1}* mice were mated to generate F₃ *Dstn^{corn1}* mice, which were also recombinant for Chr3 and used for the analysis.

Immunohistochemistry

Immunohistochemistry on whole cornea was performed as described previously (Verdoni et al. 2008a). The primary antibody for CD31 (BD Pharmingen) was used at a dilution of 1:50. Corneas were transferred to block solution containing phalloidin (1:50; Invitrogen) and an Alexa Fluor 488 conjugated secondary antibody (1:200; Invitrogen). Corneas were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:200; Sigma-Aldrich).

Imaging

Images acquired on sections and whole mount cornea were captured on an Eclipse E600 microscope (Nikon; Tokyo, Japan) using a SPOT camera (Spot Diagnostics; Sterling Heights, MI).

Quantification of the vascularized area of the cornea

Digital images of all corneal flat mounts were collected using the Spot Image Analysis system. Vascularized area and total corneal area were measured using ImageJ software with a method similar to that of Bock et al (Bock et al. 2008). Filters were applied to subtract background, reduce noise, and enhance contrast. The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the area of CD31-positive vessels within the cornea was then calculated and normalized to the total corneal area (expressed as the percentage of vascularized cornea) using thresholding analysis.

QTL and statistical analysis

We initially performed a scan across the whole genome using 66 N₂ *Dstn^{corn1}* mice. We selected 65 markers throughout the genome to distinguish between A.BY and B6 alleles. Vasculature in the cornea of N₂ *Dstn^{corn1}* mice was phenotyped prior to genotyping to prevent bias. The R/qtl package was used to perform statistical analyses (<http://www.rqtl.org/>) (Arends et al. 2010). We calculated LOD scores using the multiple-imputation method with 1-cM steps, 1000 joint genotype distribution imputations, and an assumed genotyping error rate of 0.01 (Sen and Churchill 2001). A permutation test with

10000 replications was used to determine the significance of the results. After determining the presence of a possible QTL on Chr3, an additional 135 (for a total of 201) N₂ mice were genotyped with additional Chr3 markers. For comparing the phenotypic distribution of the N₂, F₂, and F₃ genotypes, one-way ANOVAs were performed, followed by pairwise *t*-tests with a Bonferroni correction if the ANOVA resulted in *P*<0.05. GraphPad Prism software (GraphPad, San Diego, CA) was used for statistical analysis and to create all graphs reporting numerical values. **P*<0.05, ***P*<0.01, ****P*<0.001.

RESULTS

Dstn^{corn1} neovascularization is modified by genetic background

In a previous study, we reported that introgression of the *Dstn^{corn1}* allele onto the C57BL/6J (B6) genetic background results in a significant dampening of *Dstn^{corn1}* phenotypes. Specifically, the neovascularization observed in A.BY *Dstn^{corn1}* mice is significantly less when observed on the B6 background (Kawakami-Schulz et al. 2012). A cross between A.BY *Dstn^{corn1}* and B6.A.BY- *Dstn^{corn1}* mice results in F₁ mice whose level of *Dstn^{corn1}* neovascularization is intermediate to the parental strains (Figure 1A, B, C) (Kawakami-Schulz et al. 2012). This background-specific phenotypic variation led us to speculate the existence of modifier loci affecting the level of *Dstn^{corn1}* neovascularization. In order to investigate this possibility, we crossed F₁ mice back to the B6.A.BY- *Dstn^{corn1}* parental strain to obtain N₂ *Dstn^{corn1}* mice. The neovascularization phenotype of N₂ *Dstn^{corn1}* mice falls into unimodal distribution from a higher level of neovascularization, similar to the A.BY *Dstn^{corn1}* cornea (average 37.4%), to a lower level, similar to the B6 *Dstn^{corn1}* cornea (average 19.9%) (Figure 1A, C). The observed vasculature phenotype and mean value is similar to that of the F₁ parent group (Figure 1A, C). This unimodal distribution suggests polygenic regulation of *Dstn^{corn1}* neovascularization.

A QTL modifying *Dstn^{corn1}* neovascularization is located on Chromosome 3

To test the genetic basis of the variation we observed, phenotypic and genotypic data from 66 N₂ mice, aged 58 days, were gathered to conduct a genome-wide scan using R/qtl. A major QTL region was identified on Chr3 (Figure 2A). While characterizing additional markers and animals, we further confirmed the existence of a significant QTL associated with modification of corneal neovascularization in *Dstn^{corn1}* mice (Figure 2B). The peak is near to marker D3Mit315 (LOD score=3.51, *P*=0.0071, 3 LOD support interval = 8 cM-75.65 cM). The A.BY allele of this genetic modifier has a significant effect in increasing the amount of neovascularization (Figure 2C), which is consistent with our observation that the A.BY background consistently exhibits higher levels of corneal neovascularization in *Dstn^{corn1}* mutant mice. We have named this modifier locus ‘modifier of *Dstn^{corn1}* neovascularization 1’ (*Modn1*).

Consomic mice are used to demonstrate the allelic effect of *Modn1*

In order to test the solo effect of Chr3 on neovascularization in *Dstn^{corn1}* mice, we took advantage of the availability of consomic mice that have Chr3 from A/J mice and the rest of the genome from B6 mice (B6 Chr3^{A/J}). Since A.BY mice are very closely related with A/J mice (<http://jaxmice.jax.org/strain/000140.html>), we expect that A/J mice and A.BY mice share the same alleles for the modifier loci. By crossing B6 Chr3^{A/J} to B6.A.BY- *Dstn^{corn1}* mice, we generated F₂ and F₃ *Dstn^{corn1}* mutant mice with three different genotypes at a potential QTL on Chr3 (AA, AB and BB). In this cross, we are able to generate mice with all possible combinations for B6 and A/J alleles of genes within the region of interest and are able to eliminate allelic effects from genes that are not located on Chr3. We observed significant increase of vascularization in mice carrying one or two A/J alleles at the modifier locus (Fig. 3).

DISCUSSION

The search for genetic modifiers can unearth a locus or loci that interact with a known gene or disease genotype. A modifier screen serves as powerful tool to link observed phenotypic variation, which is characteristic of both normal and diseased tissues, with genetic diversity that exists among groups of individuals. Differences in the genetic background of laboratory mice have been shown to significantly affect both normal and disease condition traits (Johnson et al. 2008; Jurisic et al. 2010; Le Roy et al. 1999; Lively et al. 2010; Rogers and D'Amato 2011; Wang et al. 2007; Yuan et al. 2009). Our mouse model utilizes a spontaneous genetic mutation called *Dstr^{corn1}* to study the spontaneous formation of vasculature in the cornea and identify novel genetic modifiers. In this study, a QTL affecting the degree of neovascularization in *Dstr^{corn1}* mice was identified on Chr3 and named 'modifier of *Dstr^{corn1}* neovascularization 1' (*Modn1*). At this locus the A.BY allele of the modifier gene could increase the severity of neovascularization in a semi-dominant manner. Our studies using consomic mice demonstrated that the modifier locus has an individual effect on the severity of neovascularization. While the effect of *Modn1* is significant it does not account for all of the variation we observe, indicating that there may be additional genetic factors, either outside the QTL region on chromosome 3 or on other chromosomes, that influence the amount of corneal neovascularization in *Dstr^{corn1}* mice. In previous studies, several QTL involved in neovascularization in the cornea have been identified (Rogers and D'Amato 2011; Rogers et al. 2004; Rogers et al. 2003). However, these studies required that the cornea be altered via external methods. Our QTL on Chr3, *Modn1*, is a new locus identified as a QTL associated with spontaneous corneal neovascularization that develops *in vivo*. Interestingly, the trend that we observe, that is, more vasculature in non-pigmented cornea and less vasculature in pigmented cornea, is opposite to the effect of a gene recently found to quantitatively modify induced corneal neovascularization (Rogers et al. 2013). Considering that the allelic effect of this QTL appears only with the *Dstr^{corn1}* mutation, the locus involved in this study may interact with factors in the pathway through which the *Dstr^{corn1}* mutation leads to vasculature in the cornea.

There are multiple genes of interest in the *Modn1* region that may contribute to the phenotypic variation we observe for neovascularization in the *Dstr^{corn1}* cornea. Although the *Modn1* region is still too large to determine the gene(s), we set criteria for choosing candidate genes. These candidate genes are listed in Table 1.

First, we used the Mouse Genome Informatics (MGI) resource (<http://www.informatics.jax.org/>) to compare sequence information for the B6 and A/J inbred strains, and selected genes in the *Modn1* region with sequence differences that lead to non-synonymous mutations (NS SNPs). These genes are likely candidates since the nature of the sequence difference has the potential to change the structure and function of the protein product. Alteration of a protein that is involved in angiogenesis may modify its ability to bind to other molecules, including actin and/or SRF. Changes in protein structure and/or function may lead to variability in the tissue remodeling that is required for a network of blood vessels to form.

Second, genes that are known to contribute to angiogenesis, or the formation of vasculature, can vary between strains and may be responsible for the genetic modification we observe in this study. They may play a role in the establishment and variability of the vascular network when comparing different mouse strains. Genes of interest in these regions include: endothelial cell-derived fibroblast growth factor -2 (*Fgf2*), which is involved in the guided migration of cells and may influence the forged pathway of the vascular network (Qazi et al. 2009). Additionally, sequence differences in the sequence for epidermal growth factor (*Egf*),

which is important for angiogenesis and blood vessel development, could influence the degree of vasculature that forms as a result of the *Dstn^{corn1}* mutation (Schreier et al. 2013).

Third, due to the nature of the gene *Dstn*, which has been deleted in our model, it is possible that other genes that are involved in the establishment and regulation of the actin cytoskeleton influence the phenotypic severity of abnormalities. *Arghgef11* is a guanine nucleotide exchange factor capable of activating small GTPases, which regulate the actin cytoskeleton.

Fourth, we have previously demonstrated that SRF is a major regulator of *Dstn^{corn1}* phenotypes and that genetic ablation of *Srf* from the corneal epithelium in *Dstn^{corn1}* mice rescues the neovascularization and other phenotypes in the cornea (Verdoni et al. 2010). There are multiple genes in the region whose expression levels are hypothesized to be directly affected by SRF, including *Nes*, *Apoa1bp*, and *Cyr61* (Benson et al. 2011; Sun et al. 2006). Since SRF overexpression is necessary for the establishment of *Dstn^{corn1}* phenotypes, proteins that are downstream from SRF are likely candidates that may play a role in *Dstn^{corn1}* neovascularization.

Fifth, genes that are known to be up- or down-regulated in *Dstn^{corn1}* cornea when compared to WT cornea are good candidates. These genes could potentially be in the pathway through which *Dstn* mutation induces neovascularization. We previously performed microarray analysis comparing the gene expression in the cornea between A.BY WT and *Dstn^{corn1}* mutant mice (Verdoni et al. 2008a). The genes *Etv3*, *Rhbfg*, *Mtbp*, and *Adh7* mapped to the region of interest on Chr3.

Genes that belong to multiple categories are of particular interest. For example, sequence differences between the A/J and B6 alleles of *Vcam1* result in amino acid differences. Additionally, *Vcam1* has been shown to play a primary role in the induction of neovascularization in the retina in response to oxidative stress and could contribute to the inflammatory response that results in changes in actin dynamics (Dong et al. 2011; Lin et al. 2013). Although there are no NS SNPs between the A/J and B6 backgrounds that lead to amino acid changes in its gene sequence, Cysteine-rich protein 61 (*Cyr61*) induces angiogenesis, has been shown to directly interact with SRF, and is significantly up-regulated in *Dstn^{corn1}* (Hanna et al. 2009; Kim et al. 2003).

In this study, we took advantage of the availability of a consomic line and demonstrated that a single QTL is responsible for modification of neovascularization in the *Dstn^{corn1}* cornea that we reported previously (Kawakami-Schulz et al. 2012). The consomic line is a valuable tool to eliminate most background effects and is less time-intensive than creating congenic lines. Our F₂ intercross using the Chr3 consomic line could be used to collect recombinant mice to narrow the QTL region as previously described (Ohno et al. 2012; Rogers et al. 2013). Identification of the modifier gene, *Modn1*, will allow us to further understand how the *Dstn^{corn1}* mutation and abnormal actin regulation cause neovascularization in the cornea.

Acknowledgments

The authors would like to thank Zhen Zhang for development and technical support of E-mouseLab, Katie Clowers and Zak Lemmon for feedback and assistance with R/qtl, and the University of Wisconsin-Madison Genetics Confocal Facility for the use of the confocal microscope. This work was supported by a grant from the National Institutes of Health (NIH R01EY016108) and a core grant to Waisman Center (NIH P30HD03352). Support for S.V.K-S was partially provided by the NIH pre-doctoral training program in Genetics (NIH 5T32GM07133).

References

- Arends D, Prins P, Jansen RC, Broman KW. R/qtl: high-throughput multiple QTL mapping. *Bioinformatics*. 2010; 26:2990–2992.10.1093/bioinformatics/btq565 [PubMed: 20966004]
- Benson CC, Zhou Q, Long X, Miano JM. Identifying functional single nucleotide polymorphisms in the human CArGome. *Physiological Genomics*. 2011; 43:1038–1048.10.1152/physiolgenomics.00098.2011 [PubMed: 21771879]
- Bock F, Onderka J, Hos D, et al. Improved semiautomatic method for morphometry of angiogenesis and lymphangiogenesis in corneal flatmounts. *Exp Eye Res*. 2008; 87:462–470.10.1016/j.exer.2008.08.007 [PubMed: 18789928]
- Dong A, Shen J, Zeng M, Campochiaro PA. Vascular cell-adhesion molecule-1 plays a central role in the proangiogenic effects of oxidative stress. *Proceedings of the National Academy of Sciences*. 2011; 108:14614–14619.10.1073/pnas.1012859108
- Hanna M, Liu H, Amir J, et al. Mechanical regulation of the proangiogenic factor CCN1/CYR61 gene requires the combined activities of MRTF-A and CREB-binding protein histone acetyltransferase. *Journal of Biological Chemistry*. 2009; 284:23125–23136.10.1074/jbc.M109.019059 [PubMed: 19542562]
- Ikeda S, Cunningham LA, Boggess D, et al. Aberrant actin cytoskeleton leads to accelerated proliferation of corneal epithelial cells in mice deficient for destrin (actin depolymerizing factor). *Human Molecular Genetics*. 2003; 12:1029–1037. [PubMed: 12700171]
- Jakobsson L, Franco CA, Bentley K, et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nature Publishing Group*. 2010; 12:943–953.10.1038/ncb2103
- Johnson BA, Aoyama N, Friedell NH, et al. Genetic modification of the schisis phenotype in a mouse model of X-linked retinoschisis. *Genetics*. 2008; 178:1785–1794.10.1534/genetics.107.084905 [PubMed: 18245825]
- Jurisc G, Sundberg JP, Bleich A, et al. Quantitative lymphatic vessel trait analysis suggests Vcam1 as candidate modifier gene of inflammatory bowel disease. *Genes Immun*. 2010; 11:219–231.10.1038/gene.2010.4 [PubMed: 20220769]
- Kawakami-Schulz SV, Verdoni AM, Sattler SG, et al. Differences in corneal phenotypes between destrin mutants are due to allelic difference and modified by genetic background. *Mol Vis*. 2012; 18:606–616. [PubMed: 22419854]
- Kim KH, Min YK, Baik J-H, et al. Expression of angiogenic factor Cyr61 during neuronal cell death via the activation of c-Jun N-terminal kinase and serum response factor. *J Biol Chem*. 2003; 278:13847–13854.10.1074/jbc.M210128200 [PubMed: 12576482]
- Kushner EJ, Bautch VL. Building blood vessels in development and disease. *Curr Opin Hematol*. 2013; 20:231–236.10.1097/MOH.0b013e328360614b [PubMed: 23567339]
- Le Roy I, Perez-Diaz F, Cherfouh A, Roubertoux PL. Prewaning sensorial and motor development in laboratory mice: quantitative trait loci mapping. *Dev Psychobiol*. 1999; 34:139–158. [PubMed: 10086232]
- Lin C, Hinds A, Burns CJ, et al. Serum response factor controls transcriptional network regulating epidermal function and hair follicle morphogenesis. *J Invest Dermatol*. 2013; 133:608–617.10.1038/jid.2012.378 [PubMed: 23151848]
- Lively GD, Jiang B, Hedberg-Buenz A, et al. Genetic dependence of central corneal thickness among inbred strains of mice. *Investigative Ophthalmology & Visual Science*. 2010; 51:160–171.10.1167/iops.09-3429 [PubMed: 19710407]
- Nakao S, Maruyama K, Zandi S, et al. Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains. *FASEB J*. 2010; 24:504–513.10.1096/fj.09-134056 [PubMed: 19858096]
- Ohno T, Hata K, Baba T, et al. Establishment of consomic strains derived from A/J and SM/J mice for genetic analysis of complex traits. *Mamm Genome*. 2012; 23:764–769.10.1007/s00335-012-9435-x [PubMed: 23052825]

- Pakneshan P, Birsner AE, Adini I, et al. Differential Suppression of Vascular Permeability and Corneal Angiogenesis by Nonsteroidal Anti-inflammatory Drugs. *Investigative Ophthalmology & Visual Science*. 2008; 49:3909–3913.10.1167/iovs.07-1527 [PubMed: 18487370]
- Qazi Y, Maddula S, Ambati BK. Mediators of ocular angiogenesis. *J Genet*. 2009; 88:495–515.10.1007/s12041-009-0068-0 [PubMed: 20090210]
- Qazi Y, Wong G, Monson B, et al. Corneal transparency: Genesis, maintenance and dysfunction. *Brain Res Bull*. 2010; 81:198–210.10.1016/j.brainresbull.2009.05.019 [PubMed: 19481138]
- Ribatti D, Crivellato E. “Sprouting angiogenesis,” a reappraisal. *Developmental Biology*. 2012.10.1016/j.ydbio.2012.09.018
- Rogers M, D’Amato R. *Common Polymorphisms in Angiogenesis*. Cold Spring. 2011
- Rogers MS, Adini I, McBride AF, et al. The albino mutation of tyrosinase alters ocular angiogenic responsiveness. *Angiogenesis*. 2013.10.1007/s10456-013-9342-0
- Rogers MS, Birsner AE, D’Amato RJ. The mouse cornea micropocket angiogenesis assay. *Nat Protoc*. 2007; 2:2545–2550.10.1038/nprot.2007.368 [PubMed: 17947997]
- Rogers MS, Rohan RM, Birsner AE, D’Amato RJ. Genetic loci that control vascular endothelial growth factor-induced angiogenesis. *FASEB J*. 2003; 17:2112–2114.10.1096/fj.03-0246fje [PubMed: 12958152]
- Rogers MS, Rohan RM, Birsner AE, D’Amato RJ. Genetic loci that control the angiogenic response to basic fibroblast growth factor. *FASEB J*. 2004; 18:1050–1059.10.1096/fj.03-1241com [PubMed: 15226265]
- Schreier B, Rabe S, Schneider B, et al. Loss of epidermal growth factor receptor in vascular smooth muscle cells and cardiomyocytes causes arterial hypotension and cardiac hypertrophy. *Hypertension*. 2013; 61:333–340.10.1161/HYPERTENSIONAHA.112.196543 [PubMed: 23248150]
- Sen S, Churchill GA. A statistical framework for quantitative trait mapping. *Genetics*. 2001; 159:371–387. [PubMed: 11560912]
- Smith RS, Hawes NL, Kuhlmann SD, et al. Corn1: a mouse model for corneal surface disease and neovascularization. *Investigative Ophthalmology & Visual Science*. 1996; 37:397–404. [PubMed: 8603845]
- Sun Q, Chen G, Streb JW, et al. Defining the mammalian CArGome. *Genome Res*. 2006; 16:197–207.10.1101/gr.4108706 [PubMed: 16365378]
- Verdoni AM, Aoyama N, Ikeda A, Ikeda S. Effect of destrin mutations on the gene expression profile in vivo. *Physiological Genomics*. 2008a; 34:9–21.10.1152/physiolgenomics.00285.2007 [PubMed: 18381839]
- Verdoni AM, Schuster KJ, Cole BS, et al. A Pathogenic Relationship Between a Regulator of the Actin Cytoskeleton and Serum Response Factor. *Genetics*. 2010; 186:147–157.10.1534/genetics.110.117309 [PubMed: 20610412]
- Verdoni AM, Smith RS, Ikeda A, Ikeda S. Defects in Actin Dynamics Lead to an Autoinflammatory Condition through the Upregulation of CXCL5. *PLoS ONE*. 2008b; 3:e2701.10.1371/journal.pone.0002701.g005 [PubMed: 18628996]
- Wang SS, Shi W, Wang X, et al. Mapping, genetic isolation, and characterization of genetic loci that determine resistance to atherosclerosis in C3H mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2007; 27:2671–2676.10.1161/ATVBAHA.107.148106
- Yuan Z, Pei H, Roberts DJ, et al. Quantitative trait locus analysis of neointimal formation in an intercross between C57BL/6 and C3H/HeJ apolipoprotein E-deficient mice. *Circ Cardiovasc Genet*. 2009; 2:220–228.10.1161/CIRCGENETICS.108.792499 [PubMed: 19718279]

Figure 1.

Corneal neovascularization resulting from the *Dstn*^{corn1} mutation is modified by genetic background. (A) Immunofluorescence using CD31 highlights blood vessels that infiltrate the cornea in A.BY *Dstn*^{corn1}, B6.A.BY- *Dstn*^{corn1}, and B6 N₂ *Dstn*^{corn1} mice at P58. Bar, 200 μ m. (B) Visual representation of quantification of the vascularized area in *Dstn*^{corn1} cornea. Representative images of B6.A.BY, F₁ and A.BY *Dstn*^{corn1} corneas are shown. The total corneal area, that is, the entire area used in measurement, is encased by the red line. The vascularized area, which is positive for CD31 staining, is black. White areas indicate CD31 negative areas of the cornea. The vascularized area was calculated by dividing the vascularized area by the total cornea area. (C) Quantification of neovascularization in the

cornea of A.BY *Dstr^{corn1}*, B6.A.BY- *Dstr^{corn1}*, F₁ *Dstr^{corn1}* (B6.A.BY x A.BY) and B6 N₂ *Dstr^{corn1}* (F₁ x B6.A.BY) mice at P58 reveals that mean values between lines vary significantly.

Figure 2.

QTL mapping of modifiers of *Dstn^{corn1}* neovascularization. (A) Whole genome scan LOD score distribution for association of neovascularization in 66 B6 N₂ P58 *Dstn^{corn1}* mice. Two horizontal lines represent significant linkage as assessed by permutation testing (bottom, $P < 0.05$; top, $P < 0.01$). (B) LOD score distribution on Chr3 for all B6 N₂ mice. After a QTL was identified, an additional 135 mice were genotyped and phenotyped for Chr3. The LOD score distribution for all 201 B6 N₂ mice is shown. The genetic map was constructed in R/qtl on the basis of our cross. Upper ticks on the horizontal axis represent markers used in this analysis. Only a selection of the total markers used are labeled on the LOD score curve. Three horizontal lines represent significant linkage (bottom, $P < 0.05$; middle, $P < 0.01$; top, $P < 0.001$). (C, D) Quantification of the vascularized area shows that cornea from mice homozygous for the B6 allele (BB) at D3Mit315 have significantly less vasculature than cornea from heterozygous (AB) mice ($P < 0.0001$). Sample sizes: BB, $n=80$, AB, $n=104$. Error bars, SEM * denotes statistical significance resulting from a t-test, *** $P < 0.001$.

Figure 3.

Quantification of corneal neovascularization in P58 F_2 and F_3 *Dstr^{corn1}* Chr3 recombinants. The mean level of vasculature in the cornea of mice homozygous for the allele of either strain (BB or AA) at D3Mit315 is significantly different. Mice homozygous for the A/J allele (AA) have significantly more vasculature than heterozygous (AB) mice. Sample sizes: BB n=18, AB n=33, AA n=21. Error bars, SEM * denotes statistical significance resulting from ANOVA. ** $P < 0.01$, *** $P < 0.001$, and the absence of a bar denotes no significance.

Table 1

Markers and genes of interest located in the region defined by QTL analysis. Rows in gray indicate Chr3 markers, and their Mb positions, used in this study. Genes of interest are listed with the number of NS SNPs that have been identified when comparing the B6 and A/J backgrounds. A check indicates whether the gene belongs to the group named in the column title (see text for further details).

Marker Name/Gene Symbol	Gene Name	NS SNPs	Mb position	Angiogenesis	Actin dynamics	SRF Target	Gene expression difference
D3Mit268			28.72				
D3Mit151			31.24				
<i>Fgf2</i>	Endothelial cell-derived fibroblast growth factor-2	1	37.31	✓			
D3Mit64			50.16				
<i>Tiparp</i>	TCDD-inducible poly(ADP-ribose) polymerase	2	65.53	✓			
D3Mit137			78.83				
<i>Gucyl a3</i>	guanylate cyclase 1, soluble, alpha 3	1	82.09	✓			
<i>Fhdc 1</i>	FH2 domain containing 1	5	84.44		✓		
D3Mit40			87.30				
<i>Etv3</i>	ets variant gene 3	2	87.53				✓
<i>Arhgef11</i>	Rho guanine nucleotide exchange factor (GEF) 11	3	87.62		✓		
<i>Nes</i>	nestin	14	87.97			✓	✓
<i>Apoa1bp</i>	apolipoprotein A-I binding protein	1	88.06			✓	✓
<i>Rhhg</i>	Rhesus blood group-associated B glycoprotein	2	88.24				✓
D3Mit175			88.33				
D3Mit315			115.84				
<i>Vcam1</i>	vascular cell adhesion molecule 1	1	116.11	✓	✓		
D3Mit216			123.43				
<i>Ank2</i>	ankyrin 2, brain	3	126.92	✓	✓		✓
D3Mit348			127.04				
<i>Egf</i>	Epidermal growth factor	2	129.68	✓			
D3Mit14			132.03				
D3Mit291			136.37				
<i>Mtp</i>	microsomal triglyceride transfer protein	1	138.09				✓
<i>Adh7</i>	alcohol dehydrogenase 7 (class IV)	1	138.22				✓

Marker Name/Gene Symbol	Gene Name	NS SNPs	Mb position	Angiogenesis	Actin dynamics	SRF Target	Gene expression difference
D3Mit351			139.60				
D3Mit38			142.83				
<i>Cyr61/CCN1</i>	cysteine rich protein 61	0	145.65	✓		✓	✓
D3Mit44			148.14				
D3Mit128			150.76				