Junctional Adhesion Molecule-A Regulates Vascular Endothelial Growth Factor Receptor-2 Signaling-Dependent Mouse Corneal Wound Healing

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Abstract

Inflammation and angiogenesis are integral parts of wound healing. However, excessive and persistent wound-induced inflammation and angiogenesis in an avascular tissue such as the cornea may be associated with scarring and visual impairment. Junctional adhesion molecule A (Jam-A) is a tight junction protein that regulates leukocyte transmigration as well as fibroblast growth factor-2 (FGF-2)-induced angiogenesis. However its function in wound-induced inflammation and angiogenesis is still unknown. In this study, we report spontaneous corneal opacity in Jam-A deficient mice associated with inflammation, angiogenesis and the presence of myofibroblasts. Since wounds and/or corneal infections cause corneal opacities, we tested the role of Jam-A in wound-induced inflammation, angiogenesis and scarring by subjecting Jam-A deficient mice to full thickness corneal wounding. Analysis of these wounds demonstrated increased inflammation, angiogenesis, and increased number of myofibroblasts thereby indicating that Jam-A regulates the wound-healing response by controlling wound-induced inflammation, angiogenesis and scarring in the cornea. These effects were not due to inflammation alone since the inflammation-induced wound-healing response in Jam-A deficient mice was similar to wild type mice. In order to determine the molecular mechanism associated with the observed aberrant corneal wound healing in Jam-A deficient mice, we assessed the expression of the components of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor-2(VEGFR-2) signaling pathway. Interestingly, we observed increased levels of VEGF-A mRNA in Jam-A deficient eyes. We also observed nuclear localization of phosphorylated SMAD3 (pSMAD3) indicative of TGFβ pathway activation in the Jam-A deficient eyes. Furthermore the increased wound-induced corneal inflammation, angiogenesis, and scarring in Jam-A deficient mice was attenuated by treatment with DC101, an anti-vascular endothelial growth factor receptor-2 (VEGFR-2) antibody. Our results suggest that in the absence of Jam-A, the VEGF-A/VEGFR-2 pathway is upregulated, thereby augmenting wound induced corneal inflammation, angiogenesis, and myofibroblast accumulation leading to scarring.

Citation: Chatterjee S, Wang Y, Duncan MK, Naik UP (2013) Junctional Adhesion Molecule-A Regulates Vascular Endothelial Growth Factor Receptor-2 Signaling-Dependent Mouse Corneal Wound Healing. PLoS ONE 8(5): e63674. doi:10.1371/journal.pone.0063674

Editor: Christina Lynn Addison, Ottawa Hospital Research Institute, Canada

Received November 29, 2012; Accepted April 7, 2013; Published May 8, 2013

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Funding: This work was supported by National Institutes of Health grant HL63960 to UPN, EY12221 to MKD, and INBRE program grant P20 RR16472 supporting the University of Delaware Core Imaging facility. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Wound healing is a dynamic process critical to restore tissue structure following damage. Wound healing is often divided into three over-lapping stages [1]. First the inflammatory stage, which results in the recruitment of macrophages and neutrophils to the site of injury [2,3]. Macrophages help clear pathogens and cellular debris from the site of the wound. Second, the proliferative and fibrotic phase. The inflammatory cells release matrix metalloproteinases (MMPs), cytokines, and growth factors that lead to the proliferative and fibrotic stage of wound healing along with angiogenesis (also known as neovascularization) at the injured site [4,5,6]. This is an important step in wound healing in many tissues since these new blood vessels transport oxygen, other nutrients, and additional inflammatory cells needed for faster wound healing. Finally, at the third stage, once the wound is closed, the tissue is remodeled in an attempt to re-establish normal tissue architecture. However, myofibroblast persistence and/or excess angiogenesis can lead to scarring and compromised tissue function [7].

VEGF-A is known to promote angiogenesis during wound healing and often works in concert with cell adhesion molecules such as integrin αvβ3 and integrin αvβ5 in endothelial cells to mediate this function [9,9]. VEGF-A signals by binding to its receptors VEGFR-1 (FLT-1) and VEGFR-2 and is known to also promote inflammation and epithelial to mesenchymal transition [10]. VEGF-A signaling through its receptors FLT-1 and VEGFR-2 also regulates TGFβ expression that in turn regulates corneal wound healing [11,12,13]. In contrast with the pro-angiogenic properties of VEGF-A and its receptors, soluble VEGF-1 (sFLT-1) is anti-angiogenic, acting as a VEGF-A trap, which inhibits VEGF-A signaling.
Blood vessels consist of quiescent vascular endothelial cells, which form tight junctions, and maintain the vessel integrity. JAM-A is a tight junction protein that is involved in tight junction permeability [14], leukocyte transmigration [15] and FGF-2-induced angiogenesis [16]. JAM-A associates with the integrin αβ₃ and is essential for FGF-2 induced endothelial cell migration on vitronectin [17,18]. Although Jam-A deficient mice have an apparently normal vasculature, FGF-2 induced angiogenesis is defective in these mice as assayed by an in vivo Matrigel plug assay [16]. We observed that a significant percentage of Jam-A deficient animals on a C57Bl/6NHSd genetic background developed spontaneous corneal opacities as they age. Histological investigation of the eyes of these mice revealed increased corneal angiogenesis, inflammation, and myofibroblast accumulation. We also found that Jam-A deficient mice on this inbred background have profound defects in the healing of full thickness corneal wounds due to the increased VEGF-A levels in the eye. This upregulation of VEGF-A was found to be functionally relevant since treatment of Jam-A deficient mice with a function blocking VEGF-R antibody resulted in a partial rescue of the observed wound-healing defect. These results suggest that in the absence of Jam-A, pathways regulating VEGF-A/VEGFR-2 signaling are upregulated, resulting in heightened inflammation, angiogenesis and scarring.

Materials and Methods

Animals

Generation of Jam-Agt/gt (F11f2pGT1p;F11f2pGT1p;ES1Upn) mice has been previously described [16]. The mice were genotyped by the polymerase chain reaction (PCR). C57Bl/6NHSd (Harlan Laboratories), wild type (WT) and Jam-Agt/gt mice of both genders were used in this study.

Ethical statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Delaware Institutional Animal Care and Use Committee (AUP no: 1094). All surgery was performed under the ketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

Antibodies

A monoclonal rat anti-mouse PECAM-1 (CD31) antibody was obtained from BD Biosciences Pharmingen (San Diego, CA; catalog # 550274), rabbit anti-mouse phosphorylated VEGFR-2 (pVEGFR-2) antibody was obtained from Cell Signaling Technology (Beverly, MA; catalog # 2478), the FITC conjugated mouse monoclonal anti-α-smooth muscle actin (αSMA) antibody was obtained from Sigma (St Louis, MO; catalog # F3777), rat anti-mouse Ly-6B.2 alloantigen antibody which detects polymorphonuclear (PMN) cells was obtained from Serotec (Raleigh, NC; catalog # MCA771G) and rabbit anti-mouse MMP-9, rabbit anti-mouse collagen I, rat anti-mouse CD11b, and pSMAD3 antibodies were purchased from AbCam (Boston, MA; catalog # ab38898, ab299, ab8878, ab52903 respectively). Goat anti-rabbit Alexa Fluor 568 or donkey anti-mouse Alexa Fluor 488 labeled secondary antibodies were obtained from Life Technologies (Grand Island, NY). Draq5 was purchased from Biostatus Limited (Leicestershire, United Kingdom; catalog # DR30200). Rat anti-mouse DC101 was obtained from BioXCell (West Lebanon, NH). Rat IgG was obtained Santa Cruz Biotechnology, Inc (Santa Cruz, CA; catalog # sc2026).

Silk suture-induced corneal angiogenesis

Silk suture-induced corneal angiogenesis assays were performed as previously described [19]. Briefly, following ketamine/xylazine anesthesia, an 8–0 silk suture was inserted into the center of the cornea of 8–10 week old mice. Erythromycin ophthalmic ointment was applied immediately after suture. The mice were observed every day for the appearance of a gross scar. The mice were sacrificed 0 hour, 3 days, 7 days, and 12 days post surgery and the corneas were analyzed by immunohistochemistry.

Full thickness corneal wounds

Mice aged between 8–10 weeks were anesthetized with ketamine/xylazine and a full thickness wound was inflicted in the central region of the cornea using a Feather Surgical No 11 blade. The wound was closed with a 10-0 nylon suture (Ethicon) and erythromycin ophthalmic ointment was applied immediately. The mice were observed for gross signs of scarring. The mice were sacrificed at 0 hour, 3 days, 7 days, and 12 days post surgery and the cornea were analyzed by immunohistochemistry.

Anti-VEGFR-2 (DC101) treatments

Mice aged between 8–10 weeks that underwent full thickness corneal wounds were intraperitoneally injected with 200 μg/Kg DC101 diluted in saline (DC101 concentration optimized on its ability to inhibit tumor growth in C57Bl/6NHSd mice) or rat IgG every alternate day starting from 0 hour post surgery and sacrificed on the 12th day post surgery.

Immunofluorescence

Enucleated eyes were directly embedded in optimal cutting temperature (OCT) media obtained from Sakura Finetek (Torrence, CA) without prior fixation. Cryosections (16 μm) were cut, mounted on slides, and subjected to immunofluorescence studies as described previously [20]. In brief, the sections were fixed in either in chilled acetone/methanol (1:1) for 20 minutes or paraformaldehyde and 0.25% Triton X-100 and then blocked in 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (blocking buffer) for 1 hour at room temperature (RT). Anti-pSMAD3 stained sections were blocked in 5% BSA with goat and horse serum for 1 hour at RT. The sections were then incubated with appropriate primary antibodies diluted in blocking buffer at 4°C overnight. The slides were washed and the primary antibodies detected by incubation with the appropriate secondary antibodies, diluted in blocking buffer along with a (1:1000) dilution of the DNA specific nucleic acid stain, Draq5 to detect the cell nucleus. The sections were analyzed and imaged using either a Zeiss LSM 510 confocal microscope. Immunofluorescence quantitation was performed using Velocity 5.2 software (Perkin Elmer Boston, MA). A ratio of the area occupied by the individual immunostain to the total corneal area in each image was determined.

Quantitative Real Time Polymerase Chain Reaction (Q-rtPCR)

To perform Q-rtPCR, RNA from whole eyes except for the lens of 32–48 week old WT and Jam-Agt/gt mice were used. The RNA was isolated using the RNeasy kit obtained from Qiagen (Valencia, CA); One μg of RNA was used from both WT and Jam-Agt/gt mice to prepare cDNA using the high capacity cDNA reverse transcription kit obtained from Applied Biosystems (Foster city, CA). The primers used for Q-rtPCR are shown in Table-1. Quantitative analysis of mRNA expression was performed using ABI Prism 7300 and SYBR green. Data is presented as a fold.
increase compared to WT values (2ΔΔct) relative to β-Actin expression.

Quantitation and statistical analysis
Statistical analysis of the data was performed using Student’s t-test (mean ± SEM value; SEM, standard error of the mean). P<0.05 is regarded as statistically significant at a 95% confidence interval.

Results

Jam-A<sup>gt</sup> eyes with spontaneous corneal opacity exhibit inflammation, angiogenesis and scarring

We have previously reported that the corneas of Jam-A<sup>gt</sup> (F11<sup>G<sub>gt</sub>TJtg</sup>) mice are transparent and exhibit no differences in corneal epithelial debridement wound healing compared to wild type mice (WT) [21]. Since these animals were on a mixed C57Bl/6-129 background (B6.129P2-F11<sup>G<sub>gt</sub>TJtg</sup>), we backcrossed them to C57Bl/6Ndsl for 10 generations to move the F11<sup>G<sub>gt</sub>TJtg</sup> allele to an inbred background. We have observed that approximately 16% of congenic C57Bl/6Ndsl Jam-A<sup>gt</sup> mice develop spontaneous corneal opacities (Fig. 1A) with an incidence that increases with age, while this phenotype was never observed in strain matched WT controls (Table 2). Histological analysis of the paraffin embedded eyes of these mice revealed that the opaque corneas exhibited thickening and increased cellularity of the corneal stroma and disorganization of the corneal epithelial layer. However this phenotype was absent in both WT mice of the same strain and Jam-A<sup>gt</sup> mice with transparent corneas (Fig. 1B).

The eyes of affected Jam-A<sup>gt</sup> mice also often exhibited anterior subcapsular cataracts typified by a multiflared lens epithelium (Fig. 1B). The corneal opacities of Jam-A<sup>gt</sup> mice could be attributed to a scarring response since some cells of the corneal stroma were found to express αSMA, indicative of the presence of myofibroblasts, which are absent from the normal cornea (Fig. 1C). Furthermore, the opaque corneas from Jam-A<sup>gt</sup> mice exhibited enhanced neovascularization (as measured by PECAM-1 expression). There was also a robust upregulation of MMP-9 expression (Fig. 1D), which is known to be expressed in corneas exhibiting scarring. The enhanced MMP-9 expression in scarred corneas is due to the infiltration of inflammatory cells [22,23]. The upregulated MMP-9 expression observed in the abnormal Jam-A<sup>gt</sup> eyes co-localized with Ly-6B.2 staining (Fig. 1E) and not αSMA (Fig. S1), indicating that the source of MMP-9 was from the inflammatory cells such as neutrophils and not myofibroblasts.

However since all of the MMP-9 expressing cells did not colocalize with Ly-6B.2 expressing cells, we believe that some of the MMP-9 expression observed could be by the other inflammatory cells such as macrophages [24].

*Jam-A<sup>gt</sup>* mice show increased wound-induced corneal angiogenesis

Corneal wound healing has been studied by inducing corneal trauma by various methods such as burns, the placement of corneal sutures, epithelial scrape wounds, and full thickness wounds [19,21,25,26]. Corneal scarring is often observed in response to a corneal injury or inflammation to the cornea [19,27]. A full thickness corneal wound causes a break in the Descemet’s membrane leading to extracellular matrix (ECM) remodeling [28]. This is associated with inflammation as well as neovascularization [29,30]. Since our data suggested that Jam-A<sup>gt</sup> mice were inappropriately sensitized to spontaneous corneal scarring, we next tested their ability to heal full thickness corneal wounds. In both WT and Jam-A<sup>gt</sup> mice, corneal transparency was compromised by the 12<sup>th</sup> day post injury; however, Jam-A<sup>gt</sup> corneas were more severely affected than controls (Fig 2A).

Hematoxylin and eosin (H&E) staining showed that injured Jam-A<sup>gt</sup> corneas appeared more abnormal by the 12<sup>th</sup> day post injury than WT controls with an increased corneal thickness and stromal cellularity (Fig. 2B). Full thickness wounds caused a significant (P<0.0001) increase in inflammation as assessed by PMN accumulation in the Jam-A<sup>gt</sup> corneas by the 12<sup>th</sup> day post wounding compared to the WT corneas (Fig. 2C&D). This data was further supported by a similar increase in MMP-9 and CD11b (a marker for neutrophils) [31] expression observed in the Jam-A<sup>gt</sup> corneas by the 12<sup>th</sup> day post wounding compared to the WT corneas (Fig. S2A). MMP-9 staining pattern was found to co-localize with CD11b staining indicating that the MMP-9 expressing cells also express CD11b (Fig. S2A&B). Co-localization of Ly-6B.2 with MMP-9 confirmed that MMP-9 expressing cells are PMN. Since the MMP-9 expressing cells express both Ly-6B.2 as well as CD11b, these observations collectively suggest that these inflammatory cells are neutrophils (Fig. S2C&D). Jam-A<sup>gt</sup> corneas also exhibited significantly (P<0.0001) more neovascularization than controls by the seventh day post injury as measured by the extent of PECAM-1 staining (Fig. 2E&F).

Wounded Jam-A<sup>gt</sup> corneas also demonstrated upregulated myofibroblast accumulation as shown by αSMA expression by the seventh day post injury, which continued to increase until at least the 12th day post injury, while injured WT corneas exhibited limited αSMA expression (Fig. 2G). Quantification revealed a significant (P<0.0005) difference in αSMA expression between Jam-A<sup>gt</sup> and WT mice (Fig. 2H).

Along with the increased αSMA expression observed, the wounded Jam-A<sup>gt</sup> corneas also exhibited extensive collagen I deposition indicative of fibrosis (Fig S3). αSMA is expressed in both pericytes as well as myofibroblasts [32,33]. To determine the source of αSMA expression, we performed co-immunostaining experiments with anti-αSMA and PECAM-1 antibodies. We found minimal αSMA expression in close proximity to PECAM-1 expression suggesting that pericytes were not the major source of αSMA (Fig. S4A). Since TGFβ activation leads to expression of αSMA [34], we investigated if the TGFβ pathway was activated in the Jam-A<sup>gt</sup> eyes. We observed nuclear localization of pSMAD3 indicative of TGFβ activation in the Jam-A<sup>gt</sup> corneas that was not observed in WT corneas (Fig. 2I&J). These abnormalities associated with the excessive wound-healing response in the Jam-A<sup>gt</sup> cornea indicate that, in WT mice, Jam-A negatively regulates wound-induced inflammation, angiogenesis as well as myofibroblast accumulation in the cornea following full thickness wounds.
However, we observed no difference in Jam-A expression in the WT eyes at different time points post injury suggesting that Jam-A is not upregulated as a result of wounding (Fig. S5A).

No difference in inflammation-induced corneal angiogenesis between Jam-A^gt/gt and WT eyes

Inflammation is known to induce angiogenesis in a variety of tissues including cornea [19,35]. Silk suture placement is routinely used to study inflammation induced corneal angiogenesis since silk proteins induce a foreign body immune response [19,36,37]. Since

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**Table 2. Incidence of scarring in Jam-A^gt/gt and WT mice.**

| Age         | Jam-A^gt/gt | WT  |
|-------------|-------------|-----|
| 0–3 months  | 6/30        | 0/36|
| 3–6 months  | 4/72        | 0/50|
| 6–12 months | 9/17        | 0/9 |

doi:10.1371/journal.pone.0063674.t002
Jam-Agt/gt corneas showed an increased inflammatory response in response to full thickness corneal injury than WT mice (Fig. 2G&H), we next tested whether inflammation itself was sufficient to induce the enhanced angiogenesis and myofibroblast accumulation observed in injured Jam-Agt/gt corneas. We induced inflammation using the silk suture model of corneal injury by introducing silk suture into the stroma of the cornea without disturbing the Descemet’s membrane (Descemet’s membrane is broken in the full thickness wounds). First, in both WT and Jam-Agt/gt mice, placement of a central silk suture in the cornea did not produce overt corneal opacities by the 12th day post injury (Fig. 3A). This was supported by H&E staining of cryosections.
which did not reveal any obvious morphological differences between Jam-A<sup>agt/gt</sup> and WT corneas (Fig. 3B). Also the Jam-A<sup>agt/gt</sup> mice did not show increased stromal cellularity upon introduction of a silk suture in contrast to what was observed in Jam-A<sup>agt/gt</sup> mice after full thickness corneal wounding (Fig. 2B). As expected, the silk suture did induce extensive corneal inflammation as measured by elevated Ly-6B.2 staining in both Jam-A<sup>agt/gt</sup> and WT mice. (F) Quantification of PECAM-1 staining from E. The number of Jam-A<sup>agt/gt</sup> and WT mice used: 0 hours (n = 3); Day 3 (n = 3); Day 7 (n = 5); Day 12 (n = 6). There was no significant difference observed in inflammation or angiogenesis between the Jam-A<sup>agt/gt</sup> and WT mice. Scale bar 100 μm. CE: Corneal epithelium; CS: Corneal stroma; I: iris.

doi:10.1371/journal.pone.0063674.g003

Figure 4. Increased VEGF-A expression in Jam-A<sup>agt/gt</sup> eyes. mRNA expression was analyzed in Jam-A<sup>agt/gt</sup> and WT mice by Q-rtPCR using whole eye tissue minus the lens. mRNA expression was quantified for VEGF-A, VEGFR-2, FLT-1 and sFLT-1 (n = 4) (*)P = 0.03) (ns: not significant).

doi:10.1371/journal.pone.0063674.g004

Figure 3. No difference in inflammation induced corneal angiogenesis between Jam-A<sup>agt/gt</sup> and WT mice. (A) Photographic images of WT and Jam-A<sup>agt/gt</sup> eyes. (B) H&E staining of frozen sections prepared from Jam-A<sup>agt/gt</sup> and WT eyes 12 days post suture. (C&E) Confocal images. Nuclear staining using Draq5 is shown in blue. (C) Corneal inflammation demonstrated by Ly-6B.2 staining. (D) Quantification of Ly-6B.2 staining shows no difference in inflammation between Jam-A<sup>agt/gt</sup> and WT mice. (E) Angiogenesis depicted by PECAM-1 staining in Jam-A<sup>agt/gt</sup> and WT mice. (F) Quantification of PECAM-1 staining from E. The number of Jam-A<sup>agt/gt</sup> and WT mice used: 0 hours (n = 3); Day 3 (n = 3); Day 7 (n = 5); Day 12 (n = 6). There was no significant difference observed in inflammation or angiogenesis between the Jam-A<sup>agt/gt</sup> and WT mice. Scale bar 100 μm. CE: Corneal epithelium; CS: Corneal stroma; I: iris.
Figure 5. Jam-A$^{gt/gt}$ eyes reveal increased VEGFR-2 signaling during wound-induced corneal angiogenesis. (A) Photographic images of WT and Jam-A$^{gt/gt}$ mice exhibiting corneal scar observed 12 days post corneal injury and DC101 treatment. (B) H&E staining of OCT embedded eyes of Jam-A$^{gt/gt}$ and WT mouse treated with IgG and DC101 demonstrate corneal morphology 12 days post wound. (C, E, G, & I) Confocal images of eye sections of IgG and DC101 treated WT and Jam-A$^{gt/gt}$ mice. Scale bar 100 μm except for (I), which is 10 μm. Nuclear staining using Draq5 is shown in blue. (C) Ly-6B.2 staining showed inflammation in Jam-A$^{gt/gt}$ and WT mice. (D) Quantitation of Ly-6B.2 staining of C. (E) PECAM-1 staining to indicate angiogenesis. (F) Quantitation of PECAM-1 levels upon DC101 treatment compared to IgG control. (G) αSMA expression indicative of presence of
myofibroblasts in Jam-A<sup>−/−</sup> and WT mice. (H) Quantitation of αSMA staining in G. (I) pSMAD3 staining indicative of TGFβ activation in IgG and DC101 treated eyes. (J) Quantification of pSMAD3 staining shown as a ratio of area of pSMAD3 to the total area occupied by nuclei. (n = 3) (*P < 0.05, **P < 0.0001). CE: Corneal epithelium; CS: Corneal stroma; t: iris.

doi:10.1371/journal.pone.0063674.g005

Enhanced wound-induced inflammation, angiogenesis and scarring are dependent on VEGFR-2 signaling

Since VEGF-A signaling is known to be involved in inflammation, neovascularization as well as accumulation of myofibroblasts, we then investigated whether the VEGF-A/VEGFR-2 pathway [38] is upregulated in Jam-A<sup>−/−</sup> mice [39]. qRT-PCR analysis of RNA isolated from Jam-A<sup>−/−</sup> and WT eyes indicated that VEGF-A mRNA levels were significantly (P = 0.03) elevated in Jam-A<sup>−/−</sup> eyes (Fig. 4). Further, the mRNA levels of FLT1 (VEGFR-1) or VEGFR-2 appeared to be slightly increased in Jam-A<sup>−/−</sup> compared to WT mice although these differences were not statistically significant (Fig. 4). The mRNA levels of sFLT1-1, which is a soluble form of VEGFR-1 recently shown to regulate corneal avascularity by acting as a VEGF-A trap [40], was also apparently downregulated in Jam-A<sup>−/−</sup> eyes compared to the controls, however the difference also did not reach significance at 95% confidence.

In order to test whether the increase in VEGF-A expression in Jam-A<sup>−/−</sup> eyes could cause the dysregulation of corneal wound healing in Jam-A<sup>−/−</sup> mice by signaling through VEGFR-2, we first determined activation of VEGFR-2 as assessed by pVEGFR-2 staining in the full thickness injury WT and Jam-A<sup>−/−</sup> corneas. We observed minimal pVEGFR-2 staining in the WT corneas. However, extensive pVEGFR2 staining was observed in the corneal epithelium of the Jam-A<sup>−/−</sup> eyes 12 days post injury with some staining in the stromal cells (Fig. S6). We also blocked VEGFR-2 signaling by treating the mice post injury with DC101, which is known to block VEGFR-2 function [41]. Treatment with DC101 reduced corneal opacity following full thickness corneal injury as compared to IgG treated mice (Fig. 5A). H&E staining showed that DC101 treatment also attenuated the increase in corneal thickness and stromal cellularity compared to IgG treated Jam-A<sup>−/−</sup> mice 12 days after full thickness corneal wounds (Fig. 5B). DC101 treatment was also associated with a down regulation in the number of PMN cells in the corneal stroma (Fig. 5C&D). We also observed a significant decrease in neovascularization as measured by the extent of PECAM-1 staining upon DC101 treatment in both WT and Jam-A<sup>−/−</sup> corneas (Fig. 5E&F). DC101 treatment also inhibited the accumulation of myofibroblasts following full thickness corneal injury of Jam-A<sup>−/−</sup> and WT mice (Fig. 5G&H). The presence of pericytes was also diminished in these corneas as shown by the reduced expression of αSMA that is associated with PECAM-1 (Fig. S4B). We further observed the absence of TGFβ activation in the DC101 treated Jam-A<sup>−/−</sup> eyes as compared to the IgG treated Jam-A<sup>−/−</sup> eyes as indicated by the lack of nuclear pSMAD3 staining (Fig. 5I&J). The above results show that Jam-A negatively regulates wound-induced inflammation, angiogenesis and scarring by modulating the VEGF-A/VEGFR-2 pathway.

Discussion

The process of wound healing involves inflammation, neovascularization, and tissue remodeling. We have observed that a significant percentage of Jam-A<sup>−/−</sup> mice develop spontaneous corneal scarring, inflammation, and angiogenesis. This was mimicked in experimentally-induced full thickness corneal wounds in Jam-A<sup>−/−</sup> mice. We observed that this corneal scarring phenotype is not due to inflammation alone and requires TGFβ activation. Finally, we show that the resulting increased VEGF-A dependent VEGFR-2 signaling contributes to the observed propensity for corneal scarring in Jam-A<sup>−/−</sup> mice. This demonstrates a novel function for Jam-A in modulating the wound-healing response.

Corneal trauma such as a full thickness wound disturbs the stromal architecture and induces ECM remodeling as the stromal keratocytes are further primed to attain a myofibroblast-like phenotype [28] [42] [43]. Mechanistically, corneal scarring is believed to be caused by secretion of TGFβ2 by corneal epithelial cells into the underlying stroma, activating stromal keratocytes to undergo a myofibroblast-like transformation [32,44]. This TGFβ-induced myofibroblast-like transformation is stabilized by topographic cues provided by the surrounding extracellular matrix (ECM). Consistent with this, we observed that introduction of silk suture does not cause corneal scarring despite the prevalence of inflammation and neovascularization. The increased Ly-6B.2 staining in the silk sutured Jam-A<sup>−/−</sup> corneas at a later time point

Figure 6. Proposed mechanism by which Jam-A regulates corneal inflammation, neovascularization, and scarring. Jam-A negatively governs VEGF-A expression thereby regulating VEGFR-2 signaling pathway. The VEGF-A/VEGFR-2 pathway leads to angiogenesis as well as recruits inflammatory cells. The VEGF-A/VEGFR-2 pathway also has a positive feedback loop with TGFβ pathway that along with ECM remodeling leads to scarring. Introduction of silk suture results in inflammation leading to neovascularization and no scarring due to the presence of an intact Descemet’s membrane. Full thickness wounds that cause a break in the Descemet’s membrane lead to ECM remodeling and TGFβ pathway activation that together can lead to scarring. Jam-A deficient mice exhibit increased VEGF-A/VEGFR-2 signaling and demonstrate increased inflammation, angiogenesis as well as scarring compared to WT mice.

doi:10.1371/journal.pone.0063674.g006
could be due to the inability of the Jam-A<sup>gt/gt</sup> neutrophils to egress since JAM-A has been shown to be involved in neutrophil transmigration [15]. Along with corneal wounds or inflammation, corneal scarring can also occur in the absence of an external agent as observed in congenital stromal dystrophy caused by mutations in the gene for decorin. In this case, loss of decorin yields a loss of corneal transparency due to inappropriate activation of the TGFβ pathway leading to myofibroblast accumulation [45,47,48].

VEGF-A is required for the process of wound healing [49] however excess VEGF-A/VEGFR-2 signaling can cause inappropriate wound healing responses that leads to scarring [50]. While VEGF-A/VEGFR-2 signaling is often thought to mostly regulate angiogenesis [51,52], it also contributes to recruitment of inflammatory cells leading to inflammation [53]. Further, VEGF-A induced signals have been shown to induce expression of αSMA in cancer cells [54] which could explain the scarring phenotype observed in the Jam-A deficient corneas due to the inappropriate accumulation of myofibroblasts. In the case of Jam-A<sup>gt/gt</sup> mice, the increased VEGF-A could further increase the activity of the TGFβ pathway resulting in the heightened scarring phenotype observed in the Jam-A deficient mice. This is consistent with the observed positive feedback loop between VEGF-A and TGFβ [10,12] that contributes to VEGF-A dependent scarring.

VEGF-A/VEGFR-2 expression is modulated by FGF-2 signaling in blood vessel endothelial cells (BVECs) [55,56]. It is possible that the increased VEGF-A/VEGFR-2 signaling in the Jam-A deficient mice is due to a compensatory mechanism for the loss of FGF-2 signaling. However VEGF-R2 expression is reduced in the absence of FGF-2 signaling, while an increase in FGF-2 signaling causes increased VEGF-A signaling in BVECs [55,56] ruling out the possibility of a compensatory mechanism. It is known that integrin α<sub>v</sub>β<sub>3</sub> regulates NFκβ dependent VEGF-A expression in BVECs [33]. It is also known that integrin α<sub>v</sub>β<sub>3</sub> associates with JAM-A in unstimulated BVECs and dissociates upon FGF-2 stimulation [57]. It can be speculated that JAM-A associated with integrin α<sub>v</sub>β<sub>3</sub> keeps the integrin in an inactive conformation thereby keeping VEGF-A expression by BVECs in check. Alternatively, since JAM-A and VEGF-A are expressed in many ocular cell types besides endothelial cells [14,58], JAM-A may be regulating VEGF-A expression in the eye via as yet unknown mechanisms.

Based on the data presented in this paper, we propose a model (Fig. 6) for the role of JAM-A in corneal wound healing. JAM-A suppresses VEGF-A expression in the cornea. In a full thickness wound, there is ECM remodeling and TGFβ signaling in addition to inflammation and neovascularization leading to corneal scarring. Introduction of a silk suture results in corneal inflammation and neovascularization, but no scarring due to the intact Descemet’s membrane. All these events are caused by increased VEGF-A/VEGFR-2 signaling, since blocking VEGFR-2 signaling after full thickness corneal wounding attenuated all the three processes associated with wound healing. These observations are consistent with previously published effects of VEGF-A [51,53]. In summary, here we demonstrate that Jam-A can control basal VEGF-A expression levels in the eye. Further, we show that this control of VEGF-A expression is important to regulate the appropriate levels of wound-induced inflammation and angiogenesis for optimum healing of full thickness corneal wounds. However, further investigation is needed to understand the mechanisms by which Jam-A regulates basal levels of VEGF-A expression levels in the eye.
Author Contributions
Conceived and designed the experiments: MKD UPN. Performed the experiments: SC YW. Analyzed the data: SC MKD UPN. Wrote the paper: SC MKD UPN.

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