WFDC1 Is a Key Modulator of Inflammatory and Wound Repair Responses

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WFDC1/ps20 is a whey acidic protein four-disulfide core member that exhibits diverse growth and immune-associated functions in vitro. In vivo functions are unknown, although WFDC1 is lower in reactive stroma. A Wfdc1-null mouse was generated to assess core functions. Wfdc1-null mice exhibited normal developmental and adult phenotypes. However, homeostasis challenges affected inflammatory and repair processes. Wfdc1-null mice infected with influenza A exhibited 2.75-log-fold lower viral titer relative to control mice. Wfdc1-null infected lungs exhibited elevated macrophages and deposition of osteopontin, a potent macrophage chemokine. In wounding studies, Wfdc1-null mice exhibited an elevated rate of skin closure, and this too was associated with elevated deposition of osteopontin and macrophage recruitment. Wfdc1-null fibroblasts exhibited impaired spheroid formation, elevated adhesion to fibronectin, and an increased rate of wound closure in vitro. This was reversed by neutralizing antibody to osteopontin. Osteopontin mRNA and cleaved protein was up-regulated in Wfdc1-null cells treated with lipopolysaccharide or polyinosinic-polycytidylic acid coordinate with constitutively active matrix metallopeptidase-9 (MMP-9), a protease that cleaves osteopontin. These data suggest that WFDC1/ps20 modulates core host response mechanisms, in part, via regulation of osteopontin and MMP-9 activity. Release from WFDC1 regulation is likely a key component of inflammatory and repair response mechanisms, and involves the processing of elevated osteopontin by activated MMP-9, and subsequent macrophage recruitment. (Am J Pathol 2014, 184: 2951—2964; http://dx.doi.org/10.1016/j.ajpath.2014.07.013)

We have previously reported the growth inhibitory functions of ps20 in vitro,1,2 purified the secreted ps20 protein,3 and cloned the rodent and human WFDC1 genes encoding ps20.4,5 Rodent and human mRNA encoding ps20 are highly conserved, exhibit a canonical four-disulfide core domain typical of the whey acidic protein (WAP) family, and localize to human chromosome 16q24.4,5 By contrast, many of the other WAP family members are clustered on chromosome 20q13, suggesting WFDC1 had a divergent evolutionary origin.4,5 Eighteen human WAP proteins have been identified; most are secreted factors that exhibit protease inhibitory activity and contain at least one canonical four-disulfide core domain.7 The WAP family members exhibit several diverse biological functions associated with immune responses and antimicrobial activity.6 However, the biology and function of WFDC1/ps20 in vivo is poorly understood.

Several in vitro studies suggested WFDC1/ps20 functions in stromal—epithelial interactions and tissue homeostasis.1—3 Further study showed that WFDC1/ps20 expression was reduced in reactive stroma in human prostate cancer, and this was predictive of prostate cancer progression.5 Reactive stroma in cancer is very similar to wound repair reactive stroma generally observed at sites of altered tissue homeostasis,

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including wound repair, fibrosis, cancer, and cardiovascular disease.\textsuperscript{9-11} Conversely, \textit{in vivo} studies showed that overexpression of WFDC1/ps20 in a recombined cancer/stromal cell mixed xenograft model resulted in elevated tumorigenesis attributable to elevated angiogenesis.\textsuperscript{12} Together, these studies suggest that WFDC1/ps20 functions in adult tissue homeostasis by modulating cell proliferation, adhesion, and migration.

Additional studies support the notion that WFDC1/ps20 functions during infection processes and immune responses. Human WFDC1/ps20 promoted HIV infection in CD4 T cells by up-regulating intercellular adhesion molecule 1,\textsuperscript{13} whereas murine Wfdc1 limited murine hepatitis virus infectivity and neutrophil recruitment\textsuperscript{14} in our mouse model, suggesting differential innate immune functions. Other studies have shown that mutations in WFDC1 were associated with macular degeneration leading to blindness,\textsuperscript{15-17} although mechanisms were not addressed.

In summary, previous studies suggest that WFDC1/ps20 may function as a regulator of diverse cell responses associated with general homeostasis involving tissue repair and immunity. To evaluate this hypothesis directly, we generated a Wfdc1-null mouse to assess the null phenotype and to pinpoint changes in host response mechanisms during homeostasis challenges. We report here that Wfdc1-null mice challenged with viral infection or physical wounding exhibit clear biological alterations. Wfdc1-null mice exhibited marked resistance to influenza virus infection and more robust wound repair responses with elevated and earlier recruitment of macrophages. These homeostasis responses were associated with elevated Spp1/osteopontin and constitutively active matrix metallopeptidase-9 (MMP-9) that mediated some of Wfdc1-null associated biology. Together, these data suggest that Wfdc1 represses emergency response mechanisms during resting, normal adult tissue homeostasis and that release from Wfdc1 regulation at sites of reactive and repair tissue biology is a key permissive event in coordination of core inflammatory and repair processes.

Materials and Methods

\textbf{Wfdc1 Knockout/Knockin Mouse}

A Wfdc1-null mouse was generated via knockout of exon 1 and insertion of lacZ in frame with the Wfdc1 ATG start codon. The 5' homology arm using the Wfdc1 genomic clone AF169632.1 was digested with Spel, resulting in a fragment containing nucleotides +2542 to +418 (relative to the ATG) (that includes exon 1) cloned into an Spel site in pSK- (Stratagene, Santa Clara, CA). An NcoI sense primer 5'-GCCACCAGCCTGCCATGGTGATCTCGG-3' (NcoI site underlined) along with the anti-sense complement was used in the Stratagene QuikChange Mutagenesis Kit to mutate the Wfdc1 ATG to an NcoI site to generate the construct pSpelNcoImutant. We then mutated the lacZ ATG to an NcoI site in the construct pPD46.21, obtained from Dr. Franco DeMayo [Baylor College of Medicine (BCM)], that contains a 3.3-kb lacZ fragment with an SV40 nuclear localization site. A lacZ sense primer 5'-CCGAGCTACA-GAAACATGCGTCCTCAAAAGAAGAGCG-3' (NcoI site underlined) along with the anti-sense complement was used in the Stratagene QuikChange Mutagenesis Kit to mutate the ATG from lacZ to start an NcoI site to generate pPD46.21NcoImutant. The NcoI mutant constructs were digested with NcoI and SalI, and the 3.3-kb lacZ NcoI mutant fragment cloned into the pSpelNcoImutant construct at the Wfdc1 ATG site to produce pSpelIgal that contains −2542 to +1 of Wfdc1 and a 3.3-kb lacZ gene. To generate a 3' homology arm using a Xhol digest of MGL5-I11S4, a Wfdc1 genomic clone containing exon 1 and part of intron 1, which generates a fragment from +549 to +3883, was used. This 3334-bp 3' homology arm was cloned in a 3-way ligation with the 5' homology arm from the pSpelIgal construct that was digested with ApaI and Xhol to generate a fragment containing +1170 to +1 of the Wfdc1 promoter with the 3.3-kb lacZ gene and a 1.7-kb Xhol fragment from PGKNeo that contains the phosphoglycerate kinase (PGK) promoter and neo gene cassette. All three fragments were cloned into pSK- that was linearized with ApaI to generate the Wfdc1KO/KI construct. The orientation of the PGK-neo cassette was cloned in the anti-sense direction relative to the lacZ gene. The Wfdc1KO/KI construct was sequenced to verify position and orientation. The Wfdc1KO/KI construct was then linearized with ApaI and injected into AB2.2 p.13 embryonic stem cells by the GEM core at BCM.

Chimeric mice were mated to C57Bl/6 mice for four to five generations and given the name Wfdc1\textsuperscript{tm1LacZBCM}. Use of mice was carried out according to the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals,\textsuperscript{18} and the policies of BCM.

Screening Wfdc1 Knockout Mice

The Wfdc1 knockout/knockin (KO/KI) mice were screened by Southern blotting using a 1.3-kb probe spanning nucleotides +2542 (Spel) to +1170 (ApaI). The introduction of the lacZ gene produced an extra EcoRV site that on digestion of the genomic DNA with EcoRV and hybridization with the 1.3-kb Spel-ApaI probe yielded a 6.5-kb knockout allele and a 9.5-kb wild-type allele. Subsequently, mice were screened by PCR using two reactions each containing primers to mouse β-actin and either primers to Wfdc1 exon1 sense (5'-GGAGGTACACTCTGGCA-3') and Wfdc1 antisense (5'-CAGGATTCGGCTCGG-3') or β-galactosidase (β-gal) sense (5'-TTCACCTGCGTTGCTTACAGG-3') and β-gal antisense (5'-ATGGACGGAGTAAACCCGGTCATTCT-3'). Screening with the Wfdc1 exon1 and β-gal primers yields 133-bp and 364-bp bands, respectively.
β-Gal Enzyme Staining of Wfdc1-Null Tissue

Tissues stained for β-gal enzyme were fixed in 4% paraformaldehyde for 1 to 2 hours, incubated in graduated 10% to 20% sucrose (in Hanks’ balanced salt solution) over 3 days at 4°C, and then frozen in optimal cutting temperature compound. Frozen sections (10 μm) were postfixed in 0.2% glutaraldehyde, washed seven times in PBS (pH 8.3), and stained with X-Gal at 37°C overnight (O/N). Following staining for β-gal, some frozen tissues were also stained for CD34, Mac-3, and pro-SP-C.

Immunostaining after β-Gal Enzyme Staining

For immunostaining after β-gal enzyme staining, the tissue was evaluated for vessels using anti-mouse CD34 (#119301; BioLegend, San Diego, CA) at 1:100 dilution, for macrophages using anti-mouse Mac-3 (BD Pharmingen #553322; BD Biosciences, San Jose, CA) at 1:100 dilution, or for type II pneumocytes using anti-mouse pro-SP-C (WRAB-9337; Seven Hills Bioreagents, Cincinnati, OH) at 1:1600 dilution for 1 hour at room temperature. Streptavidin-AP followed by Vector Red substrate (Vector Laboratories, Burlingame, CA) was used to visualize the immunostaining. Images were acquired using a CRi multispectral deconvolution camera (N-MSI-420-FL) feeding a Macintosh Mac Pro computer (Apple, Cupertino, CA) running Nuance image analysis software version 3.0.0 (CRi, now PerkinElmer, Waltham, MA).

Immunostaining of Human Prostate

Human prostate sections were acquired from the Human Tissue Acquisition and Pathology Core Laboratory (Dan L. Duncan Cancer Center, BCM). An IgY antibody to a peptide (CVKQRRQADGRILRHKLYKEY) at amino acids 180 to 200 of the human ps20 protein was made by Aves Laboratories (Tigard, OR), tested for specificity, and used at 1:1200 dilution together with a rabbit anti-CD31 (#ab134168; Abcam, Cambridge, MA) at 1:400 dilution O/N at 4°C for colocalization of ps20 and CD31. Secondary antibodies [biotin-conjugated anti-chicken from Jackson Immuno-Research Laboratories (West Grove, PA) #103-065-155 and the horseradish peroxidase (HRP)-conjugated goat anti-rabbit from Abcam #ab137913], were incubated for 45 minutes at 37°C. To develop the chicken anti-ps20 antibody, Streptavidin-AP (Vector Laboratories) was incubated (30 minutes at room temperature) followed by incubation with the Vector Blue substrate (Vector Laboratories) (30 minutes at room temperature). To develop the rabbit anti-CD31 antibody, the NovaRED substrate was incubated for 15 minutes at room temperature. Methyl Green (Vector Laboratories) was used as a counterstain. Images were converted to optical density (OD) and deconvoluted to separate blue (anti-ps20) from the red (anti-CD31) and green counterstain.

Electroretinogram

Four Wfdc1-heterozygous and four Wfdc1-null mice at 9 months of age for a total of eight eyes per group were tested for a blindness phenotype using an electroretinogram protocol as described previously.19

Influenza Virus Infections

Wild-type, heterozygous, and null Wfdc1 KO/KI littermates (12 to 16 weeks of age) were anesthetized (with isoflurane), and 50 μL of approximately 10 median tissue culture infective doses of influenza A/Texas (H1N1) virus was inoculated intranasally. Mice were sacrificed at 4 days postinfection for infectivity assays and at days 7 and 14 to assess histopathology and immunohistochemistry (IHC). Lungs were harvested and lobes were frozen in 1 mL of minimal essential medium in tubes with glass beads, bead beaten, and then the supernatant collected for virus titering or fixed in 4% paraformaldehyde and embedded in paraffin for IHC.

Influenza Virus Titering

Round-bottom plates (96 well) were prepared with MDCK cells (CCL64; ATCC, Manassas, VA) 24 hours before titering. The medium was changed to 100 μL of minimal essential medium + Worthington Trypsin (0.5%) to prepare cells for viral infection. Frozen infected lungs were thawed and bead beaten for 60 seconds. The homogenate was centrifuged (5 minutes at 2000 × g), and a 1:10 dilution in minimal essential medium was made. Each mouse lung was titered in duplicate with a control. Diluted lung homogenate (50 μL) was added to 100 μL of medium, then serial dilutions (1:3) were performed across the plate and the plate incubated for 5 days at 37°C in 5% CO2. Virus titer was determined by adding 50 to 100 μL of red blood cells/well and incubating for 30 minutes at room temperature. Statistical analysis of the virus titer comparing infected heterozygous versus null lungs was performed using an unpaired t-test with Welch’s correction with the GraphPad Prism version 5 program (GraphPad Software, La Jolla, CA).

IHC of Influenza-Infected Tissue

IHC analysis of both uninfected and influenza-infected mouse tissues was performed as previously described20,21 with modifications. Tissues were fixed in Zinc fixative (BD Pharmingen #550523; BD Biosciences) and processed for IHC without antigen retrieval to assess macrophage, osteopontin, and MMP-9 immunostaining. All secondary antibody incubations were at 37°C for 1 hour. To stain for macrophages, IHC was performed on tissue from wild-type, Wfdc1-heterozygous, and Wfdc1-null mice using rat monoclonal to macrophage (Abcam #ab56297) 1:500 dilution at 4°C O/N and with biotin-conjugated goat anti-rat IgG, (Invitrogen #D-20697; Life Technologies, Carlsbad, CA) 1:200 dilution.
To stain for MMP-9, IHC was performed on infected lung tissue from wild-type, Wfdc1-heterozygous, and null mice using rabbit anti-mouse MMP-9 (Abcam #ab38898) at 1:200 dilution at 4°C O/N and with biotin-conjugated goat anti-rabbit IgG, (Invitrogen #B-2770) at 1:500 dilution. To stain for osteopontin, IHC was performed on infected lung tissue from wild-type, Wfdc1-heterozygous, and null mice using goat anti-mouse osteopontin (#AF808; R&D Systems, Minneapolis, MN) at 1:400 dilution at 4°C O/N and with biotin-conjugated donkey anti-goat IgG, (Invitrogen #D-20698) at 1:500 dilution. Streptavidin-HRP (#RPN1239V; GE Healthcare, Chalfont St Giles, UK) at 1:1000 dilution also was used. Hybridization and digital analysis were performed according to the manufacturer’s protocol.

RT-PCR and Quantitative PCR Analysis

For reverse transcription of mRNA, 500 ng of stromal cell RNA was reverse-transcribed using the Superscript III kit (Invitrogen) in 20 μL of reaction. For quantitative PCR analysis, the RT reaction was diluted 1:5, and 5 μL was used in a 96-well plate with the FastStart Universal SYBR Green Master (Roche, Basel, Switzerland ) in 25 μL of final volume. An ABI 7700 instrument (Life Technologies) was used with the following program: 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Zymography and Western Blotting

Three Wfdc1-heterozygous stromal cell lines (HetMP1, HetMP3, and HetMP5) and two Wfdc1-null stromal cell lines (312#1L1 and 312#2P1) were treated with LPS or poly I:C as described above for 24 hours. Conditioned medium was collected and concentrated in an Amicon Ultra-4 centrifugal filter for zymography assays or concentrated by 10% trichloroacetic acid precipitation for Western blotting. For zymography, one-third of the concentrated medium was run on Bio-Rad ready-cast 10% gelatin gels (Bio Rad Laboratories, Hercules, CA), and stained with Coomassie Blue R-250 (Bio Rad Laboratories). For Western blotting, one-third of the trichloroacetic acid–precipitated conditioned medium was resuspended in 1 x Nupage LDS sample buffer (Invitrogen) and run on a 10% Nupage Bis-Tris precast gel (Invitrogen) under reducing conditions. Following transfer to a PVDF membrane using the iBlot transfer apparatus (Invitrogen), the membrane was blocked in Roche Western blocking reagent for 1 hour, incubated in anti-mouse osteopontin (R&D Systems #AF808) at 0.2 μg/mL at 4°C O/N, followed by incubation in biotin-conjugated donkey anti-goat antibody (Invitrogen #D20698) at 1:1000 dilution for 1 hour at room temperature, and then incubated in Streptavidin-HRP (#RPN1239V; GE Healthcare, Chalfont St Giles, UK) at 1:5000 dilution for 45 minutes at room temperature. The blot was visualized using the Pierce ECL substrate and scanned with a Kodak Image Station 4000R.
(Kodak, Rochester, NY) with Carestream version 5 software (Carestream Health, Rochester, NY).

**In Vivo Wounding Assay and Immunostaining of Skin Wounds**

Two 3-mm-diameter punch biopsy lesions were made on the flanks of Wfdc1-heterozygous and null mice. A total of at least five animals with two biopsies each were used to evaluate the wound healing over 7 days in each group. From day 1 to 7, the wounds were measured with calipers. The subsequent measurements were analyzed using a two-way analysis of variance analysis in GraphPad Prism version 5.0a. Additional measurements were analyzed using a two-way analysis of variance test with Bonferroni post-tests for each group. From day 1 to 7, the wound healing over 7 days in each group. From day 1 to 7, the wound healing over 7 days in each group.

For the anti-osteopontin migration inhibition assay, two Wfdc1-null stromal cell lines 312#2P1 and 312#2L4 were used in a scratch assay with the addition of either 10 μg of IgG isotype control, or 10 or 20 μg of anti-osteopontin (R&D Systems #AF808) added at time 0 hours following the scratch. At 10 hours post scratch, images of the wound width were photographed and analyzed as above. The anti-osteopontin migration inhibition assay was performed in triplicate with three scratches per well two independent times. The scratch width in each image was measured in at least three different places along the scratch. Individual t-tests were performed comparing the control IgG with either 10 or 20 μg of anti-osteopontin IgG with both cell lines.

For the adhesion assay, two Wfdc1-heterozygous cell lines (HetMA3 and Het MP1) and two Wfdc1-null cell lines (312#2P1 and 312#2P4) were plated at 1000 cells/well on 96-well strips from a Millicot ECM Screening Kit (Millipore #ECM101-105) that contains individual 8-well strips coated with either fibronectin, laminin, vitronectin, collagen type I, or collagen type IV. The assay was performed according to the manufacturer’s directions and read at OD 540 nm with a Bio-Rad iMark microplate reader. The adhesion assay was performed in duplicate and repeated three independent times. Two-way analysis of variance test with Bonferroni post-tests was performed to compare the OD 540-nm measurements of the extracellular matrix component for each Wfdc1-heterozygous cell line versus each Wfdc1-null line.

**Results**

**Phenotype of Wfdc1-Null Mice and Spatial Expression of Wfdc1**

To generate Wfdc1-null mice, exon 1 of the Wfdc1 gene was deleted and the bacterial lacZ gene knocked in to the Wfdc1 locus to trace expression using homologous recombination as described in Figure 1A. The resulting Wfdc1-heterozygous and homozygous null mice were evaluated for altered developmental or adult tissue phenotypes. Wfdc1-heterozygous and null mice exhibited no overt gross morphological or abnormal tissue phenotypes during either embryonic development or postnatal maturation. Reproductive rate, litter size, and sex ratios of Wfdc1-null mice were each normal. This normal-appearing phenotype is similar to null mice generated for other WAP family members.25–27

Owing to reports of mutant WFDC1/ps20 being associated with blindness, eye function was carefully evaluated. A complete electroretinogram analysis showed no overt physiological differences in retina function in Wfdc1-null and heterozygous mice (Supplemental Figure S1 and Supplemental Table S1). The spatial and temporal patterns of Wfdc1 expression were evaluated by tracking the knockin nuclear β-gal reporter, constructed with a SV40 nuclear localization signal. Positive nuclear β-gal was observed in the adult mouse
retina (Figure 1B), prostate (Figure 1C), skin (Figure 1D), bladder (Figure 1E), testis (Figure 1F), lung (Figure 1G), and brain (Figure 1H), and in the human prostate gland (Figure 1I). Other mouse tissues such as the liver, spleen, thymus, kidney, and bone did not exhibit obvious nuclear β-gal staining. Of interest, β-gal staining was observed in cells within the walls of the microvasculature in many tissues.

Colocalization of β-gal expression with anti-CD34 demonstrated that Wfdc1 expression was spatially associated with endothelial cells in small vessels in the brain, lung, and testes (Figure 1, F–H). Wfdc1 expression was not spatially associated with either macrophages or type II pneumocytes in the lung (Supplemental Figure S2). In the retina, most cells in the inner nuclear layer and a few cells in the outer nuclear layer exhibited intense nuclear β-gal (Figure 1B). Epidermal cells of the skin and smooth muscle cells of the bladder exhibited sporadic expression of nuclear β-gal (Figure 1, D and E). Prostate gland exhibited a more general nuclear localization of β-gal in the acinar epithelial cells (Figure 1C). This spatial pattern of β-gal activity is consistent with immunolocalization of actual Wfdc1/ps20 in the human prostate gland, where ps20 is spatially localized in prostate acinar epithelial cells and vascular, CD31-positive endothelial cells (Figure 1I). Together, these data are consistent with the notion that WFDC1/ps20 is localized to cells with a relatively high adhesive and barrier function. This includes endothelial cells where there is a blood—tissue barrier (brain and testis); lung, where the endothelial barrier is critically important; retina layers that exhibit high adhesion; and epithelial cells associated with barrier function in the prostate gland.

WFDC1 Affects Influenza Virus Infection

Owing to our previous studies that showed WFDC1/ps20 exhibits an immunomodulatory role that promotes HIV infection in vitro,13 we investigated whether WFDC1/ps20 status affects influenza A infection in lung tissue. Wild-type, Wfdc1-heterozygous, and Wfdc1-homozygous null mice were subjected to lung infection with influenza A/TX. Remarkably, viral titers in lung tissue at 4 days postinfection showed a 2.75-log-fold lower titer in Wfdc1-null mice as compared to Wfdc1-heterozygous mice (Figure 2A).
(P < 0.005). Viral titers in the wild-type mice were similar to Wfdc1-heterozygous mice. To assess whether levels of viral infectivity were associated with differences in immune cell infiltration, the 7-day postinfection lungs were evaluated for macrophage and neutrophil recruitment. A considerable elevation in macrophage recruitment was observed in Wfdc1-null mice as compared to the infected wild-type and heterozygous lung tissue (Figure 2, B–D). No differences were observed in neutrophil immunoreactivity.

**WFD1C Affects Rate of Wound Closure**

In addition to reports implicating the WAP-family members, Slpi and ela in wound repair, we have published that WFD1C/ps20 is down-regulated during reactive stroma formation in the progression of prostate cancer, where reactive stroma exhibits hallmarks of a wound repair response.

Accordingly, the Wfdcl-null and heterozygous mice were evaluated for alteration in wound repair subsequent to 3-mm–diameter full-thickness skin wounds. Evaluating wound gaps over a 7-day skin wound healing assay, Wfdcl-null mice exhibited a significant increase in rate of wound closure from days 5 to 7. By day 6, the wound gap in Wfdcl-null mice had significantly decreased to 46% of the starting wound gap as compared with a decrease to 62% of the starting gap in Wfdcl-heterozygous mice (P < 0.05) (Figure 3A). By day 7, Wfdcl-null mice exhibited 28% wound gap as compared with 46% in Wfdcl-heterozygous mice (P < 0.05). All tissues were harvested at day 7 and processed for IHC. Evaluation of wound repair tissues stained with Masson’s trichome, to assess reactive stroma, exhibited significant differences (Figure 3B). Measurements from microscopic evaluation of transverse sections showed that Wfdcl-null mice averaged a 1086-μm wound gap as compared with a 1400-μm wound gap for Wfdcl-heterogeneous mice (P < 0.05) (Figure 3B). IHC followed by deconvolution microscopy and quantitative image analysis showed a significant elevation and considerably more heterogeneity in macrophage recruitment in the Wfdcl-null mice (587 median OD value) as compared with the Wfdcl-heterozygous mice (200 median OD value) (P < 0.05) (Figure 3C). Similar to the lung infection experiments, no significant differences were observed in neutrophil infiltration. To determine the stage of wound repair, day 7 wounds were evaluated for Ki-67 staining by deconvolution microscopy and quantitative image analysis. During classical wound healing, cellular proliferation occurs during the second, or tissue formation, stage, whereas a decrease in proliferative cells occurs during the third, or tissue regeneration, stage.

A significant decrease in Ki-67 staining intensity was observed with the Wfdcl-null mice (13,772 median OD value) as compared with Wfdcl-heterozygous mice (24,537 median OD value) (Figure 3D). The lower Ki-67 staining intensity in both the epithelium and transitional stroma at day 7 in the Wfdcl-null mouse is consistent with a more advanced wound closure as compared with heterozygous mice.

**Elevated Spp1 Osteopontin Expression in Wfdcl-Null Cells**

To assess mechanisms of WFD1C/ps20 action in mediating inflammatory responses, fibroblast cell lines were derived from Wfdcl-null and heterozygous mice. To evaluate differential inflammatory responses, the cell lines were treated with either LPS or poly I:C to simulate bacterial and viral infections, respectively. Gene expression profiling revealed an enhanced inflammatory gene expression signature (Supplemental Figure S3). Genes differentially up-regulated in Wfdcl-null cells included tumor necrosis factor-α (TNF-α), IL-1β, IL-12β, granulocytic macrophage colony stimulating factor 3 (GM/CSF3), and Cox 2 (prostaglandin E synthase, Pges). Of interest, expression of keratinocyte chemokine (KC) (murine homolog of IL-8) in response to both poly I:C and LPS was not induced nearly as high in Wfdcl-null cell lines (approximately 40-fold less) as compared to Wfdcl-heterozygous cell lines (Supplemental Figure S4). The FGFR1 gene did not show an increased expression in response to the inflammatory agents LPS and poly I:C.

We have reported previously that WFD1C/ps20 regulates differential cell piling and cell shape in prostate smooth muscle cell lines. In addition, elevated WFD1C/ps20 expression was associated with cell adhesion in spheroid formation in cultures of prostate stromal cells. Accordingly, we evaluated expression profiles of genes encoding cell adhesion proteins, both in the context of a proinflammatory stimulus and in the context of spheroid formation, to assess alterations associated with Wfdcl-null conditions. This
revealed that in Wfdc1-null conditions, Opn (Spp1) was the only adhesion-related gene that was up-regulated in both inflammatory conditions (poly I:C treatment) and during spheroid formation (hanging-drop cultures) (Figure 4A and B). Opn expression was up-regulated 62-fold higher in Wfdc1-null cells relative to Wfdc1-heterozygous cells after exposure to poly I:C (Figure 4A). Western blot analysis showed that Wfdc1-heterozygous cells secreted very little osteopontin, whereas Wfdc1-null fibroblasts secreted both more full-length and cleaved osteopontin protein, known to be the activated forms (Figure 4B). Osteopontin levels also correlated with the ability to form spheroids in hanging-drop cultures. Wfdc1-heterozygous cells formed typical spheroids in hanging-drop cultures (Figure 4C). However, Wfdc1-null cells were completely restricted from spheroid formation in hanging-drop cultures, were negative for nuclear reporter β-gal during spheroid culture conditions, and exhibited a 2.3-fold elevation in Opn message relative to Wfdc1-heterozygous cell spheroids (Figure 4C).

Owing to the elevated expression in Wfdc1-null cells, IHC for osteopontin was evaluated in the influenza A-infected lungs and in day 7 wound repair tissues to determine whether elevated osteopontin protein associated with the null phenotype. Consistent with the in vivo data, Wfdc1-null cells in vitro exhibited significantly elevated migration in wounding scratch assays in culture, as compared with Wfdc1-heterozygous cells (Figure 5A). At 9 hours, Wfdc1-null cells exhibited a significant increase in closure and had migrated an average of 506 μm as compared with an average of 244 μm with the heterozygous cell lines (P < 0.005). Wfdc1-null cells achieved near full closure by 16 hours (655-μm migration), whereas the Wfdc1-heterozygous lines displayed significantly less closure (516 μm) (P < 0.005) (Figure 5A). To assess whether the increased migration of Wfdc1-null cells was attributable to elevated osteopontin activity, neutralizing antibody was added to two different null cell lines (P1 and L4) subsequent to scratch wounding. Osteopontin neutralizing antibody inhibited wound closure in the P1 Wfdc1-null line relative to control at both 10 μg (358-μm migration)
and 20 μg (303-μm migration) concentrations relative to control conditions (458-μm migration) (Figure 5B) (P < 0.01). In the L4 cell line, osteopontin neutralizing antibody again significantly inhibited at 10 μg (257-μm migration) and 20 μg (179-μm migration) relative to control conditions (395-μm migration) (Figure 5B). No significant differences were found in cell proliferation of the Wfdc1-null cells as compared with the Wfdc1-heterozygous cells in vitro.

Together, these results suggest that Wfdc1-null cells exhibit elevated cell migration and that this was mediated by elevated osteopontin secreted by the Wfdc1-null cells.

Because wound repair migration is affected by differential cell adhesion and contraction, adhesion to several matrix proteins that are expressed at sites of wound repair was evaluated. Of these, the Wfdc1-null cell lines exhibited significantly increased adhesion to both fibronectin and laminin relative to Wfdc1-heterozygous cells (Figure 5C).

However, no changes were observed in cell adhesion to vitronectin, collagen I, or collagen IV.

Externalized MMP-9 Activity

Several studies have implicated MMP activity in osteopontin-mediated adhesion and migration.34-36 Moreover, MMP-9 has been shown to cleave osteopontin into fragments that promote cell migration and the metastatic potential of cancer cells.37,38 Hence, we evaluated whether MMP activity was altered in Wfdc1-null cells. Elevated levels of biologically active forms of MMP-9 were found in Wfdc1-null cell lines (312#1L1 and 312#1P1) as compared with Wfdc1-heterozygous cell lines (HetMP1, -3, and -5) as shown by gelatin zymography (Figure 6A). By contrast, the levels of MMP-2 were similar in all cell lines, irrespective of Wfdc1 status. Interestingly, the levels of active MMP-9 in Wfdc1-null cells in steady-state conditions were similar to...
levels in LPS-treated conditions, showing that Wfdc1-null cells not only exhibit a higher level of osteopontin, but also express constitutively active MMP-9 as compared with Wfdc1-heterozygous cell lines. Although activation levels were different, there appeared to be similar levels of total protein irrespective of Wfdc1 status. IHC analysis of MMP-9 in infected lung tissue showed that immunoreactivity was approximately the same in the wild-type, Wfdc1-heterozygous, and Wfdc1-null influenza-infected lung tissue (Figure 6B), suggesting the level of total protein expression does not differ in the infected lung tissue. These data suggest that MMP-9 protein is constitutively active in Wfdc1-null conditions even though total protein is similar to Wfdc1-heterozygous conditions. These data may explain the elevated levels of cleaved (activated) osteopontin observed in Wfdc1-null conditions, because MMP-9 is known to cleave and produce osteopontin fragments that promote cell migration/invasion.

Discussion

Interestingly, knockout of Wfdc1 resulted in no overt developmental or adult tissue phenotype, similar to what has been reported for other WAP family members. However, differential biological responses were evident during challenges to homeostasis in adult tissues. Importantly, Wfdc1-null mice exhibited a significant elevation in resistance to influenza A virus infection and a more rapid cutaneous wound repair response. Both these responses were associated with elevated recruitment of macrophages and elevated expression of osteopontin. Moreover, Wfdc1-null cells exhibited elevated secretion of constitutively active MMP-9, an enzyme that is important in osteopontin processing to active fragments among other biological activities. Consistent with this notion, elevated expression of both full-length and cleaved osteopontin was observed in the Wfdc1-null condition. The cleaved osteopontin fragments have been associated with elevated migration and metastasis in cancer. Wfdc1-null cells in vitro also exhibited altered adhesion and rapid closure in scratch assays, consistent with in vivo results. Together, these results suggest that Wfdc1 is not likely involved in embryological development or adult normal steady-state biology; however, it is a key repressor and modulator of critical aspects of response mechanisms that operate during disrupted tissue homeostasis. As such, Wfdc1 may be one of the genes best described as being associated with modulating emergency biology. Consistent with this suggestion, osteopontin has also been shown to be associated with a wide variety of emergency biology responses.

Osteopontin is a matricellular protein expressed by a number of cell types, including macrophages, smooth muscle, endothelial cells, and retinal Müller cells. In addition, osteopontin is important in wound repair, fibrosis resolution, and host responses to microbial infection.
a potent chemokine in the recruitment of macrophages during inflammatory reactions and in modulating cell migration/invasion. The biological responses noted in Wfdc1-homozygous null conditions are fully consistent with elevated osteopontin activity, including altered cell migration, adhesion, spheroid formation in vitro, and recruitment of macrophages. This was particularly evident in the day 6 and day 7 wound repair study, where the timeline of more rapid closure was congruent with elevated osteopontin and macrophage recruitment. Osteopontin has not been previously associated with WAP family members. Considerable heterogeneity was observed in the elevated macrophage recruitment in the Wfdc1-null cells at the blood–brain barrier, endothelial cells at the blood–testis barrier, skin epidermal cells, prostate epithelium, retina layers, and smooth muscle in the bladder. Corroborating the brain endothelial–specific expression, another study identified Wfdc1 as both a blood–brain barrier and endothelial-enriched transcript relative to peripheral endothelial cells and pericytes. The expression in the retinal inner nuclear layer is consistent with an additional study that showed that Wfdc1 is in the top 11% of genes that are expressed in the inner nuclear layer–specific Müller cells. In addition, localization of WFDCl/p20 changed during retinal development in Chx10-null mice, where Chx10 has been shown to be a retinal progenitor cell and Müller cell–specific gene. Together, these data suggest that WFDCl/p20 is likely an important regulator of retina biology, although we did not observe vision-related phenotypes associated with Wfdc1-null conditions.

WFDCl is unique among the human WAP family members. WFDCl is not located in chromosome 20q13, which contains 19 genes with either a WAP or Kunitz-type domain in a region shown to undergo strong genetic selective pressure. Most of the 20q13 WAP genes, such as SLPI and PI3 (SKALP, elafin), exhibit antimicrobial activity and antiprotease activity. Most of the 20q13 WAP genes are up-regulated following changes in tissue

**Figure 6** Increased gelatinase activity in untreated Wfdc1-null cells. A: Three Wfdc1-heterozygous (Het) stromal cell lines express MMP-2 at nearly similar levels when untreated or treated with lipopolysaccharide (LPS), whereas Wfdc1-null stromal cell lines also express increased MMP-9 activity in both untreated and LPS-treated conditions. B: IHC for MMP-9 (red) in Wfdc1 wild-type, heterozygous, and null influenza–infected lungs shows similar staining intensity and localization of staining in vessels (asterisks), bronchioles (double daggers), and macrophages (arrows). Original magnification, ×200 (B).
homeostasis and appear to attenuate the innate host response. 58–60 By contrast, WFDC1/ps20 is down-regulated in cancer, specifically in the stroma, 8,51,61–64 and stimulates response mechanisms. WFDC1/ps20 was down-regulated in two models of in vivo activation of hepatic stellate cells, which are cells most responsible for liver fibrosis. 65 We have now shown that Wfdc1-null mice exhibited augmented tissue repair/inflammatory responses during both influenza infection and skin wound repair. It could be surmised that down-regulated expression or activity of WFDC1/ps20 is required in barrier cells to signal-activated tissue responses leading to recruitment of infiltrating cells. In addition to expressing more proinflammatory chemokines, like osteopontin, these cells also express other WAP family members such as SLPI and P13. Wfdc1-null cells also expressed an increased inflammatory gene profile, however with a conspicuous lack of KC overexpression. The other WAP family members that have been studied seem to regulate IκB degradation and subsequent NF-κB signaling. 66 The lack of a full proinflammatory gene profile argues against WFDC1/ps20 regulating the NF-κB signaling pathway in the same way as the other WAP family members, although this requires further study.

The present report suggests that WFDC1/ps20 is a fundamental regulator of diverse inflammatory and wound repair responses. Elevated or basal levels of WFDC1/ps20 would be expected to sustain adult differentiated biology, whereas down-regulated WFDC1/ps20 expression or activity would induce a permissive switch to a repair/inflammatory biology that is required to clear microorganisms, and to affect a repair response typified by elevated stromal cell migration and matrix remodeling. Homeostasis after the repair might then be associated with a resumed expression or activity of WFDC1/ps20 and a return to an inflammatory-quiescent, adult differentiated biology state. Hence, determining the mechanisms that regulate Wfdc1 expression and functions will help further elucidate how this protein and the downstream biology affects core responses and modulation of tissue homeostasis. These mechanisms will be important to the design of novel therapeutic approaches to treating microbial disorders, inflammatory responses, and altered wound repair responses.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.07.013.

References


