A New Role for the Muscle Repair Protein Dysferlin in Endothelial Cell Adhesion and Angiogenesis

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Objective—Ferlins are known to regulate plasma membrane repair in muscle cells and are linked to muscular dystrophy and cardiomyopathy. Recently, using proteomic analysis of caveolae/lipid rafts, we reported that endothelial cells (EC) express myoferlin and that it regulates membrane expression of vascular endothelial growth factor receptor 2 (VEGFR-2). The goal of this study was to document the presence of other ferlins in EC.

Methods and Results—EC expressed another ferlin, dysferlin, and that in contrast to myoferlin, it did not regulate VEGFR-2 expression levels or downstream signaling (nitric oxide and Erk1/2 phosphorylation). Instead, loss of dysferlin in subconfluent EC resulted in deficient adhesion followed by growth arrest, an effect not observed in confluent EC. In vivo, dysferlin was also detected in intact and diseased blood vessels of rodent and human origin, and angiogenic challenge of dysferlin-null mice resulted in impaired angiogenic response compared with control mice. Mechanistically, loss of dysferlin in cultured EC caused polyubiquitination and proteasomal degradation of platelet endothelial cellular adhesion molecule-1 (PECAM-1/CD31), an adhesion molecule essential for angiogenesis. In addition, adenovirus-mediated gene transfer of PECAM-1 rescued the abnormal adhesion of EC caused by dysferlin gene silencing.


Key Words: adhesion molecules ■ angiogenesis ■ endothelium ■ growth factors ■ nitric oxide ■ vascular biology

Dysferlin and myoferlin are members of the ferlin family of proteins. The name ferlin is derived from FER-1, a protein required for the correct fusion of specialized membranous organelles with the plasma membrane of sperm during spermiogenesis in Caenorhabditis elegans. In mammalian cells, dysferlin was the first ferlin shown to regulate membrane fusion events at the plasma membrane of skeletal muscle cells. Akin to the application of patches at sites of damage, endovesicle fusion occurs at the sarcomere of skeletal muscle following physical injury; the triggering effect of unregulated extracellular calcium (Ca\(^{2+}\)) entry into cells is believed to activate the Ca\(^{2+}\)-binding C2 domains of ferlins and lead to specific interactions with membrane phospholipids and fusion of membrane vesicles. Interestingly, a growing number of peripheral functions were subsequently attributed to the presence of ferlins in various settings; changes in dysferlin activity or expression have been reported in preeclampsia, cardiomopathy, Alzheimer disease and multiple sclerosis, whereas myoferlin, another ferlin family member, plays a role in the pathogenesis of both muscular dystrophy and cardiomyopathy. Another salient example of growing functions for ferlins is otoferlin, which is linked to a recessive form of deafness in humans and mice.

Recently, we documented the unexpected expression of myoferlin in cultured vascular endothelial cells (EC) and intact blood vessels though proteomics identification of caveolae and lipid raft–resistant proteins. Loss of myoferlin results in attenuation of proliferation, migration, and nitric oxide (NO) release following vascular endothelial growth factor (VEGF) challenge, and this coincides with a near-complete loss of surface expression of VEGF receptor-2 (VEGFR-2) due to increased polyubiquitination and degradation. This, combined with our recent characterization of myoferlin in EC endocytosis raises the possibility that the involvement of ferlins in nonmuscle systems may reside in their as yet poorly described ability to regulate both vesicle fusion and client protein trafficking as cargos of putative membrane-bound patches.

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In this study, we found that cultured EC also expressed dysferlin and that in stark contrast to myoferlin, dysferlin did not participate in VEGFR-2 expression. Instead, we show that dysferlin gene silencing caused near-complete inhibition of proliferation in subconfluent EC due to EC detachment from their growth surface, and most intriguingly, confluence or sufficient cell-cell contact could provide complete protection against such adhesion defect. Dysferlin protein expression could be detected in the aorta and the mesenteric and coronary arteries of rodent or human origins, and agonist-induced angiogenic challenge of dysferlin-null mice resulted in deficient angiogenesis, supporting an active role for dysferlin in endothelial homeostasis in vivo and in vitro. Mechanistically, we show that the loss of dysferlin in subconfluent cells caused mislocalization followed by polyubiquitination and proteasomal degradation of platelet endothelial cellular adhesion molecule (PECAM)-1, a transmembrane protein essential for angiogenesis. Furthermore, the adhesion defect caused by dysferlin silencing could be rescued by adenoviral overexpression of PECAM-1. Together, these data identify a novel pathway for PECAM-1 regulation and confirm that dysferlin participates in vascular homeostasis. We also propose that ferlins are profoundly heterogeneous in their capacity to regulate different membrane remodeling events, and this is likely attributable to the fusion of membrane vesicles containing unique protein cargo.

Materials and Methods

Cell Culture
Native bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) were isolated from bovine aorta and purchased from Lonza (Basel, Switzerland), respectively. Cells were grown in high-glucose Dulbecco’s modified Eagle’s medium or M199 medium (Invitrogen) supplemented with FBS (HyClone, South Logan, Utah), L-glutamine, endothelial cell growth supplement (ECGS), and penicillin-streptomycin (Sigma, St. Louis, Mo). Full-length PECAM-1-coding adenovirus was amplified and used as previously described. Transwells (0.4 μm diameter or M199 medium) were from Corning.

RNA Isolation, RT-PCR, and Northern Blot Analysis
Total RNA was isolated from confluent cells with a RNA extraction kit (Qiagen). cDNA synthesis was performed using dT oligos (Superscript, Invitrogen). Polymerase chain reaction was performed using the following degenerated bovine-human dysferlin-specific polymerase chain reaction oligos: 5'-GGAAACGAGTCTGCGGGAAGC-3' and 5'-TGTGGAGGAGGAGCAGGAG-3'. Northern blot analysis was performed using a 269-bp human dysferlin probe synthesized with the 5'-GGAAACGAGTCTGCGGGAAGC-3' and 5'-GTCCAGCAGAGTCCGCGGAGG-3' oligos with high homology to bovine and human dysferlin cDNA.

Plasmids, Cell Transfection, and Visualization
BAEC were transfected with 2 μg of a β-galactosidase (β-Gal), hemagglutinin (HA)-ubiquitin (a kind gift from Dr. Alex Toker; Beth Israel Deaconess Medical Centre), or a previously characterized green fluorescent protein (GFP)–dysferlin-fused plasmid (N terminus–tagged; kindly provided by Dr. Kate Bushby; Newcastle University) for 6 hours with Opti-MEM I and Lipofectamine 2000 and visualized by using a Leica AOB5 confocal microscope. Confocal z-stack images were acquired at 1-μm intervals for 15 μm in accordance with the Nyquist criterion (Volocity software).

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Western Blot Analysis, Immunoprecipitation, Immunofluorescence, and Cholesterol-Enriched Microdomain/Lipid Raft Isolation
The antibodies used for Western blot (WB) are β-cotamer protein (β-COP, rabbit, ABR), phospho- and total ERK-1/2 (rabbit and mouse, Cell Signaling Technology), VEGFR-2 and Cav-1 (rabbit, Santa Cruz Biotechnology), HSP90 (mouse, Becton Dickinson), dysferlin Hamlet 1 and 2 (mouse, Novocasta), PECAM-1 (mouse and rabbit, Santa Cruz Biotechnology), PECAM-1 and VE-cadherin (Cell Signaling Technology), and HA (rat, Roche). Immunoprecipitation (IP) was performed with protein-G-coated beads (Sigma). For cell immunofluorescence, PECAM-1 antibody was from Cell Signaling Technology and used on paraformaldehyde-fixed HUVEC as described. Preparation of cholesterol-enriched microdomains/lipid rafts (CEM/LR) was performed as described. Short Interfering RNA Treatment
Bovine dysferlin cDNA target sequences (Dharmacon) were 5'-3': NNGGAAAGAAAUCGGUGAUGA (Dysf1), NNGGCAAGAGCGCAGGACUU (Dysf2), and their respective scrambled nonsilencing controls NNAUGAGGACUGGGAAACG (NS1) and AAGGCCCGGCCCCAUAUC (NS2). Human dysferlin DNA target sequences were synthesized with the 4-for-silencing program (Xeron/Qiagen) and target the following DNA sequences: Dysf1, CAGCGTGAACCTGTATGGAA; Dysf2, CAGATTTCTCCTGATATTGCA; Dys3, CTTGGTGCATCATTGACACA; Dys4, CTCCCTTGGCGCCCTCTTA. Proliferation, Viability, Apoptosis Assays, NO Release Measurement, and Phosphorylation Assay
BAEC treatment for proliferation and survival assays and for NO chemiluminescence were performed as described. Ear and In Vitro Angiogenesis Assay
Animal experiments were performed in accordance with the University of British Columbia Animal Care Committee. Adenoviruses encoding murine VEGF-A164 (approximately 7 × 10^5 viral particles in 15 μL) were injected into the right ear of dysferlin-null mice or age and sex-matched wild-type (WT) mice (JAX, Bar Harbor, Maine). The left ear of mice was injected with a β-Gal virus. After 6 days, animals were euthanized, and ears were isolated, cut longitudinally in two, and embedded in O.C.T. medium to obtain full-length sections of the central ear area. For in vitro angiogenesis assays, rat tail collagen gel (Becton Dickinson) was prepared by mixing 7 volumes of collagen I solution (final concentration, 2 mg/mL in 0.02 N acetic acid) with 1 volume of 10× MEM and 2 volumes of NaHCO3 (1.17 mg/mL final). A 200-μL cell-free layer of collagen mixture was allowed to gel, followed by addition of a 400-μL top layer of collagen mixture containing 5,000 HUVEC treated with short interfering RNA (siRNA). Cells were then grown by adding a third layer of M199 medium containing 20% FBS. Tube-like structures and cell numbers were visually counted. Non-embedded HUVEC time 0 controls treated with siRNA showed greater than 90% viability.

Immunohistochemistry
Human coronary artery specimens were obtained from the James Hogg icAPTURE Biobank tissue collection. Vascular tissues fixed and subjected to antigen retrieval (20 minutes, 120°C, 30 psi) in citrate (Dako, Mississauga, Ontario, Canada), quenched, blocked in 10% normal rabbit serum, incubated with anti-dysferlin goat antibodies (Santa Cruz Biotechnology), and processed using the Vectastain biotin-avidin detection kit (Vector Laboratories) and NovaRED HP substrate reagent (Vector Laboratories). Image digitization of positive and negative (IgG) conditions was done at the same time using an Aperio ScanScope digital slide scanner. For ears, frozen sections (5 μm thick) were immunostained with rat monoclonal anti-mouse PECAM-1 or VE-cadherin primary antibodies (BD Biosciences) and Cy3-conjugated secondary antibodies. Posi
before reverse transcription caused loss of amplification (lanes labeled RNAse), thereby confirming mRNA dependence. Using a dysferlin cDNA probe and BAEC and HUVEC mRNA (Figure 1B, left), Northern blot analysis revealed the presence of a 7.5- to 9.9-kbp transcript (arrow, right). WB using BAEC or HUVEC protein lysates confirmed dysferlin protein expression with both dysferlin antisera (Figure 1C, 250-kDa marker). Skeletal muscle protein and β-COP were used as positive and loading controls, respectively.

To characterize dysferlin subcellular localization, we used a GFP-tagged version of dysferlin shown to behave similarly to endogenous dysferlin.17 GFP-positive signal was detected throughout live (unfixed) BAEC transiently expressing GFP-dysferlin (Figure 1D; gradient color plane image). Immunofluorescence allowed the identification of nuclear and Golgi structures (4′,6-diamidino-2-phenylindole and GM130, respectively; Supplemental Figure 1A), and high GFP-dysferlin expression was found in x-z-plane sectional views of the nucleus and Golgi apparatus (Figure 1D, x-z planes).

Finally, enrichment of dysferlin in specialized CEM/LR of the plasma membrane was determined by sucrose fractionation as previously described for myoferlin.13 Dysferlin was found to be enriched in BAEC and HUVEC light, cholesterol-rich CEM/LR fractions (Figure 1E); these fractions were also enriched in the caveolae protein Cav-1 and lacked Golgi/post-Golgi contaminants (β-COP, heavy fractions) and bulk plasma membrane markers such as HSP90.13 Similarly to myoferlin, cholesterol disruption using methyl-β-cyclodextrin increased dysferlin solubility in Triton X-100-based buffers (data not shown),13 an additional sign that dysferlin is a CEM/LR resident protein. Collectively, these data identify dysferlin as an EC protein enriched in cholesterol-rich microdomains.

**Loss of Dysferlin Does Not Impair VEGFR-2 Expression or Signaling in EC**

Because myoferlin was shown to regulate VEGFR-2 expression and signaling, gene silencing techniques were used to perform loss-of-function studies. Transfection of near confluent BAEC or HUVEC cultures with bovine (Dysf1, Dysf2) or human (Dysf1 to Dysf4) dysferlin siRNA (75 nmol/L; Dysf1 and Dysf2 shown) caused decreases of up to 52%, 68%, and 81% (BAEC) and 62%, 82%, and 94% (HUVEC) in dysferlin protein expression at 24, 48, and 72 hours, respectively, compared with 2 scrambled nonsilencing siRNA (NS1, NS2) (Figure 2A and 2B; 72 hours shown). Surprisingly, IP and WB analysis revealed that Dysf1 and Dysf2 (72 hours) did not cause downregulation of VEGFR-2 and Tie-2 (another angiogenic tyrosine kinase receptor) (Figure 2A and 2B) or VEGF/VEGFR-2-induced NO release and Erk1/2 phosphorylation (Supplemental Figure 1B and 1C). Together, these data indicate that dysferlin gene silencing does not decrease VEGFR-2 expression or downstream signaling and document specific cargo proteins for dysferlin versus myoferlin-dependent trafficking events.

**Figure 1.** Cultured EC express dysferlin. A, Detection of a 1.5-kbp dysferlin-specific amplicon by RT-PCR with primary BAEC or HUVEC mRNA as a template. Amplification was sensitive to RNase treatment before reverse transcription. B, Northern blot analysis using BAEC and HUVEC total mRNA (left) showed positive dysferlin mRNA expression (arrow). C, WB analysis of dysferlin protein expression in HUVEC, BAEC, and mouse skeletal muscle lysates using 2 antisera (dysferlin antibodies 1 and 2 [Dysf Ab #1 and 2]). Loading control was β-COP. D, Confocal optical section (x-y plane) and sectional views (x-z plane of nuclei and Golgi) showing GFP-dysferlin expression in live BAEC. Color gradient is shown to illustrate GFP signal intensity levels. Scale bar = 10 μm. E, Enrichment of dysferlin in CEM/LR (light fractions) from HUVEC and BAEC lysates. Blotting against HSP90 and β-COP was performed to show lack of bulk and Golgi apparatus proteins in CEM/LR.
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Figure 2. Dysferlin gene silencing causes loss of proliferation through impaired adhesion in subconfluent but not confluent EC. A and B, Downregulation of dysferlin (Dysf) in near-confluent BAEC or HUVEC did not decrease VEGFR-2 or Tie-2 expression. Cells were treated with bovine or human-specific dysferlin (Dysf1 and Dysf2) or scrambled nonsilencing siRNA (NS1 and NS2). WB analysis against dysferlin (top) and VEGFR-2 or Tie-2 following IP (bottom) was performed. C, Dysferlin gene silencing decreases BAEC proliferation. BAEC were seeded (approximately 5% confluence), transfected with siRNA sequences, and starved in 0.1% FBS, and the average length of tube-like structures per area (length mm/mm²) and proliferation (HUVEC/mm²) were quantified at 24 and 48 hours postembedding. Typical data are shown, presented as mean ± SEM.

Dysferlin Regulates Proliferation and Adhesion of Subconfluent EC

To determine whether dysferlin regulates basic EC functions, such as proliferation, subconfluent BAEC (covering less than 5% of growth surface) were treated with siRNA sequences (6 hours; Day -1), starved (0.1% FBS) to induce G₀ synchronization and incubated in 10% FBS-containing medium, inducing a time-dependent increase in proliferation of control BAEC (Figure 2C, black bars, right), but dysferlin siRNA–treated cells (white bars) showed lower counts than those at day 0. This suggested that decreased proliferation is not the primary outcome of dysferlin knockdown but rather a secondary consequence of another cellular defect.

To test whether loss of dysferlin causes necrosis or apoptosis, Trypan blue exclusion and Caspase-8 activity were determined in total BAEC (adherent and nonadherent) grown under high-FBS conditions (similarly to Figure 2C). Forty-eight hours after siRNA treatment, no difference was observed between the dysferlin and nonsilencing siRNA-treated groups (Supplemental Figure IIA and IIB), suggesting that loss of dysferlin did not promote necrosis or apoptosis.

To determine whether the decreased proliferation observed in Figure 2C was a result of aberrant adhesion, the ratio of adhered versus total cells was established in BAEC grown under similar conditions. Twenty-four, 48, and 72 hours after dysferlin siRNA treatment, up to 28%, 70%, and 81% of BAEC and 25%, 34%, and 42% of HUVEC showed deficient adhesion (Figure 2D; Supplemental Figure IIC and IID; P<0.001 compared with nonsilencing siRNA). High FBS (10%) concentrations, which caused active BAEC growth, did not prevent the loss of adhesion (Figure 2D). In HUVEC, loss of adhesion peaked at 120 hours after siRNA treatment (68% deficiency; data not shown). Incubation of dysferlin siRNA–treated BAEC in normal BAEC-conditioned medium or cocultured with untreated confluent BAEC grown on Transwells, which allowed the release of soluble factors, did not rescue the adhesion defect (data not shown), arguing against aberrant soluble cell signaling as a result of dysferlin silencing. Moreover, coating of plates with gelatin or polyarginine to compensate for changes in growth matrix requirements did not improve adhesion either (data not shown).

In contrast, BAEC seeded at higher or near-confluence levels improved or completely rescued adhesion after dysferlin siRNA treatment, respectively (Supplemental Figure IIE), indicating that increased cell-cell contact likely rescues adhesion to the growth surface. Readhesion assays provided evidence that confluent (protected) cells transfected with dysferlin siRNA exhibited impaired adhesion when trypsinized and reseeded 24 and 48 hours posttransfection at low confluence levels (Supplemental Figure IIF), which suggests that the protective effect of cell-cell contact is only transient. As expected, proliferation assays performed with cells seeded at approximately 50% confluence, which allows significant cell-to-cell contact, following dysferlin siRNA treatment induced proliferation rates similar to those of the control siRNA-treated group (10% FBS; Supplemental Figure IIIA). Hence, these data depict a role for dysferlin in regulating the adhesion machinery and mitogenesis in subconfluent EC.

Angiogenesis is known to rely on the partial detachment of EC from their basal membrane followed by migration and proliferation to form new vessels. To assess the effect of dysferlin silencing on angiogenesis in vitro, HUVEC were treated with dysferlin siRNA sequences and embedded in collagen I gel. As depicted in Figure 2E, loss of dysferlin caused a decrease in tube-like structure formation compared with complete (>85%; 72 hours) inhibition of proliferation (P<0.001). Incubation of BAEC in starvation medium caused minimal proliferation of control cells (Figure 2C, black bars, right), but dysferlin siRNA–treated cells (white bars) showed lower counts than those at day 0. This suggested that decreased proliferation is not the primary outcome of dysferlin knockdown but rather a secondary consequence of another cellular defect.
Dysferlin-Null Mice Show Impaired Agonist-Induced Angiogenesis

The high expression of dysferlin in blood vessels and its role in EC biology suggest that dysferlin might influence vascular homeostasis in vivo, such as angiogenesis. To directly test this hypothesis, adenoviruses coding for murine VEGF<sub>164</sub> (AdVEGF), an endothelial-specific angiogenic agonist, or β-Gal (Adβ-Gal) were intradermally injected into the ears of dysferlin-deficient mice (Dysf<sup>−/−</sup>) and WT controls. Six days postinjection, visualization and quantification (Figure 4A) of EC-positive structures by anti-VE-cadherin or PECAM-1 immunochemistry (red channel, PECAM-1 shown) documented a 2.4-fold increase (Figure 4A, *P<0.05, **P<0.01) in total blood vessel count induced by AdVEGF in WT mice (n=7), whereas in Dysf<sup>−/−</sup> mice, the increase was only 0.8-fold and statistically different from WT mice (Figure 4A, +P<0.05) (Figure 4A), indicating decreased angiogenesis in dysferlin-null mice. However, VEGF- and β-Gal-induced edema formation, quantified by measuring the thickness of H&E-stained ears sections as an indication of VEGF/VEGFR-2 activity, was found to be similar between WT and Dysf<sup>−/−</sup> mice (Figure 4B). Moreover, quantification of VEGFR-2 expression in EC-rich lung extracts (Figure C) was also similar. These data indicate that genetic loss of dysferlin leads to blunted neovascularization without causing autonomous defects in EC or decreased VEGF/VEGFR-2 signaling.

Dysferlin Deficiency Causes PECAM-1 Downregulation in Subconfluent Cells

VE-cadherin and PECAM-1 are the 2 adhesion molecules that undergo one of the most significant relocalizations in subconfluent versus confluent EC, and we hypothesized that they could be linked to the adhesion defect that we observed in subconfluent EC. Dysferlin knockdown (24 hours) had no effect on VE-cadherin (110 to 130 kDa) levels (Figure 5A), whereas PECAM-1 expression (110 to 125 kDa) was decreased by up to 92% in subconfluent BAEC (25% confluence) but only by up to 19% in confluent BAEC. In subconfluent HUVEC (25% to 50% confluent), PECAM-1 levels were reduced by up to 60% (140 kDa; marker, 150 kDa) following dysferlin siRNA treatment (Figure 5B). PECAM-1 staining in HUVEC was characterized as numerous punctas throughout the cytoplasm, with a higher density around the perinuclear region (Figure 5B, red channel, white arrows), whereas PECAM-1 detection was weaker and less diffused following a 24-hour dysferlin siRNA treatment in the remaining adhered cells (right). In approximately 60% confluent (mostly protected) cells, the majority of PECAM-1 localized at cell junctions, covering 82% of cell-cell contact zones with “thick” PECAM-1-positive structures (Figure 5B, bottom left and inset), whereas dysferlin silencing caused a slight decrease in PECAM-1 staining at cell junctions, with 73% of cell-cell contact areas stained positive for slightly “thinner” PECAM-1-positive structures (Figure 5B, bottom right). Confluent HUVEC treated with dysferlin or nonsilencing siRNA showed similar PECAM-1 staining almost exclusively at cell-cell junctions (data not shown). Hence, loss of PECAM-1 is a likely candidate to

direct comparison. Arrows indicate endothelial staining.

Figure 3. Dysferlin is expressed in blood vessels. A, Specificity of dysferlin staining was confirmed by positive and negative dysferlin detection in WT and dysferlin-null mouse aorta paraffin sections, respectively. Scale bar, 20 μm. KO indicates knockout. B to D, Shown are 5-μm-thick adjacent sections of a human coronary artery with age-related hyperplasia (B; scale bar, 100 μm), rat aorta (C; scale bar, 25 μm), and mouse superficial femoral artery (D; scale bar, 20 μm) stained with 1 of 2 dysferlin antiserum (left image, brown color) or a nonimmune IgG (right). A indicates adventitia; M, media; NI, neointima. Countertstain was performed with hematoxylin (blue). All immunostaining and image processing were performed in parallel, allowing direct comparison. Arrows indicate endothelial staining.

control siRNA treatment (top panels, 24 and 48 hours), as well as shorter tube-like structures and decreased proliferation (bottom left and right) under high-FBS conditions, an indication of blunted angiogenesis in vitro following dysferlin gene silencing.

Dysferlin Is Expressed in Human and Rodent Blood Vessels

Because dysferlin is stably expressed in cultured EC, immunohistochemistry was performed to characterize its expression in vascular tissues by using 2 different dysferlin antisera (C19 and E20 goat; C19 shown; Figure 3A to 3D). Antibody specificity was confirmed using paraffin-embedded sections of WT and dysferlin-null mice aortas. Dysferlin (brown staining) was highly detected in the endothelial layer (intima, arrows), adventitia (including the extracellular material), and to a lesser extent the medial smooth muscle cells of WT vessels, with little staining in dysferlin-null aorta (Figure 3A). Dysferlin was also detected in human coronary arteries with age-related hyperplasia (Figure 3B, neointima), intact rat aorta (Figure 3C) and smaller vessels, such as the mouse superficial femoral artery (Figure 3D). Cell nuclei were stained in blue. Staining of adjacent sections with isotype-matched IgG antisera (right) produced little staining, supporting dysferlin expression in vivo.
rationalize the adhesion deficiency observed following dysferlin knockdown in subconfluent BAEC.

To confirm that loss of PECAM-1 causes the adhesion defect in subconfluent EC following dysferlin knockdown, PECAM-1 was overexpressed using an adenovirus encoding for mouse PECAM-1 before dysferlin gene silencing. Infection with AdPECAM-1 (multiplicity of infection, 50) caused a 3-fold increase in total PECAM-1 expression compared with Adβ-Gal-treated cells (Supplemental Figure IIIB) and rescued by 59% and 68% the adhesion defect in BAEC and HUVEC, respectively (Figure 5C and 5D, white circles versus white squares), thereby confirming PECAM-1 as the main, but likely not exclusive, deficient gene product causing cell detachment following dysferlin gene silencing.

Dysferlin Forms a Complex With PECAM-1, Which Prevents Its Polyubiquitination and Degradation

In an attempt to show that dysferlin downregulation causes PECAM-1 protein degradation, EC were transfected with a HA-ubiquitin plasmid and treated with dysferlin siRNA, and PECAM-1 polyubiquitination was visualized by PECAM-1 IP and anti-HA blotting. An acute (16-hour) treatment with dysferlin siRNA (Dysf2) in 25% confluent BAEC and HUVEC caused decreases of 49% and 55% in PECAM-1 expression compared with matching control siRNA (NS2) (Figure 5E) and induced a drastic increase in PECAM-1 HA-ubiquitination. Inhibition of protein degradation with proteasome inhibitor MG132 (6-hour pretreatment) partially rescued (68%) loss of PECAM-1 expression following a 24-hour acute treatment with bovine dysferlin siRNA (Figure 5F). Control HUVEC quickly showed signs of toxicity to MG132 (2 hours) and were not tested for PECAM-1 degradation. Dysferlin IP from similarly treated HUVEC cells resulted in a drastically greater coimmunoprecipitation of PECAM-1 than a control IgG IP (Figure 5G). Moreover, robust colocalization was observed between GFP-dysferlin (green) and PECAM-1 (red) around the Golgi apparatus and numerous cytoplasmic punctas in fixed nonconfluent HUVEC (Figure 5H, inset). Together, these data lend credence to the hypothesis that dysferlin forms a complex with PECAM-1, which could participate in preventing PECAM-1 polyubiquitination and proteasome-dependent degradation.

Discussion

The current identification of dysferlin in multiple EC lines and vascular tissues confirms the growing occurrence of ferlins in nonmuscle cells. Initially believed to repair the sarcolemma of skeletal muscle cells, the presence of ferlins has been documented to regulate biological activities in other tissues, and as such ferlins are now believed to play other roles besides relatively simple membrane patching events. We have documented its presence in multiple cellular compartments, such as CEM/LR, and in the media and adventitia of vessels, supporting the concept that dysferlin may participate in complex signaling events not only in EC but also smooth muscle cells and fibroblasts. The dysferlin protein sequence contains many predicted protein binding domains, as well as a nuclear localization signal, which further supports a role in cellular signalization.
The positive role of dysferlin in EC-driven new blood vessel formation was directly assessed in dysferlin-null mice with a relatively short (6-day) angiogenesis assay performed in conjunction with an EC-specific agonist (VEGF). VEGF is well known to elicit EC-derived capillary growth, as these spouting and newly formed vessels lack stabilizing pericytes/smooth muscle cells, are highly unstable and undergo vasooobliteration 2 weeks postinjection (data not shown), thereby limiting interferences of our observations with other cell types (smooth muscle cells, supportive tissues) and further stressing the importance of dysferlin to EC activity. This model produces no immune inflammation compared with other angiogenesis assays, such as Matrigel plugs, which eliminates potential interferences from inflammatory cells in the EC phenotype of dysferlin-null mice.

Dysferlin-Dependent Regulation of PECAM-1 Expression and Adhesion

PECAM-1 is highly expressed in the vasculature, with approximately 1 million copies reported on the surface of EC. Maintenance of the lateral localization of PECAM-1 at EC junctions requires significant cell-cell contact to allow encounters with another homophilic PECAM-1 molecule. Hence, PECAM-1’s unique spatial localization in confluent versus nonconfluent cells makes it a prime candidate to rationalize the adhesion defect observed in EC with little to no cell-cell contact following dysferlin gene silencing. Interestingly, it has been postulated that PECAM-1 might exert its effect at the EC membrane by regulating intracellular Ca\(^{2+}\) levels, which is in line with the Ca\(^{2+}\)-sensing nature of ferlins. A membrane network linked at intervals to the
junctional surface is believed to exist just below the plasma-lemma, and intracellular vesicle-like PECAM-1 stores are found in this compartment and constitutively recycle along EC borders, which supports the concept that such PECAM-1-containing vesicle trafficking is dysferlin- and Ca^2+ -regulated. Combined with evidence showing that PECAM-1 interacts with other adhesion molecules, such as αRII, β1,26, directly modulates EC adhesion and motility,27; and plays an important role in inside-out and outside-in signaling events in EC, which is reminiscent of ferlin activity, this lends credence to our observations linking low PECAM-1 expression to dysferlin silencing-induced adhesion deficiency.

**Dysferlin, PECAM-1, and Angiogenesis**

Unchallenged dysferlin or PECAM-1-null mice do not show obvious signs of vascular defects, although angiogenic re- sponse is blunted in PECAM-1-null mice.29,30 PECAM-1 is known to serve as a scaffold for various signaling molecules, such as SHIP, phospholipase C, and SHP-2, and appropriate PECAM-1 localization is essential to proper assembly and regulation of these signaling complexes.31 When the proven importance of PECAM-1 to angiogenesis is taken into the context of our data showing deficient adhesion, proliferation, and tube formation following dysferlin knockdown, this not only supports our findings describing deficient angiogenesis in mice lacking dysferlin but also suggests that more vascular defects are likely to be found in dysferlin-null mice. Angiogenesis requires EC proliferation, migration, and 3-dimensional assembly into tubular structures, and our data strongly suggest that these steps require dysferlin. It is of interest to note that signs of deficient inflammation in skeletal muscle of dysferlin-null mice have recently been reported.32 However, the pluriopicity of PECAM-1 signaling activities, along with the unknown intracellular binding partners of dysferlin complicate our understanding of the different sig- naling events that lead to aberrant versus normal adhesion. To add to the complexity of our data, most reports on PECAM-1 study confluent EC, i.e., with junctional PECAM-1 localization, rather than subconfluent EC.

**Ferlins in Protein Cargo Trafficking of EC**

The concept that ferlin membrane patches fusing at the plasma membrane contain cargo was initially proposed by data showing deficient neurotransmitter exocytosis in otoferlin-null mice.12 Synaptotagmins, which share structural similarities with dysferlin,3,33 are well-described trafficking proteins, and their C2 domains allow them to act as calcium sensors involved in neurotransmitter and hormone secretion in various cell type.34 Hence the documented function of C2 domain-containing proteins in the regulation of fusion events and cargo trafficking further support a role for dysferlin in cargo trafficking processes, and it is likely that PECAM-1 is a key but not exclusive protein cargo of dysferlin-regulated membrane patches. On the other hand, data supporting profound differences in protein trafficking between dysferlin and myoferlin-containing membrane vesicles in EC are normal VEGFR-2 expression and VEGFR-2-dependent edema in dysferlin-null mice, as well as intact VEGFR-2 expression and downstream signaling following dysferlin siRNA treat-

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**Disclosures**

None.

**References**


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Arpeeta Sharma, Carol Yu, Cleo Leung, Andy Trane, Marco Lau, Soraya Utokaparch, Furquan Shaheen, Nader Sheibani and Pascal Bernatchez

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Supplemental figure I: A) Confocal optical section (xy-plane) showing Golgi apparatus (GM130, red) and nuclei (DAPI, blue) by immuno-fluorescence in BAEC transiently transfected with a GFP-Dysferlin encoding plasmid. B-C) Dysferlin gene silencing does not decrease VEGF/VEGFR-2-induced NO release and ERK1/2. BAEC treated for 72h with siRNA sequences were stimulated with VEGF (10^{-9}M) and total nitrite accumulation was quantified by NO-specific chemiluminescence, whereas immuno-blotting was performed using total and phospho-specific ERK1/2 antibodies. Data are presented as mean +/- S.E.M. * P<0.001 compared to their respective controls.

Supplemental figure II: A,B) Loss of Dysferlin did not decrease cell viability. Sub-confluent BAEC were treated with siRNA sequences and viability of total cells (adhered and non-adhered) was assessed using Trypan blue or Caspase-8 (apoptosis) assays using a commercial kit at 48 h. Ceramide was used as a positive control. C) Representative images showing near-complete adhesion of BAEC in non-silencing siRNA-treated cells (top) and early signs of deficient adhesion in Dysferlin siRNA-treated cells (bottom) 24h post-treatment. D) Loss of Dysferlin caused rapid defect in HUVEC adhesion. Adherent vs total cell ratio was determined by collecting unadhered cells and trypsinizing adhered cells for quantification. N = 6 per group in duplicate. *P<0.001 compared to non-silencing control. E) Adhesion rate at 72 h in 0.1% FBS of Dysferlin siRNA-treated BAEC as function of their initial confluency (time 0). Arrowheads show greatest changes in adhesion properties. F) Successful re-adhesion of HUVEC at higher confluency levels following siRNA treatment. Confluent cells were treated with Dysferlin siRNA (time 0), trypsinized at 24 and 48 h, and reseeded at different confluency rates (5, 25, 50, 75, 90%). Re-adhered vs non-adhered cells were counted 24h later. There was a near 30% decrease in adhesion 24h post-transfection in cells re-seeded at below 50% confluency, whereas at 48h, deficient adhesion is observed below 75% confluency.
**Supplement Material**

**Supplemental figure III:** A) Dysferlin gene knock-down in BAEC under partial (approx. 50% confluency) cell contact conditions did not significantly decrease proliferation. BAEC were treated as in A with the exception that 40,000 cells per well were seeded. B) Adenoviral over-expression of PECAM-1 in BAEC following Dysferlin gene knock-down. Prior to Dysferlin siRNA treatment with Dysferlin siRNA #2, BAEC were pre-treated with PECAM-1 (50 MOI) or β-Gal adenoviruses for 24h. HSP90 was used as a loading control.
Supplement Material

A

BAEC-xy plane

GFP-Dysf  GM130  DAPI

B

Nitrite (pmol/10^6 BAEC)

Non-Silencing
Dysf Silencing

* * * *

NS1  NS2  Dysf1  Dysf2  NS1  NS2  Dysf1  Dysf2

VEGF

C

Time (min)

0  5  10  15  30  60

Erk1/2

pErk1/2

NS1  Dysf1  Dysf2

VEGF: - + - + - +

Dysf

Erk1/2

pErk1/2

HSP90

Supplemental Figure I
Supplemental Figure II

A. Total cell viability (%)

B. Caspase-8 activity (48h, %)

C. Adhered HUVEC (%)

D. Initial BAEC confluency (%)

E. Adhered HUVEC (%)

F. Re-adhered HUVEC (%)

Supplement Material
**Supplemental Figure III**

A

![Bar chart showing BAEC count (x10^3) over time for NS Ctrl and Dysf Sil conditions.]

- **Day 0:**
  - NS Ctrl: 100
  - Dysf Sil: 60

- **24h:**
  - NS Ctrl: 90
  - Dysf Sil: 70

- **48h:**
  - NS Ctrl: 80
  - Dysf Sil: 60

- **72h:**
  - NS Ctrl: 70
  - Dysf Sil: 50

B

- **IP:PECAM**
  - NS2: -
  - Dysf2: +

- **AdPECAM:**
  - NS2: -
  - Dysf2: +

- **Sup:**
  - NS2: -
  - Dysf2: +

- **PECAM**
  - NS2: -
  - Dysf2: +

- **HSP90**
  - NS2: -
  - Dysf2: +