2009-05-01

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Disease Models & Mechanisms 2, 267-274 (2009) doi:10.1242/dmm.002261

FSHD region gene 1 (*FRG1*) is crucial for angiogenesis linking FRG1 to facioscapulohumeral muscular dystrophy-associated vasculopathy

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SUMMARY

The genetic lesion that is diagnostic for facioscapulohumeral muscular dystrophy (FSHD) results in an epigenetic misregulation of gene expression, which ultimately leads to the disease pathology. *FRG1* (FSHD region gene 1) is a leading candidate for a gene whose misexpression might lead to FSHD. Because FSHD pathology is most prominent in the musculature, most research and therapy efforts focus on muscle cells. Previously, using Xenopus development as a model, we showed that altering *frg1* expression levels systemically leads to aberrant muscle development, illustrating the potential for aberrant FRG1 levels to disrupt the musculature. However, 50-75% of FSHD patients also exhibit retinal vasculopathy and FSHD muscles have increased levels of vascular- and endothelial-related *FRG1* transcripts, illustrating an underlying vascular component to the disease. To date, no FSHD candidate gene has been proposed to affect the vasculature. Here, we focus on a role for FRG1 expression in the vasculature. We found that endogenous *frg1* is expressed in both the developing and adult vasculature in Xenopus. Furthermore, expression of FRG1 was found to be essential for the development of the vasculature, as a knockdown of FRG1 resulted in decreased angiogenesis and reduced expression of the angiogenic regulator DAB2. Conversely, tadpoles subjected to *frg1* overexpression displayed the pro-angiogenic phenotypes of increased blood vessel branching and dilation of blood vessels, and developed edemas, suggesting that their circulation was disrupted. Thus, the systemic upregulation of the FRG1 protein shows the potential for acquiring a disrupted vascular phenotype, providing the first link between a FSHD candidate gene and the vascular component of FSHD pathology. Overall, in conjunction with our previous analysis, we show that *FRG1* overexpression is capable of disrupting both the musculature and vasculature, recapitulating the two most prominent features of FSHD.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant myopathy characterized by progressive atrophy of the facial, shoulder and upper arm muscles. In addition to the muscular dystrophy, 50-75% of FSHD patients have retinal vasculature abnormalities (Fitzsimons et al., 1987; Padberg et al., 1995b), and the upregulation of vasculature system genes is an early event in FSHD muscle pathology (Osborne et al., 2007). Recently, muscle biopsies from FSHD patients revealed that the mesoangioblasts, an adult myogenic mesodermal stem cell occupying the perivascular niche in muscle tissue, were impaired in their ability to differentiate into skeletal muscle (Morosetti et al., 2007). Thus, FSHD progression and pathology, although predominantly evident in skeletal muscle, probably have underlying contributing or causal factors that are associated with the vasculature.

The FSHD genetic lesion is a contraction of the tandem array of 3.3 kb D4Z4 repeats at chromosome 4q35 to below a threshold of 11 copies (Lunt et al., 1995; Wijmenga et al., 1992). This repeat contraction is hypothesized to affect the expression of neighboring gene(s). Based on analysis of gene expression and proximity to the D4Z4 repeats, *FRG1* (FSHD region gene 1), which is located 100 kb centromeric to the contracted D4Z4 region, is a leading candidate disease gene for FSHD (van Deutekom et al., 1996). Since FSHD prominently affects voluntary muscles, studies on FRG1 have mainly focused on its role in muscles. Previous work found that FRG1 expression is crucial to the formation of normal muscle structure and that overexpression of the FRG1 protein adversely affects muscles during development (Hanel et al., 2009). Similarly, transgenic mice with a 25–40-fold increase in FRG1 expression, specifically in the skeletal muscle, develop a dystrophic muscle phenotype (Gabellini et al., 2006). However, measurements of *FRG1* mRNA levels from the muscles of FSHD patients have varied, including a 25-fold increase in expression, unchanged expression and a 5-fold decrease in expression, when compared with control muscles (van Deutekom et al., 1996; Gabellini et al., 2002; Jiang et al., 2003; Winokur et al., 2003; Osborne et al., 2007). Thus, a correlation between *FRG1* mRNA levels in skeletal muscle and FSHD pathology remains inconclusive and controversial.

The protein encoded by the *FRG1* gene at chromosome 4q35 in humans is highly conserved in vertebrates and invertebrates [97% amino acid (a.a.) identity with the mouse, 81% a.a. identity with *X. laevis* and 46% a.a. identity with *C. elegans*] (Grewal et al., 1998). *FRG1* expression has been detected in all human tissues that have been tested, including human embryonic brain and muscle, as well as the placenta (van Deutekom et al., 1996), suggesting that it has functions outside of the muscle. Here, we show prominent expression of FRG1 in vascular tissues during Xenopus development and in adult frog muscle capillaries. Knockdown of FRG1 results in decreased angiogenesis and loss of the Xenopus vascular marker DAB2. Overexpression of the FRG1 protein resulted in an opposite phenotype of increased angiogenesis and dilated blood vessels. Identifying FRG1 as a mediator of

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angiogenesis provides insights into the mechanisms of FSHD disease pathology and further supports the FRG1 overexpression disease model for FSHD.

RESULTS

FRG1 is expressed in vascular tissues

Two of the prominent pathological effects in FSHD patients are the progressive appearance of dystrophic skeletal muscle and retinal vasculopathy. Previously, we analyzed FRG1 expression in *X. laevis* during early development of the musculature (Hanel et al., 2009). Here, we analyze the *X. laevis* vasculature to address any potential vascular role for FRG1. Embryos that were sectioned, either sagitally or transversely, and immunostained for FRG1 (Fig. 1A) show significant FRG1 expression within the pronephrous (Fig. 1B,C), posterior cardinal vein and arteries (Fig. 1C), indicating that FRG1 is in fact expressed in the vasculature. However, ubiquitous levels of *frg1* transcripts from maternal stores, which progressively decrease during development, potentially introduce an elevated non-tissue-specific background that is not indicative of de novo expression (Hanel et al., 2009).

A transgenic approach was used in order to determine the tissues in which active *frg1* expression was taking place. The proximal *frg1* promoter sequence from *X. tropicalis*, consisting of a 645 base pair (bp) upstream regulatory region and 636 bps of the transcribed 5′ untranslated region (UTR), was inserted upstream of EGFP (enhanced green fluorescent protein) into a transgene cassette that was flanked by chicken β-globin insulator [hypersensitive site 4 (HS4)] doublets (Fig. 1D) and used for transgenesis. This proximal promoter construct produced a consistent expression pattern in transgenic animals (61% of transgenic tadpoles, n=44), which was visualized by fluorescence (Fig. 1E), with 16% displaying an underlying identical pattern with additional non-uniform spotted expression and 23% showing variable small patches of expression. Overall, the predominant pattern of fluorescence that we observed suggested that promoter expression was heaviest within the eye, dorsal-anterior portion of the head, branchial arches, and at the dorsal and ventral portions of the somites. As EGFP is known for its stability, de novo transcript levels were more clearly examined by in situ hybridization using an *egfp* probe. When examined in this manner, *egfp* expression in transgenic embryos coincided largely with fluorescence at stage 32 (Fig. 1E,F). However, as the tadpoles developed, *egfp* expression became more confined to vascular and muscle tissues (Fig. 1G,H). By stage 42, when much of larval organogenesis is complete, transgenic *egfp* expression was predominantly vascular, with weak staining throughout the epaxial tail muscle and around the gut (Fig. 1H). Although potentially missing some regulatory elements, the ability of a 645 bp regulatory region of *frg1* to direct gene expression specifically within vascular tissues strongly supports a role for *frg1* in vascular development.

The expression of the FRG1 protein was characterized further after metamorphosis in cross sections of adult frog gastrocnemius muscle. FRG1 immunostaining showed strong expression in cells at the periphery of muscle fibers (Fig. 1I). This staining pattern shows that FRG1 co-localizes with the capillary marker lectin

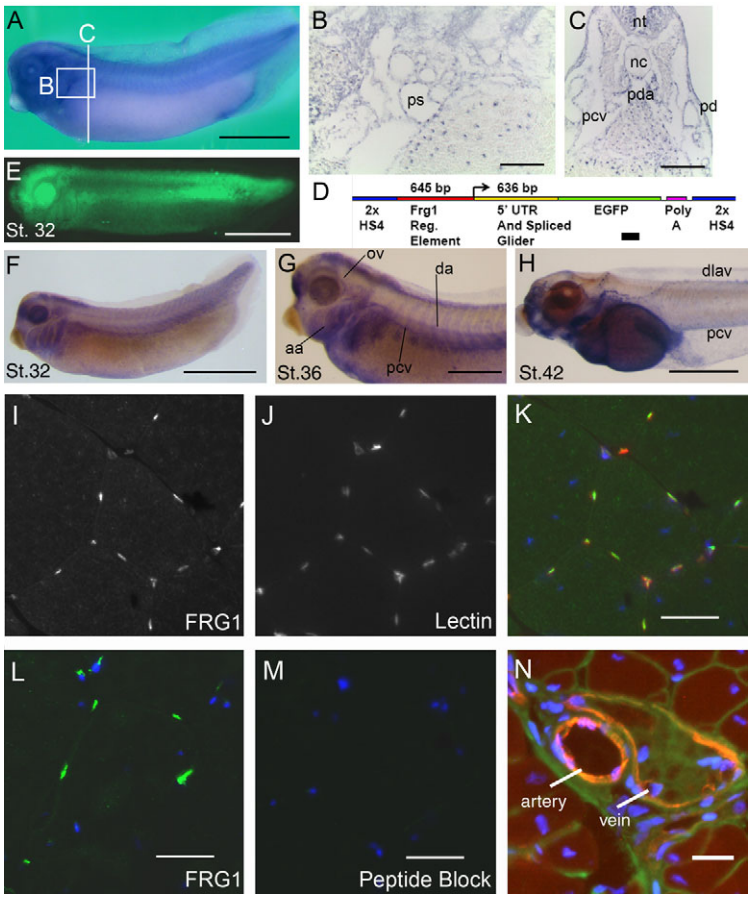

Fig. 1. Expression of the FRG1 protein during development. (A) Stage 36 whole-mount immunostaining shows the ubiquitous appearance of the FRG1 protein. FRG1-immunostained tadpoles were then sectioned either sagitally (B) or transversally (C) in the regions depicted in A. (D) Diagram of the minimal *frg1* regulatory element (FRE) construct that was used to examine tissues with active transcription. EGFP expression was observed in stage 32 transgenic animals by both whole-mount fluorescence (E) and *egfp* in situ hybridization (F). By stage 36, transgenic *egfp* expression becomes restricted to muscle and vascular cell lineages (G), and by stage 42 it is observed almost exclusively within the vasculature (H). In adult *X. laevis*, immunostaining of FRG1 [green (I,K)] is observed in the gastrocnemius muscle, where it colocalizes precisely with rhodamine-labled lectin [red (J,K)], a marker for capillaries. Antibody specificity for the FRG1 peptide in these immunohistology experiments was confirmed by immunostaining with (M), or without (L), FRG1 peptide competition. (N) Strong FRG1 staining (red) was observed in arteries and veins in gastrocnemious sections co-stained with wheat germ agglutinin (green) and DAPI (blue). Abbreviations: ps, pronephric sinus; pd, pronephric duct; pcv, posterior cardinal vein; pda, paired dorsal arteries; nc, notochord; nt, neural tube; aa, aortic arches; ov, ophthalmic vessel; da, dorsal artery; dlav, dorsal lateral vein. Bars, 1 mm (A,E-H); 30 μm (B,C); 50 μm (K-N).

(Christie and Thomson, 1989), demonstrating that the FRG1 protein is associated with capillaries within frog muscle (Fig. 1J,K). Beyond the capillaries, FRG1 was also strongly expressed in froglet arteries and veins (Fig. 1N). To ensure the specificity of the antibody, peptide competition with the antigenic peptide was performed and resulted in an absence of FRG1 staining (Fig. 1L,M). Control slides, in which the FRG1 primary antibody was omitted, were consistently negative (data not shown). Thus, similar to stage 42 transgenic tadpoles, the strongest expression of FRG1 within frog muscle is associated with the vasculature.

FRG1 is required for complete angiogenesis and expression of the vascular marker DAB2

To determine if FRG1 is required for vasculature development, FRG1 levels were reduced by specific morpholino (MO) injection. One cell from each 2-cell stage embryo was injected with an *frg1* morpholino (FMO1), which has previously been reported to adversely affect muscle development (Hanel et al., 2009), or with a non-specific control morpholino (CMO). Embryos were then examined at stage 34-36 by whole-mount in situ hybridization for *dab2*, which encodes a vascular marker that acts as an angiogenic regulator, required for sprouting of intersomitic veins (Cheong et al., 2006). FMO1-injected embryos showed a marked, dose-dependent reduction in *dab2* transcript levels (40 ng of FMO1: 100% reduction, n=42; 20 ng of FMO1: 76% reduction, n=38); the embryos were scored based on overall *dab2* expression in the areas with the clearest *dab2* signal, namely the pronephric sinus, posterial cardinal vein and vascular vitelline network (Fig. 2A-F,M). In the embryos with milder phenotypes, fewer branches of the vascular vitelline network were visible. In many embryos, this reduction was severe enough to result in a complete loss of *dab2* in the vascular vitelline network and, strikingly, from the intensely stained pronephric sinus (Fig. 2B,E). Although a small number of CMO-injected embryos displayed a mild reduction in *dab2* staining on the injected side (26% reduction, n=42), these embryos did not display the acute loss of *dab2* that was seen in FRG1 morphants (Fig. 2C,F). In addition, *dab2* loss was specific to FMO1 knockdown of FRG1 because *dab2* expression levels and patterns were successfully rescued by co-injection of FMO1 with a non-complementary mRNA encoding the *X. tropicalis* FRG1 protein (Fig. 2H,I,K,L,N).

To determine whether FRG1 knockdown led to a general loss of vasculature and, thus, of all vascular transcripts, or whether this effect was more specific to the transcript encoding FRG1, in situ hybridization was performed on FMO1 morphants for *msr*, which encodes an early vascular marker and which can be used to identify vascular endothelial cells (Devic et al., 1996). Interestingly, morphant embryo halves did not display an acute loss of *msr* transcripts, as levels within the posterior cardinal vein were comparable to those in the non-injected control half of the embryo. However, FMO1 injection was observed to cause either partial or complete (20 ng of FMO1: 76% reduction, n=21; 40 ng of FMO1: 75% reduction, n=12) inhibition of intersomitic vein sprouting (Fig. 2G,J) compared with background levels (40 ng of CMO: 18% reduction, n=27). The maintenance of the posterior cardinal vein and lack of intersomitic vein sprouting indicated that, unlike vasculogenesis, angiogenesis is disrupted by FRG1 knockdown. Together, these data suggest that FRG1 is required for the establishment of the intact vasculature, specifically in angiogenic branching from existing vessels.

Overexpression of the FRG1 protein increases vasculature levels and angiogenesis

FSHD has been associated previously with elevated levels of FRG1 (Gabellini et al., 2002); therefore, the effects of overexpression of the FRG1 protein were examined. Systemic levels of FRG1 were increased through mRNA injections using the *X. tropicalis frg1* mRNA, co-injected with a tracer mRNA. These FRG1-overexpressing embryos were compared with embryos that had

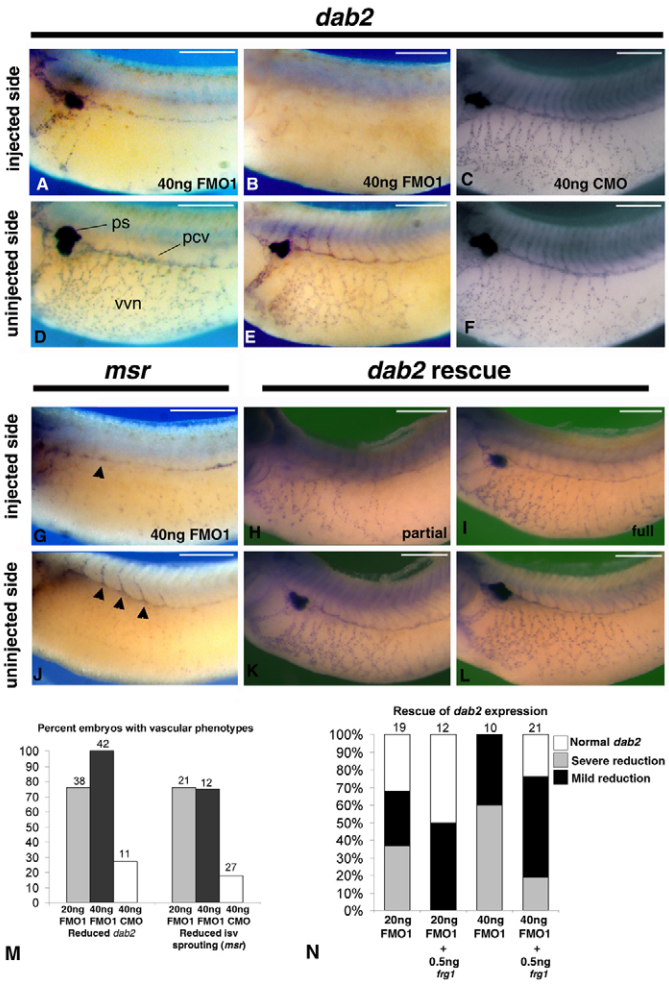

Fig. 2. Depletion of FRG1 inhibits vascular development. The mild (A) and severe (B) phenotypes of the FMO1-injected (40 ng) embryos show a reduction in vascular structures compared with the uninjected sides of the same embryos (D,E), as visualized by *dab2* expression. (C,F) CMO-injected and uninjected sides of the same embryo, respectively. (G,J) FMO1-injected (40 ng) and uninjected sides of the same embryo, stained for *msr*. The arrowheads point to intersomitic veins. (H,I) Partial and full rescue of *dab2* staining by co-injection with 40 ng of FMO1 and 500 pg of *X. tropicalis frg1* mRNA. Co-injection of 40 ng of FMO1 with 1 ng of *X. tropicalis frg1* mRNA was lethal. (M) The percentage of injected embryos displaying loss of *dab2* and *msr* staining. (N) The percentage of embryos rescued by co-injection of FMO1 with *frg1* mRNA. The numbers above the bars indicate the total number of embryos analyzed. Abbreviations: ps, pronephric sinus; vvn, vitelline vein network; pcv, posterior cardinal vein. Bars, 0.5 mm.

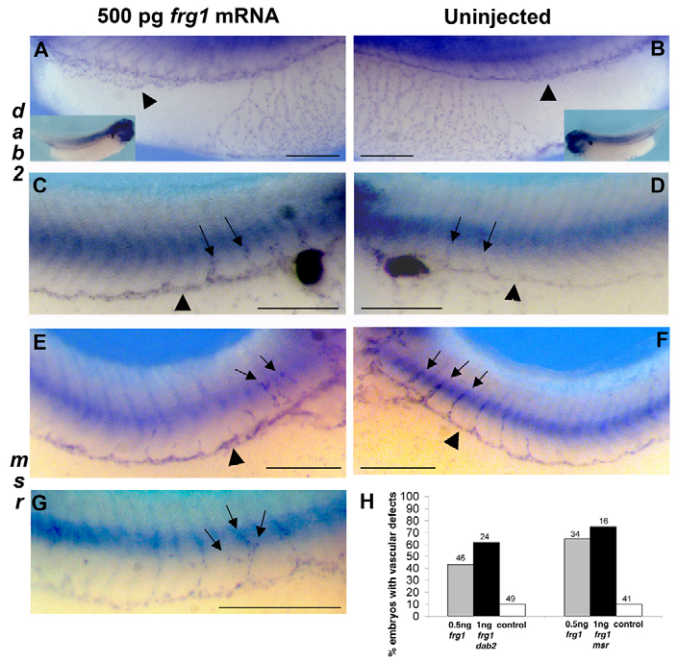

Fig. 3. Embryos with elevated FRG1 levels have increased angiogenesis and vascular dilation. (A-G) Stage 36 tadpoles injected with 500 pg of *frg1* on one side of the embryo were analyzed for vascular abnormalities by using in situ hybridization to detect *dab2* (A-D) or *msr* (E-G) transcripts. Abnormalities included branching of the posterior cardinal vein (black arrowhead in A), dilation of the posterior cardinal vein (black arrowheads in A,C,E), dilation of intersomitic veins (black arrows in C), and improper growth and branching of intersomitic veins (black arrows in E,G). For comparison, the uninjected sides of the embryos from A,C,E are shown in B,D,F, respectively. (H) Numbers of tadpole embryos displaying vascular abnormalities after injection with 500 pg of *frg1*, 1 ng of *frg1* and tracer alone. The numbers above the bars indicate the total numbers of embryos analyzed. Bars, 0.5 mm.

been injected with tracer mRNA alone. The vasculature of embryos injected at stage 34-36 was analyzed using in situ hybridization with either *dab2* or *msr* probes. Both *msr* and *dab2* staining of *frg1*-injected embryos displayed abnormally branched (compare Fig. 3A with Fig. 3B) and dilated posterior cardinal veins (compare Fig. 3A,C,E with Fig. 3B,D,F, respectively). Elevated FRG1 levels led to similar effects within the intersomitic veins, with increased branching (Fig. 3E,G) and dilation (Fig. 3C). Errors in vascular development were observed in 43% and 62% of embryos injected with 500 pg and 1 ng of *frg1*, respectively, as analyzed by *dab2* expression, and in 64% and 75% of embryos injected with 500 pg and 1 ng of *frg1*, respectively, as analyzed by probing for *msr*. Intersomitic veins were labeled more prominently by the *msr* probe than the *dab2* probe, a difference that probably accounts for the elevated level of observed abnormalities in embryos stained for *msr*. In tracer mRNA controls, a similar, but low, level of background defects was observed following use of the *dab2* (10%, *n*=49) and *msr* (10%, *n*=41) probes. Thus, elevated *frg1* levels led to dilated and overly branched vasculature. Together, this pro-angiogenic effect of increased FRG1, combined with the decreased angiogenesis in FRG1 knockdown animals, demonstrates that FRG1 levels are crucial for the proper regulation of angiogenesis.

Expression of the *frg1* transgene leads to edemas and vascular abnormalities

To analyze the vascular defects associated with consistent and tissue-specific elevations in FRG1 levels, transgenic animals that overexpress *frg1* from the *frg1* proximal promoter (FRE-FRG1) were generated and were analyzed for vascular abnormalities. The FRE-FRG1 expression cassette was flanked by HS4 and the Drosophila *Fab8* insulator sequences in order to maintain *frg1* expression upon genome integration. At stage 34, tadpoles were separated into either transgenic or non-transgenic groups based on expression of gamma crystalline-GFP within the eye. Upon examination of the embryos at stage 46, an increased number of tadpoles had developed ventral edema (Fig. 4B), a phenotype associated with lymphatic system defects or vascular defects (Inui et al., 2006; Rodrigues et al., 2008). Although background levels of this phenotype were 18% (*n*=37) for non-transgenic sperm recipients, more than a two-fold increase in this phenotype was observed in the transgenic tadpoles (44%, *n*=32). Animals from both transgenic and non-transgenic pools that appeared normal at stage 46-48 were examined for vascular defects in the tail by using fluorescein-labeled dextran injections into the heart. Animals were examined exclusively for gross vascular abnormalities, which we found to be double branching of the dorsal lateral vein (compare Fig. 4D with Fig. 4E). A two-fold increase in these defects was observed in transgenic animals (45%, *n*=20) compared with the background levels in non-transgenic animals (22%, *n*=22). These results confirm that elevated expression of FRG1 within the vascular tissues, where FRG1 is endogenously expressed, is capable of producing significant developmental vascular defects.

DISCUSSION

Epigenetic disregulation leading to the overexpression of FRG1 has been a leading candidate for the mechanism that mediates FSHD pathology; however, all of the previous studies have focused on muscle expression, muscle biopsies and muscle cell culture. Here, a role for FRG1 in the development of vascular structures has been uncovered. We find that an elevated level of FRG1 not only disrupts skeletal muscle (Hanel et al., 2009), consistent with previous work in a mouse model for FSHD (Gabellini et al., 2006), but also leads to an increase in the size and branching of the vasculature structure; both of these results are consistent with data from the tissues of FSHD patients. The vascular component of FSHD has been described in the retina of the eye where the vasculature can be readily visualized; however, the upregulation of gene transcripts in the muscle biopsies from the vascular smooth muscle and endothelial cells (Osborne et al., 2007) imply a more systemic disruption of vasculature in FSHD. One benefit of using Xenopus development for these studies is the ability to visualize intact vascular structures throughout the body and determine the systemic effects of altered FRG1 levels. Our analysis of the eye vasculature was somewhat hindered in the tadpoles that overexpress the FRG1 protein, owing to the more complex nature of the vasculature in the ocular region, which made it difficult to visualize disruption of the architecture. However, consistent with the results from elsewhere in the body, *dab2* expression was clearly reduced in the eyes of embryos injected with FMO1 (supplementary material Fig. S1), suggesting that the eye vasculature is similarly regulated. Thus, the *frg1* overexpression phenotype in Xenopus correlates strongly with both the afflicted tissues and the clinical

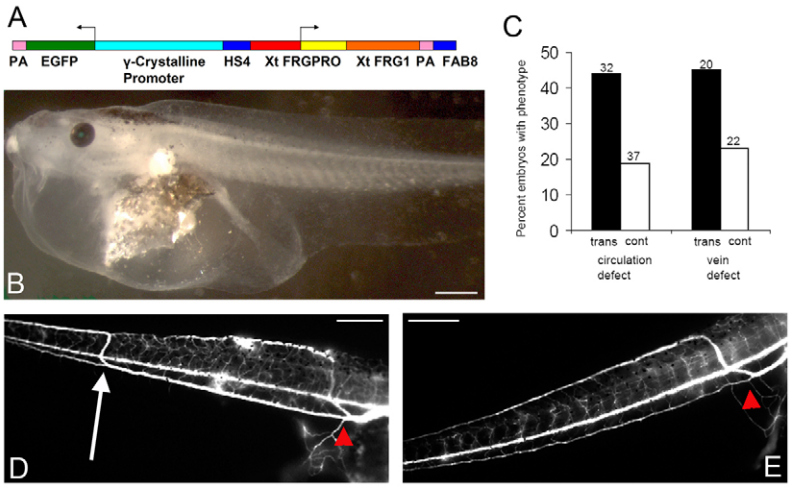

Fig. 4. Transgenic animals with FRE-specific expression of FRG1 have increased levels of vascular defects. (A) Diagram of the construct used to make transgenic tadpoles, depicting the gamma crystalline-EGFP reporter and the HS4 and *Fab8* insulator sequences flanking the *X. tropicalis* (Xt) proximal *frg1* promoter, which drives expression of the *X. tropicalis frg1* cDNA. (B,D,E) Transgenic animals displayed increased levels of ventral edema (B) or major vascular abnormalities (white arrow in D) when compared with non-transgenic controls (E) (red arrowheads indicate the normal vascular path). (C) Numbers of transgenic (trans) and non-transgenic embryos (cont) that display the ventral edema circulation defect or large vascular abnormalities. The numbers at the top of the bars indicate the total numbers of animals analyzed. Bars, 1 mm.

findings from FSHD patients, consisting of dystrophic muscle with a variable fiber size, retinal vasculopathy, and misregulation of gene transcription in the vascular smooth muscle and endothelial tissues (Fitzsimons et al., 1987; Padberg et al., 1995b; Padberg et al., 1995a; Osborne et al., 2007).

The finding that FRG1 mediates angiogenesis and not vasculogenesis was deduced from the maintenance of the posterior cardinal vein and loss of intersomitic vein sprouting in FRG1 morphants. In agreement, the opposite effect, overbranching of vascular structures, was observed in animals with elevated levels of FRG1. Similar to our FMO1 injection experiments, the reduction of DAB2 in Xenopus led to an inhibition of intersomitic vein sprouting through the loss of vascular endothelial growth factor (VEGF) induction (Cheong et al., 2006). It is possible that the loss of intersomitic sprouting in the FRG1 depletion experiments occurs as a direct consequence of the reduction in DAB2. However, FRG1 is probably not a direct regulator of *dab2*, as elevated levels of FRG1 did not appear to lead to increased levels of *dab2* staining. Furthermore, whereas overexpression of DAB2 led to a lack of intersomitic vein sprouting (Cheong et al., 2006), elevated levels of FRG1 led to improper branching and dilation of the vasculature. In fact, overexpression of the FRG1 protein in the intersomitic veins more closely resembles the effects of misexpression of *ephB4*, which encodes an ephrin receptor that is part of a second pro-angiogenic pathway involving ephrins, which are also involved in somitogenesis (Helbling et al., 2000). Our study indicates that, beyond maintaining *dab2* transcript levels, FRG1 is functioning as a pro-angiogenic factor.

The maintenance of *dab2* transcript levels by FRG1 may affect many tissues beyond the vasculature. DAB2 is highly expressed in many adult mammalian tissues and is most commonly known as a mediator of clathrin-associated endocytosis (Oleinikov et al., 2000; Kowanetz et al., 2003) and a key component of the transforming growth factor (TGF)-β signaling pathway. As a component of this signaling pathway, DAB2 has many functions including roles as a tumor suppressor (Fazili et al., 1999), a mediator of epithelial-to-mesenchymal transition (EMT) (Prunier and Howe, 2005), and in cellular migration (Hocevar et al., 2005). Furthermore, DAB2 is also involved in the inhibition of Wnt-stimulated cellular proliferation through its association with Axin (Hocevar et al., 2003; Jiang et al., 2007). In the vasculature, DAB2 functions in endodermal organization, differentiation (Morris et al., 2002; Yang et al., 2002) and in early angiogenesis through induction of VEGF by activin-like signaling (Cheong et al., 2006). Therefore, FRG1 expression is crucial for a wide variety of functions simply by its maintenance of DAB2.

A role for overexpression of the FRG1 protein in FSHD is still controversial, partly because of inconsistencies between gene expression studies in cell lines and FSHD muscle, the lack of information on the precise function of FRG1, and recent studies supporting potential roles for alternative candidate genes. Our studies strongly suggest that misexpression of the FRG1 protein is capable of producing both the musculature and vasculature pathology seen in FSHD (Hanel et al., 2009). In addition, our data are consistent with the disrupted cell signaling pathway model for FSHD that is based primarily on extending the clinical observations of FSHD patient pathology to diseases of known cause that exhibit similar pathology. Coats' disease is caused by a mutation in Norrin, a ligand to the Wnt receptor Frizzled-4 (Zerlin et al., 2008) and exhibits retinal vascular abnormalities that are highly similar to those found in FSHD. The symptoms of FSHD and Coats' disease both include the appearance of thick tortuous vessels within the retina, similar to the vessels found in this study with overexpression of FRG1. Frizzled-4 mutations, which are linked to familial exudative vitreoretinopathy, lead to similar retinal vasculopathy along with progressive auditory defects (Zerlin et al., 2008), a symptom that has also been associated with FSHD. Thus, owing to these similarities between FSHD and Coats' disease, FSHD is speculated to involve a defect in the Wnt signaling pathway, a mechanism that is supported by our data.

Conclusions

In summary, our study has shown that FRG1 is expressed in vascular structures and is essential for angiogenesis. Examination of the tissue-specific effects of *frg1* overexpression in our Xenopus system has shown that increased levels of FRG1 can account for the two prominent clinical aspects of FSHD, namely dystrophic muscle and increased angiogenesis.

METHODS

Frog husbandry

Adult *X. laevis* frogs were purchased from Xenopus Express. All procedures were carried out in accordance with established UIUC Institutional Animal Care and Use Committee (IACUC)-approved protocols for animal welfare.

In situ hybridizations, immunohistochemistry and *X. laevis* FRG1 antibody

In situ hybridizations were carried out as described (Hanel et al., 2009). Treatment and immunostaining of embryos using the XL FRG1 antibody were performed as described previously (Hanel et al., 2009). The previously characterized XL FRG1 rabbit polyclonal antibody was raised against the peptide EREAKRDDDIPNED and was produced by GenScript Corp (Hanel et al., 2009).

Sectioning and immunohistochemistry

Leg muscles dissected from froglet and adult frogs were snap frozen in isopentane, cooled in liquid nitrogen and cryosectioned into 10 µm sections. FRG1 immunostaining gave identical staining after acetone or formaldehyde fixation. Double staining with FRG1 and rhodamine-labeled *Griffonia simplicifolia* lectin I (GSL I) (Vector laboratories) was performed on sections fixed in acetone for 5 minutes. Slides were blocked in 2% donkey serum and 1% BSA. The XL FRG1 antibody was used at a dilution of 1/100 and detected with secondary donkey anti-rabbit fluorescein (1/100). After washing, GSL I was applied to slides at a concentration of 40 µg/ml and incubated for 1 hour. For peptide competition experiments, sections were treated with 0.5% Triton X for 5 minutes, followed by fixation in 10% formaldehyde in PBS for 10 minutes. Slides were blocked in 2% donkey serum, 1% BSA, 0.1% Triton X, 0.05% Tween 20. XL FRG1 antibody was incubated with, and without, a 50-fold excess of peptide at 37°C for 2 hours, followed by 16 hours at 4°C with rocking. The antibody-peptide mixture was centrifuged at 20,000 ***g*** for 15 minutes and the supernatant was diluted in the blocking solution and used for immunostaining.

Transgenic construct cloning

All PCR products were initially cloned into pGEM-T easy vectors (Promega) and sequenced. All PCR primers are listed in the supplementary material Table S1. The *X. tropicalis frg1* proximal regulatory element (FRE) was amplified by genomic PCR (using primers 1 and 2). The *X. tropicalis frg1* cDNA was amplified from total RNA by reverse transcriptase (RT)-PCR (using primers 3 and 4) and reamplified (using primers 5 and 6) for cloning.

pCAIN

The pEGFP-N1 (Clontech) vector was digested with *Ase*I and *Xho*I, filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I and self-ligated. The plasmid was digested with *Afl*II, filled in using the Klenow fragment and self-ligated. The chicken β-globin insulator (HS4) (Prioleau et al., 1999) was amplified by genomic PCR (using primers 7 and 8) from chicken blood DNA and reamplified using primers 9 and 10, or 11 and 12. The PCR products were digested with *Eco*RI and *Spe*I, or *Bam*HI and *Xba*I, and cloned by triple ligation into the modified pEGFP-N1 vector that had been digested with *Eco*RI and *Bam*HI; this process was repeated by using primers 13 and 10, or 11 and 14, and cloning into the *Bam*HI–*Not*I-digested vector, resulting in pCAIN.

pCAIN FRE-EGFP

FRE was isolated from FRE-pGEM by *Ase*I-*Bam*HI digestion and ligated into the similarly digested pEGFP-N1 vector. The resulting vector, FRE-EGFP, was digested with *Ase*I and *Afl*II, and cloned into similarly cut pCAIN.

pCAIN GC FRE-FRG1

PCR was performed on purified Drosophila genomic DNA using primers 15 and 16 to produce the *Fab8* insulator. This was then reamplified using primers 17 and 18, and 19 and 20, and cloned into pCAIN. The gamma crystalline-GFP reporter was cloned from pCGCG (a gift from Dr Yun-Bo Shi) (Fu et al., 2002). This vector was then digested with *Ase*I-*Afl*II and ligation was performed with the similarly digested FRE-FRG1 vector.

Xenopus transgenesis

Xenopus transgenesis was carried out essentially as described previously (Kroll and Amaya, 1996; Wuebbles and Jones, 2007);

TRANSLATIONAL IMPACT

Clinical issue

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common myopathy, afflicting approximately one out of 20,000 people worldwide. The most characteristic symptom is a progressive atrophy of facial, shoulder and upper arm muscles. Additionally, over half of FSHD patients have abnormal blood vessels in the back of the eye, which causes vision problems in some patients.

Over 95% of FSHD cases carry a genetic abnormality within a series of DNA repeats at chromosome 4q35. Although the unaffected population contains between 11 and 150 copies of this DNA repeat (named D4Z4), FSHD patients have deletions resulting in between one and ten D4Z4 repeats. This mutation is not in a known protein-encoding gene, thus these deletions are expected to disrupt the normal regulation of gene expression. However, there is little agreement regarding which gene or genes are misregulated in FSHD, thus complicating the creation of appropriate FSHD animal models, and hindering advances in understanding and treating this disease. Previous studies demonstrate that altered expression of FRG1disrupts the musculature in developing *Xenopus laevis*, thus supporting a role for FRG1 in FSHD pathology.

Results

In this study, the spatiotemporal expression of FRG1 is analyzed during Xenopus development, revealing prominent expression in the vasculature. The authors show that FRG1 is required for the growth of new blood vessels from pre-existing vessels, but is not needed for spontaneous blood vessel formation. Conversely, increasing FRG1 levels promotes angiogenesis and disrupts the organization of the developing vasculature. Thus, normal levels and patterns of FRG1 expression are crucial for proper vascularization.

Implications and future directions

This study, combined with previous work analyzing the effect of FRG1 on the developing musculature, demonstrates that FRG1 affects the skeletal musculature and the vasculature when its expression levels are altered in an animal model. Thus, FRG1 expression is a causal factor in FSHD pathology. Prominent expression of FRG1 in the vasculature suggests that future FSHD research should examine vascular cell types and tissue, as well as skeletal muscle.

doi:10.1242/dmm.003061

however, the sperm nuclei were not digested with restriction enzymes. Plasmids were linearized, purified and incubated (100 ng per experiment) with sperm nuclei for 5 minutes. Xenopus egg extract (10 µl) and 20 µl of sperm dilution buffer (SDB; 250 mM sucrose, 75 mM KCl, 0.5 mM spermidine, 0.2 mM spermine) were mixed and incubated at 65°C for 5 minutes, then centrifuged at 16,000 ***g*** for 3 minutes to remove the precipitate. The soluble fraction (6 µl) was diluted to 22 µl with SDB plus 10 mM of $MgCl_2$, then added to the nuclei-sperm mix and incubated for 15 minutes at room temperature. The swollen nuclei were added to 170 µl of SDB and used for microinjection at a rate of 0.586 µl/minute using a microliter syringe pump (Harvard Apparatus).

Probe constructs

The PCRs for cloning used the Triplemaster polymerase enzyme mixture (Eppendoerf); the RT-PCRs used the Superscript III HiFi one-step RT-PCR kit (Invitrogen); and restriction enzymes were purchased from New England Biolabs. All oligonucleotide primers are listed in supplementary material Table S1. Total RNA was extracted from the ovaries of *X. laevis* and *X. tropicalis* frogs with Trizol (Invitrogen), as per the manufacturer's instructions, and used for RT-PCR (with primers 21 and 22 for *dab2*, 23 and 24 for *msr*, and 25 and 26 for *egfp*) to produce cDNA. All products were cloned into pGEM T-Easy vectors (Promega) and sequenced.

RNA and morpholino microinjections

Morpholino and mRNA injections were performed as described previously (Hanel et al., 2009).

Vascular labeling in tadpoles

Fluorescein-labeled dextran (9.2 nl) was injected into the heart of stage 46 transgenic and non-transgenic tadpoles, incubated for 30 minutes at 16°C, and visualized using a Leica dissecting microscope.

ACKNOWLEDGEMENTS

We thank Jon Henry and Phil Newmark, UIUC, for technical support. This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases grant #1RO1AR055877 awarded to P.L.J. and the FSH Society Landsman Charitable Trust Fellowship #FSHS-LCT-001 awarded to M.L.H.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

R.D.W., M.L.H. and P.L.J. conceived and designed the experiments; R.D.W. and M.L.H. performed the experiments and analyzed data; R.D.W., M.L.H. and P.L.J. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.002261/-/DC1

Received 21 November 2008; Accepted 21 January 2009.

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