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MicroRNA-29a in Adult Muscle Stem Cells Controls Skeletal Muscle Regeneration During Injury and Exercise Downstream of Fibroblast Growth Factor-2

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ABSTRACT

The expansion of myogenic progenitors (MPs) in the adult muscle stem cell niche is critical for the regeneration of skeletal muscle. Activation of quiescent MPs depends on the dismantling of the basement membrane and increased access to growth factors such as fibroblast growth factor-2 (FGF2). Here, we demonstrate using microRNA (miRNA) profiling in mouse and human myoblasts that the capacity of FGF2 to stimulate myoblast proliferation is mediated by miR-29a. FGF2 induces miR-29a expression and inhibition of miR-29a using pharmacological or genetic deletion decreases myoblast proliferation. Next generation RNA sequencing from miR-29a knockout myoblasts ($Pax7^{CE/+}$; $miR\text{-}29a^{flox/flox}$) identified members of the basement membrane as the most abundant miR-29a targets. Using gain- and loss-of-function experiments, we confirm that miR-29a coordinately regulates Fbn1, Lamc1, Nid2, Col4a1, Hspg2 and Sparc in myoblasts in vitro and in MPs in vivo. Induction of FGF2 and miR-29a and downregulation of its target genes precedes muscle regeneration during cardiotoxin (CTX)-induced muscle injury. Importantly, MP-specific tamoxifen-induced deletion of miR-29a in adult skeletal muscle decreased the proliferation and formation of newly formed myofibers during both CTX-induced muscle injury and after a single bout of eccentric exercise. Our results identify a novel miRNA-based checkpoint of the basement membrane in the adult muscle stem cell niche. Strategies targeting miR-29a might provide useful clinical approaches to maintain muscle mass in disease states such as ageing that involve aberrant FGF2 signaling. STEM CELLS *2016; 00:000—000*

SIGNIFICANCE STATEMENT

Skeletal muscle mass and function is critical for the maintenance of health, and the decline of muscle mass during aging inversely correlates with mortality. Adult muscle stem cells provide an important target for strategies to maintain muscle mass, but the molecular mechanisms that control the activation of these stem cells in vivo are still incompletely understood. We demonstrate that microRNA-29a is a novel downstream target of FGF2, and that miR-29a mediates the dismantling of the basement membrane in the adult muscle stem cell niche that is required for the proliferation of myogenic progenitors during muscle regeneration. We propose the FGF2/miR-29a pathway as a novel target to prevent a decrease in muscle mass in disease states such as ageing where FGF2 signaling is overly activated.

INTRODUCTION

Skeletal muscle mass and function is critical for the maintenance of health. Exercise capacity is a powerful predictor of survival in men [1], and the decline of muscle mass during aging inversely correlates with mortality [2]. Preserving muscle mass might prolong lifespan similar to the effects of improvement of physical fitness [3]. Adult skeletal muscle has a remarkable capacity to regenerate and strategies that improve muscle regeneration could be the basis for treatment of aging as well as muscle-wasting diseases [4]. In animal models of muscle regeneration, complete destruction of myofibers induced via intramuscular (i.m.) injection of cardiotoxin (CTX) can be repaired within 2 weeks [5]. This regeneration process is driven by adult muscle stem cells, also termed satellite cells or myogenic progenitors

(MPs). These cells are quiescent in an intact muscle but can be rapidly activated upon injury and display a high proliferation rate [6]. MPs are located between the plasma membrane of muscle fibers and the basement membrane, an environment known as the adult muscle stem cell niche. The basement membrane consists of the basal and the reticular lamina which are connected by fibrillin-1 (FBN1) containing microfibrils [7, 8]. The basal lamina comprises networks of self-assembled laminins and collagen IV, which are crosslinked by nidogens and heparin sulfate proteoglycans (HSPGs). HSPGs are required for growth factor binding such as hepatocyte growth factor and fibroblast growth factor (FGF). Laminin mediates contact of the basal lamina to MPs and myofibers by binding of cell surface components, mainly integrins and α-dystroglycan. Upon injury, components of the basement membrane are degraded by proteinases [9], and important members such as collagen-IV (COL4A1), heparane sulfate proteoglycan-2 (HSPG2, perlecan), laminin gamma-1 (LAMC1), and nidogen-1 (NID1, entactin-1) are actively downregulated at the transcriptional level [10, 11]. This dismantling of the basement membrane is important for the release of growth factors and expansion of MPs. The co-ordinate breakdown and rebuilding of the basement membrane is critical for proper muscle regeneration and to avoid fibrosis, however, the molecular mechanisms are poorly understood.

One of the critical growth factors for MP proliferation that is provided by the extracellular matrix (ECM) is FGF2. FGF2 is a potent mitogen that induces proliferation of myoblasts in vitro and therefore serves as an essential growth factor in myoblast culture media [12]. In vivo, FGF2 is expressed in developing [13] and adult skeletal muscle where its expression correlates with muscle regeneration activity [14]. Accordingly, FGF2 expression is increased in skeletal muscle in different animal models of exercise, chronic nerve stimulation as well as muscle injury and has been associated with MP proliferation and fast to slow fiber type conversion [15–19]. Importantly, the exogenous application of FGF2 improves muscle regeneration and MP proliferation in vivo [20]. In cultured muscle cells as well as in MPs in vivo, the FGF receptor-1 (FGFR1) mediates the effects of FGF2 on proliferation via activation of extracellular-signal regulated kinases signaling [21, 22].

Recently, microRNAs (miRNAs), a family of noncoding RNAs, have been implicated in the process of muscle regeneration. miRNAs are short RNA molecules of 20–24 nucleotides length that bind to the 3′-untranslated region (UTR) of their target genes, which leads to mRNA degradation and inhibition of translation. A single miRNA can have important biological effects by regulating a large number of target genes that are acting in a similar pathway [23, 24]. Several mouse models harboring genetic miRNA deletion have demonstrated that miRNAs have an important role in skeletal muscle regeneration and function. The double knockout of miR-208b and miR-499 decreased type I fiber type in soleus muscle [25]. Deletion of miR-206 delayed muscle regeneration following CTX-induced muscle damage by inhibiting MP fusion to myofibers and led to a more dystrophic phenotype in the mdx mouse model of muscle dystrophy [26]. Deletion of miR-133a induced a progressive centronuclear myopathy, mitochondrial dysfunction, and a fast-to-slow myofiber switch [27]. While these models all provided evidence for an important role of miRNAs in skeletal muscle, they are based on embryonic knockouts and reflect long-term consequences of miRNA deletion. Studying the acute effects of miRNAs in skeletal muscle requires inducible miRNA knockouts, which have not been reported to date.

Here, we hypothesized that FGF2 acts through miRNAs to govern the proliferation of MPs. We report that FGF2 induces miR-29a and that miR-29a and its basement membrane targets are regulated in activated MPs during muscle regeneration in vivo. Consequently, induced deletion of miR-29a in MPs prevented efficient muscle regeneration after injury and exercise. Our results indicate that miR-29a participates in the dismantling of the basement membrane in the adult muscle stem cell niche during regeneration of skeletal muscle.

Materials and Methods

Animals

Mice were maintained in 12 hour-light/dark cycles in a pathogen-free animal facility. CTX (Sigma, Buchs, https://www.sigmaaldrich.com) was injected into the *M. tibialis* anterior (50 μl, 10 mM in PBS) of C57Bl6/6J mice (Harlan, Horst, http://old.harlan.com). Pax7$^{tm2.1}$(cre/ERT2) mice were obtained from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). Homozygous floxed miR-29ab1 mice were genotyped using polymerase chain reaction (PCR) as previously described [28]. Tamoxifen (Sigma) was dissolved in corn oil at 20 mg/ml in a 37 °C water bath for 2–3 hours and frozen as aliquots at −80 °C. 5′-Ethynyl-2′-deoxyuridine (EdU) was injected intraperitoneally at 5 μg/g body in 300 μl PBS as indicated. Mice preformed a single 90 minute bout of downhill running (−20° decline) on a treadmill (Panlab, Cornella, http://www.panlab.com) at a speed of 15 m/minute from 0 to 60 minutes, 18 m/minute from 60 to 75 minutes, and 21 m/minute from 75 to 90 minutes. Mild electrical stimulus was provided as motivation via a shock-grid at the end of the treadmill belt. To avoid any muscular priming adaptations mice were familiarized to treadmill running immediately prior to completing downhill running. This consisted of 5 minute running at 0 decline, with speed gradually increasing to 15 m/minute. Exercise tolerance was determined using an incremental treadmill test. The treadmill was set at 5° incline, and speed was increased by 2 m/minute every 2 minutes until the mouse spent >5 seconds on the shock grid without attempting to continue running. All animal studies were approved by the ethics committee of the Kantonale Veterinäramt Zürich and principles of laboratory animal care were followed.

Skeletal Muscle Histology and Immunofluorescence

Skeletal muscle was dissected and flash frozen in isopentane/liquid nitrogen. Frozen sections of 10 μm were prepared either from three different areas of the tibialis anterior, 500 μm apart, or from the whole gastrocnemius muscle. Sections were processed for immunofluorescence using the Vector M.O.M. Immunodetection kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Slides were mounted with Fluoroshield with 4′,6-Diamidin-2-phenylindol (DAPI) (Sigma). Antibodies against embryonic myosin heavy chain (eMHC) (BF-G6-s) were from Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, http://www.uiowa.edu/~dshbwww), against laminin from Sigma-Aldrich

(St. Louis, MO, http://www.sigmaaldrich.com), against FGF2 from BD Bioscience (San Diego, CA, http://www.bdbiosciences.com), and against phospho-p38 from Cell Signaling (Beverly, MA, http://www.cellsignal.com). Nuclear proliferation was detected using Click-iT Plus Edu imaging kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Images for each section were obtained using Leica confocal laser scanning microscope SP5 Mid UV-VIS. Ratio of eMHC + to laminin + fibers in regenerative areas and eMHC small fiber area were analyzed using the CellProfiler 2.1.1. program. Fiber size distribution was analyzed by laminin staining processed for segmentation analysis using the Ilastic software and fiber count using ImageJ. For routine histochemistry, cryostat sections were prepared and stained with hematoxylin and eosin (H&E), modified Gomori trichrome, and ATPase at pH 4.3, according to standard procedures. The stained preparations were evaluated in a blinded fashion by an experienced muscle pathologist (E.J.R.) for variation in myofiber diameter, the presence of atrophic and hypertrophic fibers, internalized nuclei, regenerating and degenerating fibers, myophagocytosis, inflammation, and endomysial fibrosis. The extent of fibrosis was semiquantatively graded from 1 (trace amounts in a single focus) to 3, representing multifocal and more extensive pathological changes.

RNA Isolation, Quantitative Reverse Transcriptase PCR, Northern Blotting, miRNA Microarray, and mRNA Sequencing

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). For quantitative reverse transcriptase PCR (qRT-PCR), total RNA was subjected to DNAse digestion followed by complementary DNA preparation with random hexamer primers using Super Script III Reverse Transcriptase (Invitrogen). mRNA were analyzed by quantitative real-time PCR using the FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland, http://www.roche-applied-science.com) with a 7500 Fast Start Real-Time PCR system (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Transcript levels were normalized to 18S ribosomal mRNA levels. Primer sequences are available upon request. miRNA levels were measured using qRT-PCR with the TaqMan miRNA assays (Applied Biosystems) and normalized to sno234 based on the equal distribution of sno234 levels in all samples. Northern blotting was performed as previously described in nonreducing polyacryamide gels [29]. DNA antisense probes were labeled with T4 polynucleotide kinase (NEB) and 30 μCi of [γ-32P]ATP (3,000 Cimmol-1; PerkinElmer). For miRNA microarrays and Illumina deep sequencing, RNA was processed and analyzed at LC Sciences (Houston, TX, http://www.lcsciences.com/). The sequence results were obtained as fragment per kilobase of exons per million reads for each transcript.

Primary Mouse and Human Myoblast Cultures, miRNA Transfection, and Western Blotting

Human skeletal muscle was obtained from elective surgery of the lower limb performed on two healthy donors (males, age 20 and 22 years, BMI 22 kg/m^2) at the University Hospital of Zurich. The study protocol was approved by the local ethics committees. All subjects gave written informed consent before enrollment. For isolation of primary muscle cells, skeletal muscle tissue was subjected to collagen digestion, and MPs were isolated using flow cytometry. Human isolation was based on CD56 expression and absence of CD15, CD31, and CD45 staining, while mouse MP isolation was based on the presence of α7-integrin and absence of Sca1, CD31, and CD45 staining. Mouse fibrocyte adipocyte progenitors (FAPs) were isolated based on the presence of Sca1 and absence of α7-integrin, CD31 and CD45. Cells were grown on collagen-coated plates in culture medium (1:1 v/v F10 nutrient mixture and Dulbecco's modified Eagle's medium (DMEM) (low glucose) containing 20% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S), and 5 ng/ml basic FGF (Invitrogen). Tamoxifen was dissolved in 100% ethanol and diluted 1:5,000 in growth media (final concentration 20 nM) for a 48 hour incubation period on primary myoblasts. Differentiation was initiated when myoblasts reached subconfluence by changing the media to DMEM containing 2% FBS and 1% P/S. For Western blotting, cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and proteins detected using primary (PVDF) antibodies for α-tubulin (DSHB, 12G10, 1:500), myogenin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com, sc-12732, 1:200), and adult myosin heavy chain (DSHB, MF-20, 1:100).

Proliferation Rate and Mitochondrial Activity

Proliferation rates in MPs or primary myoblasts was measured using Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies, Rockville, MD, http://www.lifetech.com). Mitochondria mass and mitochondrial membrane potential were measured using MitoTracker Green FM (MTK, Life Technologies) and tetramethylrhodamine (TMRE, Life Technologies), respectively. In brief, cells were trypsinized, washed with PBS/BSA (0.5%) and incubated for 20 minutes at 37 °C in either 100 nM TMRE or 20 nM MTK. After a final wash step cells were kept at +4 °C, and cell fluorescence was analyzed using flow cytometry.

Luciferase Plasmid, Antagomir, miRNA Mimic, and siRNA Transfection into Primary Mouse Myoblasts

For MCK luciferase assay, primary myoblasts were transfected with MCK-luciferase plasmid (gift from Robert Benezra (Addgene plasmid # 16062 [30]) and pRL-TK plasmid (Promega, Madison, WI, http://www.promega.com, #E2241) using lipofectamin 2000 (Life Technologies, #11668-027). Growth media was exchanged after 24 hours, and analysis was performed at 72 hours after transfection using the Dual-Luciferase Reporter Assay (Promega, #1960). miRNA transfection was performed on primary myoblasts using Lipofectamin RNAimax (Invitrogen) and human miRNA mimics (38 nM, Mission, Sigma-Aldrich), miRNA inhibitors (12 nM, antagomirs [29]) (Mission, Sigma-Aldrich). Cells were analyzed after the indicated time periods.

Statistical Analyses

Student's *t* tests were analyzed using excel software, one-way ANOVA with Dunnett's multiple comparison test was analyzed using the GraphPad Prism software. Fold-change regulations were analyzed using one-sample *t* test with the hypothetical means of 1. *p* values smaller than .05 were considered significant. Results are shown as means ± SEM.

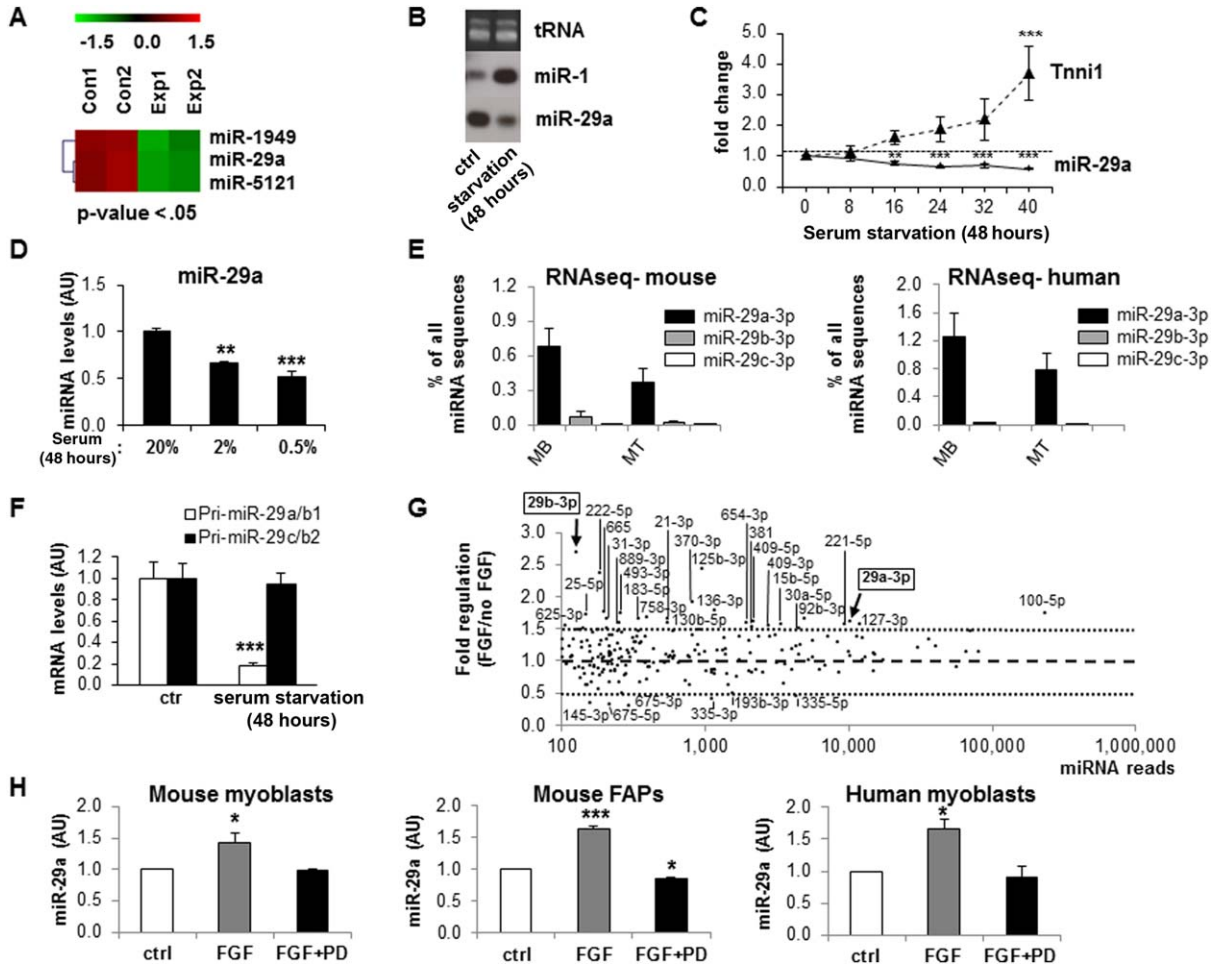

Figure 1. Regulation of miR-29a expression in primary myoblasts by serum starvation and FGF2. **(A–F)**: Primary mouse myoblasts were grown for 48 hours under growth conditions (ctrl) or serum starvation, and RNA was isolated for gene expression analysis. (A): miRNA expression was analyzed using miRNA microarrays, $n = 2$. (B): Regulation of miR-29a and miR-1 shown by northern blotting. (C, D): Time- and dose-dependent regulation of miR-29a and slow Tnni1 by serum starvation as shown by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). $n = 4$. (E): RNA sequencing results for expression levels of miR-29 members in mouse ($n = 3$) and human ($n = 2$) myoblasts before (MB) and after 4 days of serum starvation (MT). Results are shown as percentage of the sum of all detected miRNA sequences. (F): qRT-PCR for the two primary transcripts of the miR-29 family as normalized to 18s RNA. $n = 10$. **(G)**: RNA sequencing data for miRNA expression in human myoblasts incubated for 48 hours with or without FGF2, $n = 2$. Indicated are all miRNA sequences with an average fold-regulation > 1.5 or < 0.5. (H): Activation of miR-29a by FGF2 incubation for 48 hours in primary mouse myoblasts, FAPs, and human myoblasts as measured by qRT-PCR. Fgf1r was inhibited by incubations in the presence of PD173074. $n = 4$. *, $p < .05$; **, $p < .01$; ***, $p < .001$ compared to control (C, D, H: ANOVA with Dunnett's multiple comparison test, F: Student's t test). Abbreviations: FAPs, fibrocyte adipocyte progenitors; FGF, fibroblast growth factor; MB, myoblasts before 4 days of serum starvation; MT, myoblasts after 4 days of serum starvation; Tnni1, troponin 1.

Results

To identify miRNAs that are regulated by growth factors, and specifically by FGF2, in proliferating myoblasts, we established primary mouse myoblast cultures isolated from adult mouse skeletal muscle using fluorescence activated cell sorting (FACS). Myoblast cultures show uniform expression of the myoblast marker α7-integrin and efficiently differentiate into multinucleated myotubes (>90% of nuclei located in multinucleated myotubes) (Supporting Information Fig. 1A, 1B). As expected, muscle-specific miRNAs 1, 206, and 133a were induced at late stages of differentiation (Supporting Information Fig. 1C). To uncover miRNAs that are regulated at early stages of differentiation downstream of FGF2, we used two independent approaches. First, we cultured primary mouse myoblasts under growth conditions and compared their miRNA profile to myoblasts incubated for 48 hours under serum starvation and without growth factors (differentiation conditions) using miRNA microarrays. Second, we performed miRNA profiling in primary human myoblasts incubated for 48 hours under growth conditions with or without exogenous FGF2 using next generation RNA sequencing.miRNA microarrays identified only three miRNAs that were differentially regulated with a p value of $< .05$ after 48 hours of serum starvation in mouse myoblasts (Fig. 1A). MiR-29a was downregulated by 58.5%, p value .0393. Since the signals for the two other miRNAs obtained in the arrays were very low, we focused on miR-29a. Downregulation of miR-29a was confirmed by northern blotting (Fig. 1B) and was detectable by qRT-PCR already within the first 16 hours of serum starvation

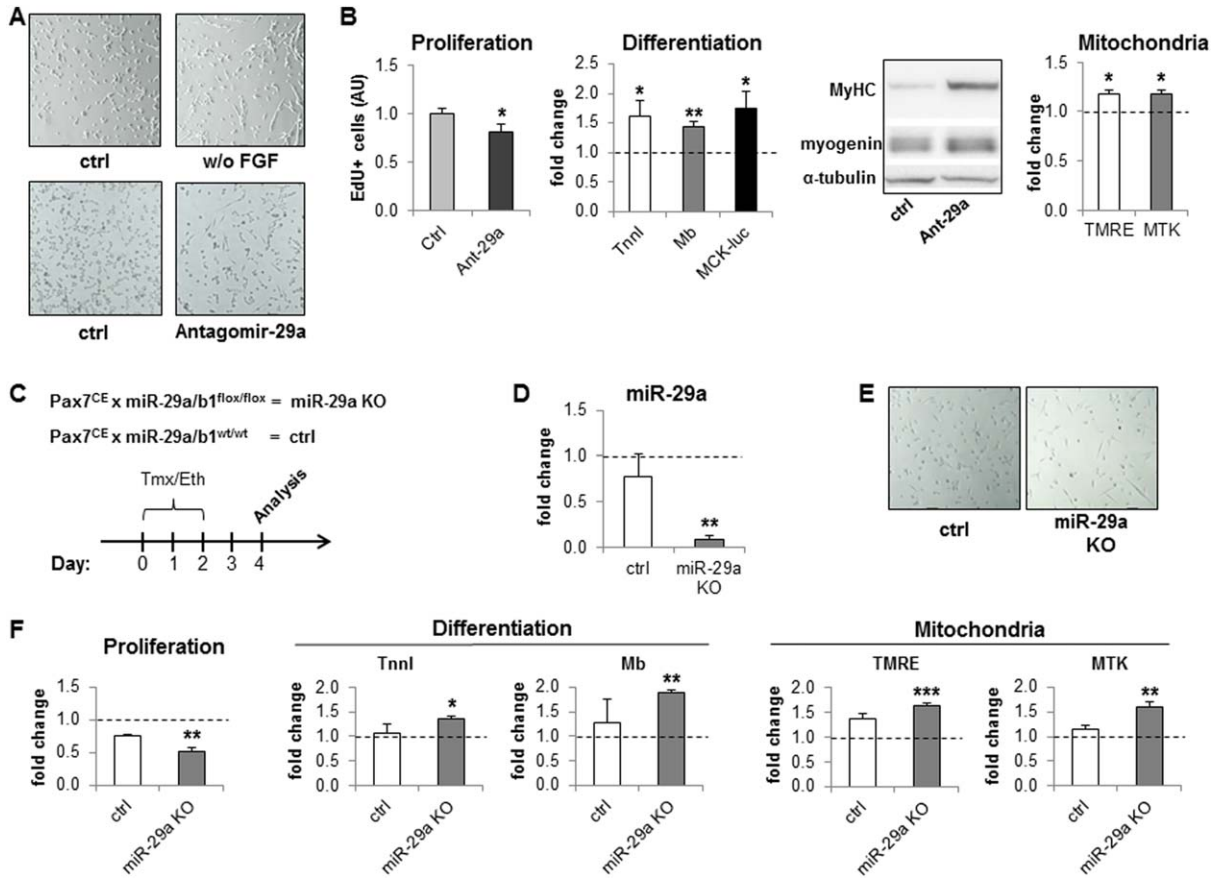

Figure 2. Loss of miR-29a decreases myoblast proliferation. Primary myoblasts were incubated with or without FGF2 or with FGF2 in the presence of a miR-29a inhibitor (antagomir-29a) or scrambled control antagomir. **(A)**: Morphological changes of mouse myoblasts after FGF2 withdrawel for 48 hours (w/o FGF) or 48 hours after transfection with antagomir-29a shown by light microscopy (×50) compared to scrambled antagomir (ctrl). **(B)**: Effect of antagomir-29a compared to control antagomirs on proliferation rate in primary myoblasts as measured by EdU incorporation and on differentiation markers TnnI and Mb as measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Induction of luciferase activity driven by the MCK promoter and MyHC expression was measured by Western blotting. Mitochondria activity was measured by staining of TMRE and MTK using flow cytometry. $n = 3–5$. For fold changes scrambled antagomir values are set as 1 and shown as dashed line. **(C)**: Strategy for genetic deletion of miR-29a in primary myoblasts isolated from two different transgenic mouse lines, miR-29a KO and control (ctrl). **(D)**: miR-29a expression levels 4 days after the start of Tmx incubations as measured by qRT-PCR. Ctrl refers to myoblasts isolated from control mice harboring only the Pax7CE transgene. **(E)**: Morphological changes 4 days after the start of Tmx shown by light microscopy (×50). **(F)**: Proliferation rate and induction of differentiation 4 days after the start of Tmx, $n = 3–6$. Fold changes compare incubations with Tmx to incubations with ethanol in the indicated myoblast cultures. Control levels are set as 1 and shown as dashed line. *, $p < .05$; **, $p < .01$; ***, $p < .001$, Student's t test. Abbreviations: EdU, 5′-Ethynyl-2′-deoxyuridine; FGF, fibroblast growth factor; KO, knockout; Mb, myoglobin; MMTK, MitoTracker Green FM; Tnnl, troponin I; TMRE, tetramethylrhodamine.

(Fig. 1C). miR-29a expression was inversely correlated to muscle-specific miR-1 (Fig. 1B) as well as to a marker of muscle differentiation, slow fiber troponin 1 (Tnni1) (Fig. 1C). The effect of serum starvation on miR-29a levels was dose-dependent (Fig. 1D). The miR-29 family consists of three members, miR-29a, -b, and -c, that derive from two different gene loci, miR-29a/b1 and miR-29c/b2 involving different promoters. To understand which member is most relevant for myoblasts, we assessed their expression using deep sequencing of small RNAs in myoblasts and fully differentiated myotubes from both mice and humans. Importantly, miR-29a was by far the most abundant miR-29 member in both cell types and species, while miR-29b and -c were almost undetectable. The sequencing results also reaffirmed that miR-29a levels were lower in fully differentiated myotubes as compared to myoblasts (Fig. 1E). The primary transcript of the gene encoding for miR-29a/b1, but not miR-29b2/c, was decreased by serum starvation, indicating that the regulation of miR-29a under these conditions occurs mainly at the transcriptional level (Fig. 1F). Together, our data demonstrate that in primary muscle cells miR-29a is suppressed at early stages of muscle differentiation.

Consistent with the results obtained in mouse myoblasts, miR-29a was induced > 1.5-fold in human myoblasts cultured in the presence of FGF2 as determined by next generation RNA sequencing (Fig. 1G). Although under these conditions miR-29b was the highest induced miRNA, it was also 100-fold less abundant than miR-29a albeit miR-29b derives from the same primary transcript as miR-29a. The induction of miR-29a by FGF2 was again confirmed by qRT-PCR in mouse and human myoblasts. Furthermore, this upregulation was suppressed by PD173074, an inhibitor of Fgfr1, indicating specificity of FGF signaling in the modulation of miR-29a expression (Fig. 1H). Importantly, regulation of miR-29a by FGF2 was also

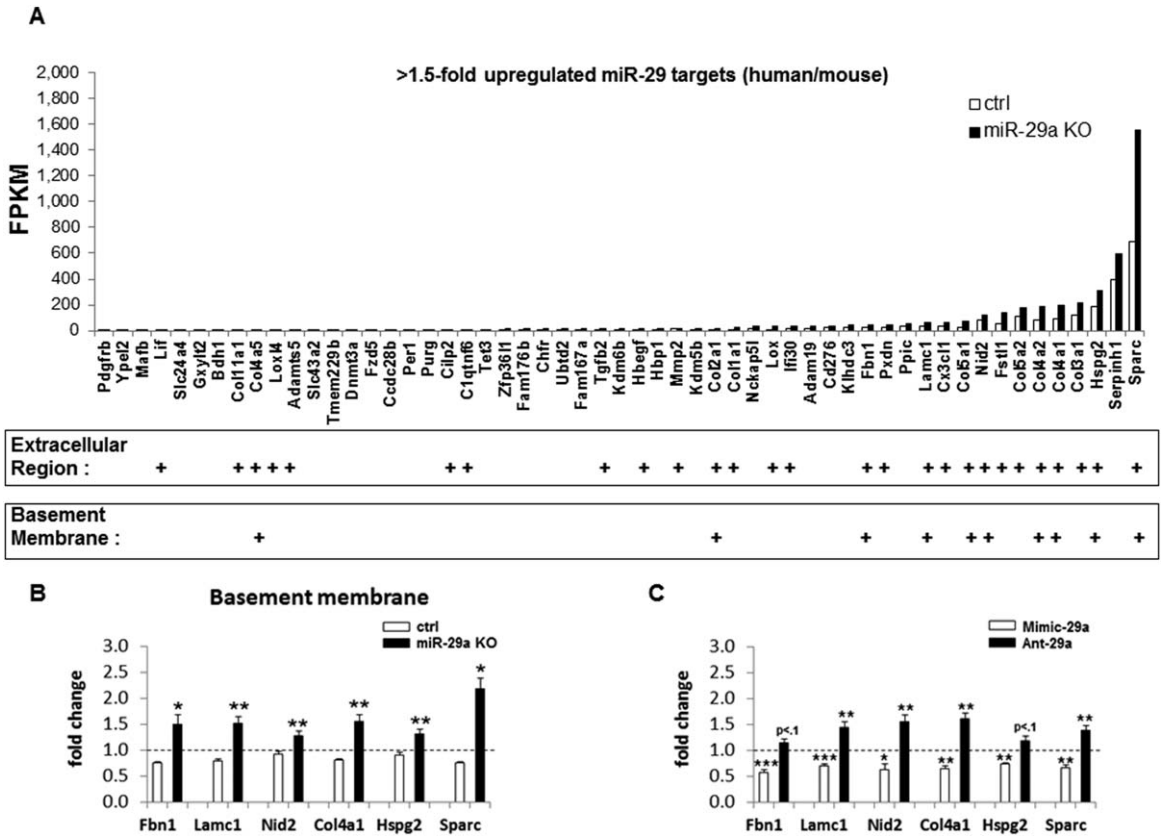

Figure 3. Components of the basemement membrane are the most abundant miR-29a targets in proliferating myoblasts. **(A)**: Proliferating myoblasts isolated from Pax7CE × miR-29a flox/flox mice were incubated with Tmx or ethanol (control), and RNA was harvested 4 days later for mRNA sequencing. Shown are all genes significantly upregulated >1.5-fold ($p < .05$) that also contain a conserved miR-29a target sequence as predicted by Targetscan (www.targetscan.org). Results are average values for Tmx (miR-29a KO) and ethanol control incubations (ctrl). $n = 3$. **(B)**: Regulation of components of the basement membrane as measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in control myoblasts ($n = 2$) and myoblasts from Pax7CE × miR-29a flox/flox mice ($n = 3$). The fold change is the effect of tmx compared to incubations with ethanol. **(C)**: Regulation of components of the basement membrane as measured by qRT-PCR 48 hours after transfection of myoblasts with either mimic-29a ($n = 5$) or antagomir-29a ($n = 9$) compared to control transfections with control mimics or scrambled antagomirs, respectively. Control levels are set as 1 and shown as dashed line. *, $p < .05$; **, $p < .01$; ***, $p < .001$, Student's t test. Abbreviations: FPKM, fragment per kilobase of exons per million reads; KO, knockout.

observed in another distinct muscle-derived lineage, the fibrocyte adipocyte progenitors (FAPs) (Fig. 1H), suggesting that regulation of miR-29a by FGF2 is not restricted to MPs and might involve progenitor lineages from other tissues. Together our results demonstrate that FGF2 regulates miR-29a in various cell types from mice and humans.

To test whether inhibition of miR-29a averts FGF2 function in primary myoblasts, we performed loss of function assays using antagomirs. Myoblasts cultured with growth media containing FGF2 display a round morphology, while FGF2 withdrawal causes them to elongate and differentiate (Fig. 2A). Inhibition of miR-29a in myoblasts grown in the presence of FGF2 leads to a similar morphological phenotype as in myoblasts grown in the absence of FGF2. Consistently, antagomir-29a decreased proliferation and increased the differentiation of myoblasts as measured by muscle differentiation markers and mitochondrial function (Fig. 2B). Cell fusion was not quantified as it was a rare event in the nonconfluent myoblast cultures. Since pharmacological inhibition of miRNAs can yield contrasting results to a genetic deletion [26, 31], we generated transgenic mice containing the floxed miR-29a/b1 gene and a Pax7CE transgene that allows for Pax7 dependent expression of a tamoxifen-inducible Cre recombinase (Fig. 2C). Tamoxifen incubation reduced miR-29a by >90% in myoblasts isolated from miR-29a floxed mice, but not from control mice (Fig. 2D). Importantly, genetic deletion of miR-29a confirmed the results obtained by the pharmacological inhibitors on myoblast morphology, proliferation, and differentiation (Fig. 2E, 2F). Together, our results demonstrate that loss of miR-29a abrogates the effects of FGF2, leading to suppressed proliferation and induction of differentiation in primary myoblasts.

To identify the relevant targets of miR-29a underlying its effects on muscle cell proliferation, we performed deep sequencing of RNA obtained from myoblasts with or without genetic deletion of miR-29a. We compared the group of upregulated genes from the RNA sequencing to a list of predicted miR-29a targets generated by the prediction program Targetscan. A total of 55 genes common to both lists were upregulated at least 1.5-fold after the miRNA knockout and were therefore identified as potential miR-29a targets (Fig. 3A). Gene ontology term clustering revealed that 50% of these genes (27/55) belong to the functional cluster "extracellular region." Importantly, the most abundant candidate targets belong to the cluster "basement membrane" and two of them (Nid2, Hspg2) represent previously unknown targets of miR-29a. Targets of the basement membrane were confirmed and validated

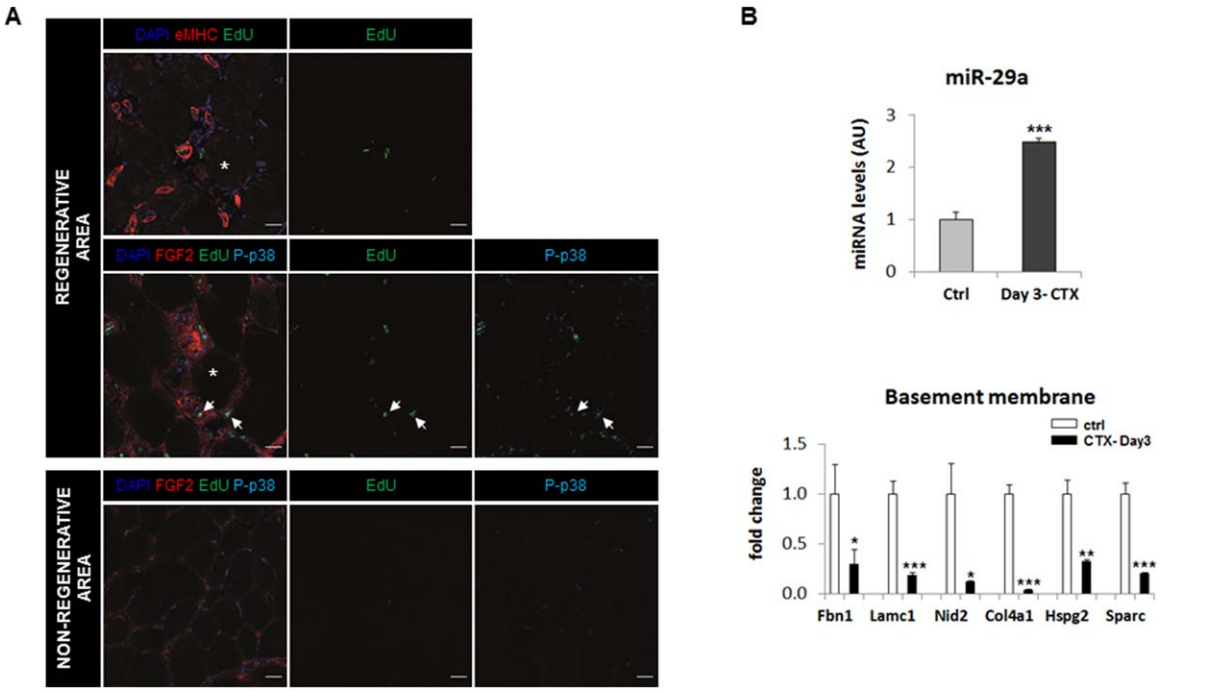

Figure 4. Regulation of FGF2, miR-29a and its targets during muscle regeneration after cardiotoxin (CTX)-induced injury. CTX was injected into TA muscle and 3–4 days later muscles were harvested. **(A)**: Frozen sections were analyzed for expression of FGF2, eMHC, P-p38, and EdU by immunofluorescence on day 4. Shown is a regenerative area with eMHC positive fibers and a nonregenerative area from the same muscle section. For the regenerative area, the asterix indicates the same position on consecutive sections. Arrows indicate nuclei positive for both EdU and P-p38. Scale bar = 50 μm. **(B)**: Activated MPs were isolated from the TA muscle 3 days after CTX injection using flow cytometry and directly used for RNA isolation. miR-29a levels and expression of components of the basement membrane were analyzed using quantitative reverse transcriptase polymerase chain reaction and compared to MPs isolated from uninjected muscle, $n = 4$. *, $p < .05$; **, $p < .01$; ***, $p < .001$, Student's t test. Abbreviations: CTX, cardiotoxin; DAPI, 4′,6-diamidin-2-phenylindol; EdU, 5′-Ethynyl-2′-deoxyuridine; eMHC, embryonic myosin heavy chain; FGF2, fibroblast growth factor 2.

using qRT-PCR in myoblasts after genetic or antagomir-mediated inhibition of miR-29a (Fig. 3B, 3C) or, conversely, after overexpression of miR-29a using miRNA mimics (Fig. 3C). Together, these results identify the major components of the basement membrane as the most abundant targets of miR-29a in myoblasts.

Next we investigated the relevance of the FGF2-miR-29a pathway for MP proliferation in vivo using CTX-induced muscle regeneration, where maximal proliferation occurs 3–4 days after CTX administration [32]. CTX was injected into the tibialis anterior muscle and after 4 days muscle sections were analyzed for FGF2 and phospho-p38 (P-p38) by immunostaining. Regenerative areas with eMHC positive myofibers were characterized by increased FGF2 staining and higher numbers of EdU and P-p38 positive nuclei compared to nonregenerative areas (Fig. 4A). Therefore, the FGF2 signaling pathway is specifically activated in regenerative muscle areas. To correlate the expression of miR-29a with FGF2 signaling, MPs were isolated using FACS 3 days after CTX injection and directly processed for RNA isolation without intermittent cell culture. miR-29a expression in MPs from CTX was higher in the treated muscle than control (Fig. 4B), indicating that miR-29a expression is induced concomitantly with activation of MPs. Conversely, the confirmed targets of miR-29a that are components of the basement membrane were downregulated (Fig. 4B). These results demonstrate that FGF2, miR-29a and its targets are reciprocally regulated during muscle regeneration. To investigate if miR-29a is necessary for the formation of new myofibers during muscle regeneration, we deleted miR-29a specifically in MPs and injected CTX into the tibialis anterior muscle of control and knockout mice (Fig. 5A). Tamoxifen-induced Cre induction efficiently reduced miR-29a levels in activated MP after CTX injection by >90% (Fig. 5A). Conversely, the expression of its basement membrane targets significantly increased in the miR-29a knockout MPs confirming that they are miR-29a targets in vivo (Fig. 5A). The deletion of miR-29a specifically decreased the number of α7-integrin + MPs and did not alter the cell number of other muscle-resident stem cell populations (Sca1 + or lin + cells) (Fig. 5B). Similar to our results obtained in vitro, deletion of miR-29a also decreased the proliferation rate of MPs in vivo (Fig. 5C), the consequences of which were the reduction of the total number of newly formed eMHC positive fibers as well as the proliferation rate of MPs merging to these newly formed fibers (percent of myofibers positive for both eMHC and EdU) 4 days after CTX injection (Fig. 5D). The effect of tamoxifen on MPs with only one Pax7 allele was excluded in a cohort of tamoxifen-fed mice harboring the Pax7CE transgene without the floxed miR-29a allele (Pax7CE/ + mice) (Supporting Information Fig. 2). The proliferation phenotype resulted in reduced skeletal muscle mass 9 days after CTX, which persisted for at least 30 days after CTX together with a decreased number of medium-sized myofibers (Fig. 6A). At day 30, fibrotic areas between fibers became frequently apparent in muscle tissue from the knockout mice (Fig. 6B). Muscles from miR-29a knockout mice also harbored more pronounced adipocyte formations compared to controls (Fig. 6B). These effects were not observed in tamoxifen-treated Pax7CE/ + control mice apart from a minor decrease in muscle mass in the long-term tamoxifen treatment protocol

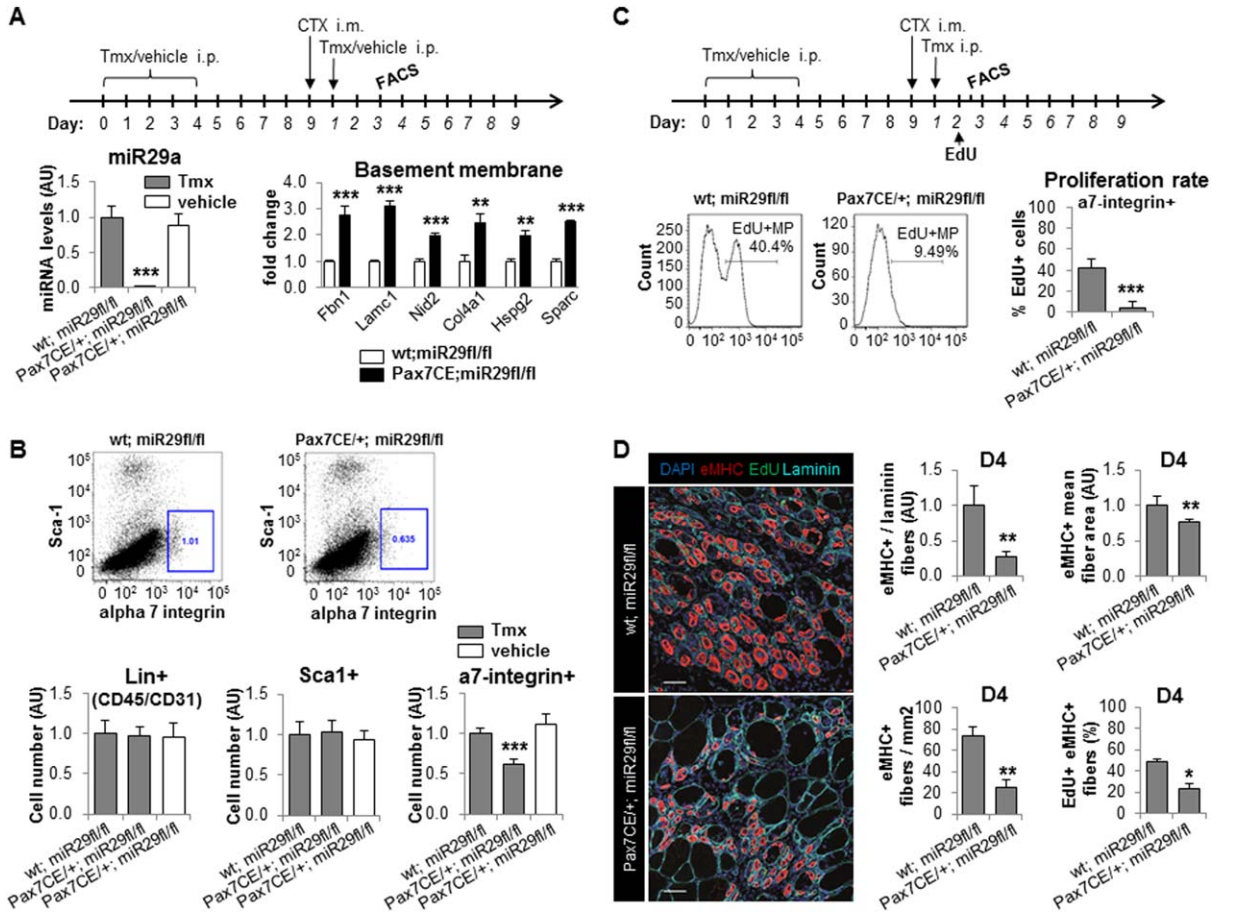

Figure 5. Tamoxifen-induced deletion of miR-29a in Pax7 + myogenic progenitors (MPs) in vivo decreases MP proliferation and prevents skeletal muscle regeneration. miR-29a was deleted in MPs using Tmx injections before muscle regeneration was induced in TA muscles. Muscle tissues were harvested at different times after CTX injection as indicated in the time lines. **(A)**: Myogenic progenitors were isolated using flow cytometry at day 3 after CTX injection for direct RNA isolation. miR-29a levels were quantified using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Grey bars indicate mice that received Tmx, white bars indicate mice that received corn oil (vehicle). $n = 4$, ANOVA with Dunnett's multiple comparison test.Gene expression of miR-29a basement membrane targets were measured by qRT-PCR in the samples from the Tmx treated mice, $n = 4$, Student's t test. **(B)**: Cell population numbers for Sca-1 + and a7-integrin + cells as analyzed as percentage of all live lin- cells. A representative FACS is shown. Lin + cells were analyzed as percentage of all live cells, $n = 8$, ANOVA with Dunnett's multiple comparison test. **(C)**: MP in vivo proliferation rate was measured by EdU incorporation and FACS analysis, $n = 4$, Student's t test. **(D)**: eMHC positive (red) and EdU positive (green) myofibers were analyzed on frozen sections from tmx-treated mice 4 days after CTX injection. Scale bar = 50 μm. Total number of myofibers was detected using laminin antibodies (blue), $n = 4$–6, Student's t test. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: CTX, cardiotoxin; DAPI, 4′,6-diamidin-2-phenylindol; EdU, 5′-Ethynyl-2′-deoxyuridine; eMHC, embryonic myosin heavy chain; FACS, fluorescence activated cell sorting; Tmx, Tamoxifen; wt, wild type.

(Supporting Information Fig. 3A, 3B). There was no evidence of persistent muscle fiber damage following deletion of miR-29a as indicated by no changes in creatine kinase activity (58 U/l ± 11 for Pax7CE/+; miR29fl/fl vs. 60 U/l ± 16 for wt; miR-29fl/fl, average ± SEM) and absence of Evans blue dye fiber staining in muscle sections (data not shown) at D30. Deletion of miR-29a also did not affect fiber type composition in the regenerated muscle areas (Supporting Information Fig. 3C). We conclude that miR-29a is critical for the proliferation rate of MPs and that loss of miR-29a leads to decreased muscle formation and fibrosis following muscle injury.

To determine whether miR-29a is involved in a more physiologic stimulation of muscle regeneration, we subjected the floxed miR-29a and Pax7CE + mice to a single bout of eccentric exercise (downhill treadmill running) (Fig. 7A). Tamoxifen-induced MP-specific deletion of miR-29a did not affect the exercise tolerance of the mice 1 week after the eccentric exercise bout (Fig. 7B) and is likely the result of the eccentric exercise inducing only small areas of muscle regeneration (Fig. 7C). Importantly, gastrocnemius muscle fiber formation following eccentric exercise was significantly reduced in mice, in which miR-29a was depleted (Fig. 7C), but not in tamoxifen-treated Pax7CE/ + control mice (Supporting Information Fig. 4). EdU positive fibers were observed in muscles from 4 out of 14 control mice and from none out of 13 miR-29a knockout mice (Fig. 7C). Together, we conclude that miR-29a is necessary for muscle regeneration not only after CTX-induced injury, but also in response to eccentric exercise.

Discussion

The results of our study reveal that miR-29a expression in MPs is required for muscle regeneration. We propose that during the proliferative phase of muscle regeneration FGF2

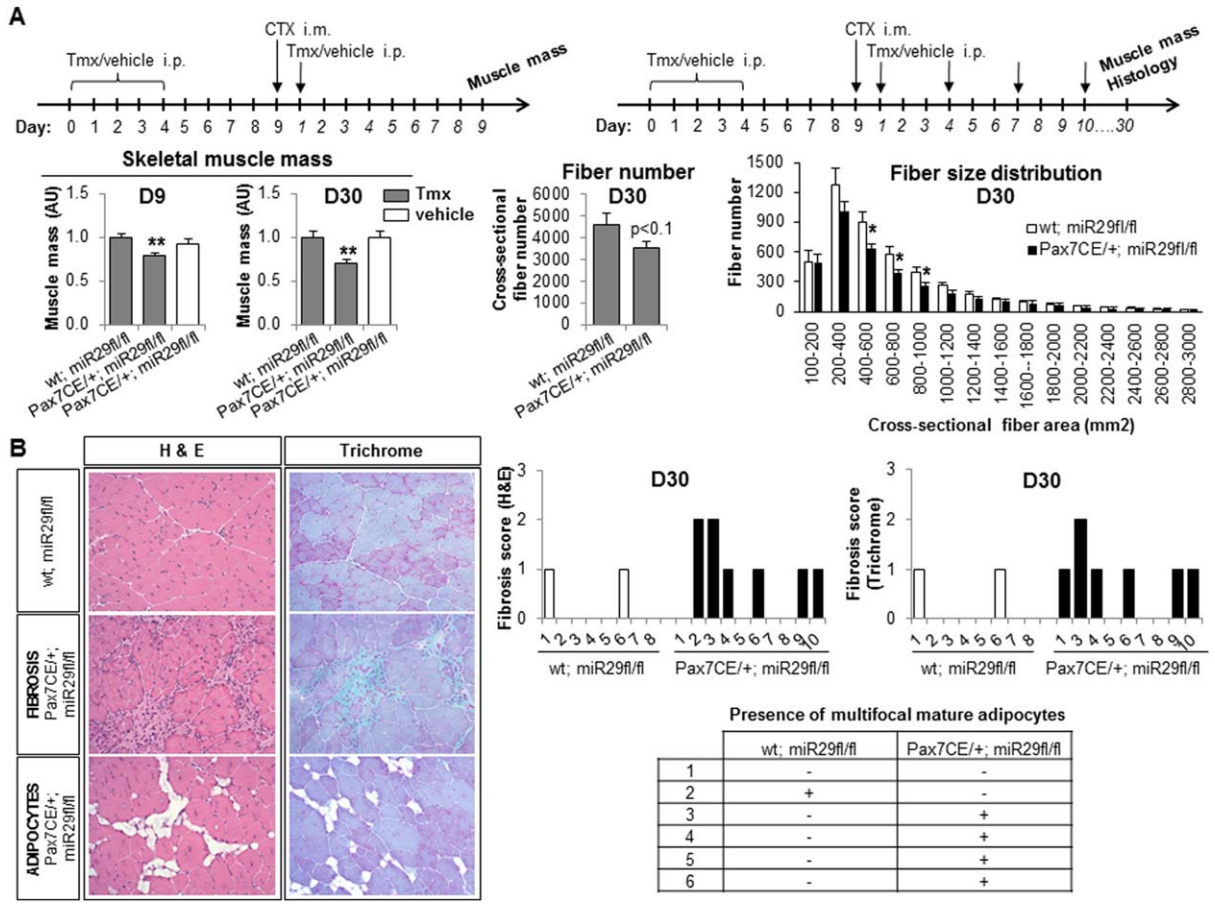

Figure 6. Tamoxifen-induced deletion of miR-29a in Pax7 + myogenic progenitors (MPs) during muscle regeneration leads to decreased muscle mass and fibrosis. miR-29a was deleted in MPs using Tmx injections before muscle regeneration was induced in TA muscles and muscle mass and histology analyzed 9 or 30 days later. **(A)**: Skeletal muscle mass was analyzed as ratio of the injected muscle compared to the uninjected muscle of the same mouse. Results are shown relative to Tmx-injected wt; miR29fl/fl control mice, $n = 5–8$ (D9), $n = 7–9$ (D30), ANOVA with Dunnett's multiple comparison test. Fiber number and size were analyzed on sections based on laminin immunofluorescence, $n = 6$, Student's t test. **(B)**: H&E staining on cryosections from Tmx treated mice at D30 after CTX injection was evaluated for fibrosis and presence of multifocal mature adipocytes within the endomysial compartment (×200). Fibrosis was also estimated using Gomori trichrome staining. *, $p < .05$; **, $p < .01$, compared to control. Abbreviations: CTX, cardiotoxin; Tmx, Tamoxifen; wt, wild type.

induces miR-29a, which leads to reduced expression of key components of the basement membrane. This in turn is followed by a further release of growth factors from the degrading basement membrane and an increase in satellite cell niche elasticity, thus providing conditions for MP proliferation (Fig. 7D). We demonstrate that miR-29a targets the major structural components of the basement membrane, including Col4a1, Lamc1, Nid2, and Hspg2, and that upregulation of miR-29a and downregulation of its targets in activated MPs within the adult muscle stem cell niche precedes muscle regeneration in vivo. This novel layer of regulation of the basement membrane by a miRNA provides a mechanism for the previously described changes in gene expression in MPs during muscle regeneration. Quiescent MPs express higher levels of the components of the basement membrane compared to activated MPs, including the miR-29a targets Col4a1, Lamc1, and Hspg2 that we identified in our study [10, 11].

Following muscle injury, preventing the dismantling of the basement membrane and its glycoprotein components could decrease MP proliferation by changing the substrate elasticity of the niche [33, 34] or by decreasing access of cellular receptors to growth factors [35–37]. We identified the targets of miR-29a based on their high abundance in myoblasts. These targets have previously been shown to promote differentiation of mouse and human primary myoblasts when added to cultured myoblasts as a soluble basement membrane [38, 39] providing strong evidence for their involvement in miR-29a function as observed in our study. Genetic deletion of miR-29a resulted in decreased proliferation of MPs in vivo and in vitro and promoted differentiation in cultured myoblasts. These defects in vivo led to failure of myofiber regeneration, consecutive fibrotic replacement and reduction in muscle mass. Importantly, this phenotype persisted even 30 days postinjury while normal muscle regenerated successfully within 14 days [5], underscoring the critical role of miR-29a for long-term recovery of skeletal muscle.

Transgenic mice overexpressing miR-499 have increased slow twitch fiber type and a longer mean running time on a treadmill [40], but the impact of genetically deleting a miRNA on the response of skeletal muscle to exercise has not been demonstrated yet. Downhill running has been used as a physiological model for the stimulation of muscle regeneration, and it is well-established that exercise can cause muscle damage especially when it involves eccentric components in both mice [41] and humans [42]. The activation of MPs is known to be

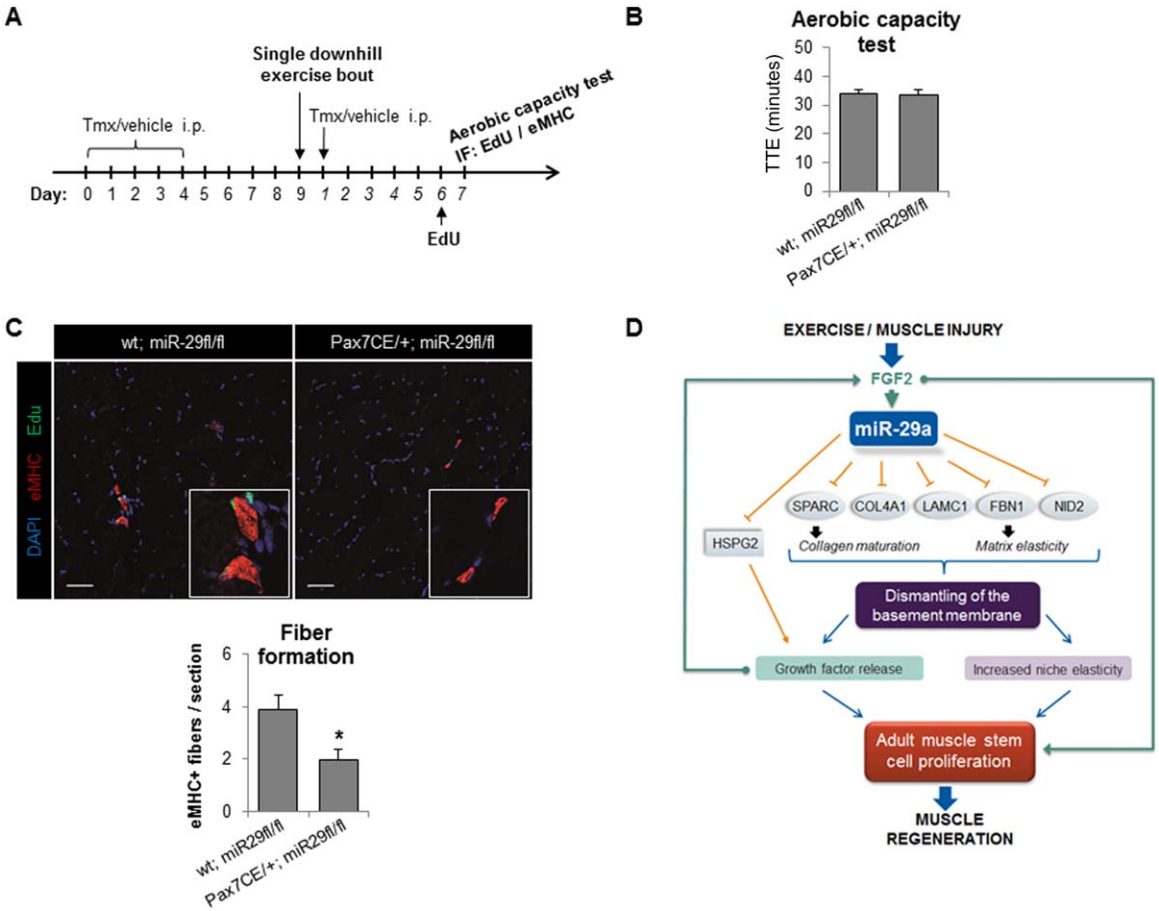

Figure 7. Tamoxifen-induced deletion of miR-29a in Pax7 + myogenic progenitors (MPs) in vivo decreases muscle fiber formation after a single bout of eccentric exercise. **(A)**: Strategy for the induction of Cre recombinase in Pax7 + MPs using Tmx injections during exercise induced muscle regeneration. **(B)**: Exercise tolerance of tmx-treated mice tested 7 days after the downhill running exercise. $n = 15$ for controls (wt; miR29fl/fl) and $n = 14$ for miR-29a knockout (KO) mice (Pax7CE/+; miR29fl/fl). **(C)**: Results for new fiber formation as determined by eMHC and EdU immunostaining on frozen sections from gastrocnemius muscles 7 days after downhill running, $n = 14$ for controls and $n = 13$ for miR-29a KO mice. Scale bar = 50 μm. *, $p < .05$, Student's t test. **(D)**: Model for the regulation of adult muscle stem cell proliferation by FGF2 and miR-29a. Abbreviations: DAPI, 4′,6-diamidin-2-phenylindol; EdU, 5′-Ethynyl-2′-deoxyuridine; eMHC, embryonic myosin heavy chain; Tmx, tamoxifen; TTE, time to exhaustion; wt, wild type.

involved in muscle hypertrophy after repeated eccentric exercise interventions [43, 44]. The mechanisms that regulate MP proliferation during eccentric exercise could involve ECM modulation. For example, a single bout of eccentric exercise causes changes in collagen IV protein and induces its degradation [45] and transgenic overexpression of skeletal muscle specific α7β1-integrin accelerated myogenesis [41]. We demonstrate that muscle regeneration after exercise depends on miR-29a and intact MP proliferation, similarly to CTX-induced muscle injury. The deletion of miR-29a in adult skeletal muscle stem cells blunted the formation of new myofibers after a single bout of exercise. As expected, the reduction in muscle fiber regeneration following downhill running did not result in any detectable impairment of exercise tolerance. We chose a more physiological exercise protocol (single bout of 90 minutes) that resulted in realistic mild muscle regeneration, whereas more strenuous and repeated running bouts are required to induce a significant detriment in performance [44]. Therefore, we conclude that miR-29a participates in MP proliferation in adult skeletal muscle following eccentric exercise.

We did not follow up on the regulation of miR-29b and miR-29c in myoblasts based on their very low abundance and since serum starvation regulated only the primary transcript of miR-29a/b, but not miR-29b/c. Contrary to our results, miR-29a–c levels were reported to be induced in the C2C12 cell line during serum starvation and differentiation, and miR-29b expression was reduced in human rhabdomyosarcoma primary cells based on qRT-PCR [46–49]. These studies are based on the regulation of the miR-29 family by the transcription factor YY1, which has binding sites in the miR-29b/c, but not the miR-29a/b promoter. The discrepancy of our results to the cancer cell line C2C12 is not surprising, since in cancers the miR-29 family plays a dual role depending on the type of cancer in which it is expressed. In hematopoietic and cervical cancer cells, for example, miR-29 acts as a tumor suppressor by targeting apoptosis, cell cycle, and proliferation pathways [50, 51]. On the other hand, miR-29a stimulates proliferation in breast cancer and mouse B cells [52, 53]. Therefore, the miR-29 family might be differentially regulated in cancerous cells and cancer cell lines compared to miR-29a in muscle MPs in vitro and in vivo. In addition, differences in miRNA expression based on qRT-PCR could also be caused by the normalization method applied. Indeed, we noticed that the RNA transcript of U6 is twofold downregulated in

differentiated versus proliferating primary myoblasts in contrast to sno234 or miR-let-7a (Supporting Information Fig. 5) and normalizing miR-29a expression to U6 RNA as previously described [46–49] failed to demonstrate the significant decrease of miR-29a levels in myoblasts after serum starvation. In our study, we have used several independent approaches to confirm downregulation of miR-29a in MP differentiation, including microarrays, northern blotting as well as next generation RNA sequencing and therefore normalizing qRT-PCR results to a gene with stable expression, such as sno234, might be more appropriate under these conditions. Indeed, results from miRNA microarray studies that do not depend on single gene normalizers support our results. A previous microarray analysis on C2C12 differentiation failed to detect induction of miR-29a [54], and miRNA microarray expression profiling in murine skeletal myocytes PMI28 and primary cultures of quail myoblasts revealed downregulation of miR-29b during muscle cell differentiation [55, 56]. Because of the discrepancies between primary myoblasts and C2C12 cells as well as overexpression artifacts, we have used two independent loss-of-function approaches, genetic deletion as well as pharmacologic inhibition of miR-29a to demonstrate the pivotal role of this miRNA for MP proliferation and differentiation. Based on the high expression of miR-29a in proliferating myoblasts and MPs, we chose to investigate miR-29a function at early stages of muscle differentiation. It is possible that deletion of miR-29a during later stages of muscle differentiation might reveal different effects as miR-29 has been assigned a promyogenic function in terminal differentiation of C2C12 myotubes by targeting YY1 [46], Rybp, and Akt3 [48, 49]. However, none of these genes were regulated in our miR-29a knockout myoblasts (Supporting Information Table 1). Lastly, attempts to use overexpression of miR-29a to stimulate MP proliferation have to consider the level of overexpression and regulation of false positive targets. Indeed, adenovirus-mediated overexpression of miR-29a decreased cell proliferation in primary murine myoblasts by targeting insulin-like growth factor 1, phosphatidylinositol 3-kinase regulatory subunit alpha p85 (PIK3R1), and B-Myb [57], which again were not regulated in our miR-29a knockout myoblasts (Supporting Information Table 1).

We identified miR-29a as part of a screen for miRNAs regulated by FGF2 signaling in myoblasts. Inhibition of miR-29a attenuated the stimulation of myoblast proliferation by FGF2, indicating that miR-29a could be involved in mediating downstream effects of this growth factor. Since the regulation of miR-29a by FGF2 was also observed in FAPs, miR-29a might also be implicated in FGF2 signaling in other tissues. In a clinical setting, suppression of miR-29a expression might offer therapeutic opportunities for pathologic conditions where FGF2 signaling is overly activated in the adult muscle stem cell niche, particularly during aging. FGF2 is the highest induced FGF ligand in aged muscle fibers, and its protein was increased specifically in the adult muscle stem cell niche under the basal lamina of muscle fibers [22]. Abnormal activation of the FGF pathway leads to increased proliferation and depletion of MPs and provides a mechanism for the decreased number of MPs in aged skeletal muscle [22]. Although a complete miR-29a inhibition will lead to defects in regeneration, a more subtle correction of miR-29a levels under these conditions would be expected to reverse some of the negative effects of increased FGF2 signaling and preserve quiescent MPs. Intriguingly, a microarray screen in skeletal muscle from 4 months old versus 28 months old rats revealed a 13-fold induction of miR-29a [57].

In several human diseases mutations in components of the basement membrane are linked to muscle weakness and myopathy emphasizing their importance for skeletal muscle. For example, mutations in laminin-2 cause at least 30% of congenital muscle diseases [58], and mutations in collagen VI [59] or collagen IV [60] are involved in a broad spectrum of myopathies. Moreover, mutations in the miR-29a target FBN1 can result in muscle weakness and decreased muscle mass presumably by poor anchoring of the myofiber basement membrane to the ECM [61]. We identified a novel role for miR-29a as a regulator of the basement membrane during muscle regeneration. The targeting of miR-29a presents a new strategy to improve disease states with aberrant FGF2 signaling and expression of basement membrane components.

Conclusion

FGF2 signalling in myoblasts and adult muscle stem cells requires miR-29a. miR-29a levels are increased by FGF2 and this miRNA coordinates the expression of several key members of the basement membrane in myogenic progenitors cells. miR-29a is necessary for muscle stem cell proliferation and formation of myofibers during muscle regeneration.

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Author Contributions

A.G. and J.K.: designed and analyzed most experiments and prepared the manuscript; A.G.: performed most experiments; J.K.: participated in animal experiments; T.L.M. and M.R.: designed and performed exercise experiments; E.L.: performed cryosections and designed and conducted all immunofluorescent analyses; E.J.R.: conducted immunohistochemistry analyses; A.M.: analyzed MP proliferation rate in vivo using FACS; K.T.: characterized myoblast cultures; A.H.: participated in qRT-PCR analysis; C.M.C.: provided knockout mice. All authors discussed and commented on the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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