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RESEARCH PAPER

Linc-RAM is required for FGF2 function in regulating myogenic cell differentiation

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ABSTRACT

Myogenic differentiation of skeletal muscle stem cells, also known satellite cells, is tightly orchestrated by extrinsic and intrinsic regulators. Basic fibroblast growth factor (FGF2) is well documented to be implicated in satellite cell self-renewal and differentiation by repressing MyoD. We recently identified a MyoD-regulated and skeletal muscle-specifically expressed long non-coding RNA Linc-RAM which enhances myogenic differentiation by facilitating MyoD/Baf60c/Brg1 complex assembly. Herein, we investigated the transcriptional regulation and intracellular signaling pathway in mediating Linc-RAM gene expression during muscle cell differentiation. Firstly, we demonstrate *Linc-RAM* is negatively regulated by FGF2 via Ras/Raf/Mek/Erk signaling pathway in muscle cells. Overexpression of MyoD significantly attenuates repression of *Linc-RAM* promoter activities in C2C12 cells treated with FGF2. Knockout of *MyoD* abolishes FGF2-mediated repression of *Linc-RAM* gene transcription in satellite cells sorted from skeletal muscle of *MyoD*$^{-/-}$*;Pax7-nGFP* mice, suggesting inhibition of MyoD is required for FGF2-mediated expression of *Linc-RAM*. For the functional significance, we show that overexpression of Linc-RAM rescues FGF2-induced inhibition of C2C12 cell differentiation, indicating inhibition of Linc-RAM is required for FGF2-mediated suppression of myogenic differentiation. Consistently, we are able to further corroborate the requirement of Linc-RAM inhibition for FGF2-modulated repression of myogenic differentiation by using an *ex vivo* cultured single fiber system and satellite cells sorted from *Linc-RAM*$^{-/-}$*;Pax7-nGFP* knockout mice. Collectively, the present study not only reveals the intracellular signaling in FGF2-mediated Linc-RAM gene expression but also demonstrate the functional significance of Linc-RAM in FGF2-mediated muscle cell differentiation.

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FGF2; MyoD; muscle cell differentiation; Long non-coding RNA; Linc-RAM

Introduction

Satellite cells are resident adult skeletal muscle stem cells that are the indispensable primary contributors to skeletal muscle regeneration [1]. In resting muscle, satellite cells are normally quiescent; in response to stress or injury, however, they activate, proliferate, and either differentiate or self-renew to replenish the quiescent stem cell pool [2]. Thus, the differentiation potential and reversible quiescence of satellite cells are critical for their functions in regulating postnatal muscle growth, regeneration, and tissue homeostasis.

These processes are tightly orchestrated by extrinsic cues from the stem cell niche and by intrinsic regulators, including intracellular signaling components and transcriptional factors [3]. The critical extrinsic signals include fibroblast growth factors (FGFs), Wnt7a, Notch ligands, transforming growth factor-β (TGF-β), brain-derived neurotrophic factor (BDNF), mechano-growth factor (MGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-I) [3–11]. These extrinsic factors act on multiple intracellular signaling pathways to modulate the self-renewal, proliferation and differentiation of satellite cells. FGFs, which are essential for skeletal muscle stem cell self-renewal, inhibit the expressions of myogenic differentiation genes, such as those encoding myogenic differentiation 1 (Myod1, MyoD) and myogenin (MyoG) [12]. Satellite cells express high levels of FGF receptors 1 and 4 [13,14]. Of the multiple FGFs, FGF2, also known as basic FGF (bFGF), has been well documented as a potent regulator of satellite cell function [5,15]. FGF2 regulates satellite cell function by activating multiple intracellular signaling pathways, including Erk MAPK, p38α/βMAPK, PI3 kinase, PLCγ, and STATs [16–20]. Interestingly, FGF2 appears to facilitate satellite cell division and muscle regeneration in dystrophic mice [21], and the transgenic delivery of FGF2 and FGF6 to damaged muscle has been shown to enhance their regeneration in mice [22]. However, the downstream target genes responsible for the FGF2-mediated regulation of satellite cell behavior have not yet been fully elucidated.

A growing number of evidence show that miRNAs play critical role in regulating satellite cell functions [23]. miR-1 and miR-206 regulate satellite cell differentiation by repressing *Pax7* [24]. miR-27a, which is expressed in differentiating skeletal muscle of the embryonic myotome and in activated SCs of adult muscles, promotes satellite cell differentiation by targeting *Pax3* [25]. We recently demonstrated that miR-431 regulates satellite cell heterogeneity by refining Pax7 expression [26]. Moreover, miR-127, which is encoded by the same

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miRNA cluster as miR-431, was shown to accelerate muscle regeneration and ameliorate muscular dystrophy by enhancing satellite cell differentiation via the targeting of sphingosine-1-phosphate receptor 3 (S1PR3) in mice [27].

lncRNAs, which are defined as being> 200 nt in length, often show spatiotemporally restricted expression patterns and have been functionally implicated in cell lineage specification and differentiation during development. For example, the brain-specific lncRNA, RMST, regulates neural fate by physically interacting with Sox2 [28], and the heart-expressed lncRNA, Braveheart, is required for cardiovascular lineage commitment [29]. Several skeletal muscle-expressed lncRNAs have been reported to control myogenic cell differentiation. For instance, Linc-MD1 functions as a competing endogenous RNA [30], and Linc-YY1 interacts with Yin Yang 1 (YY1) to regulate target gene expression [31]. The upstream regulatory region of the *MyoD* gene encodes several muscle-specific lncRNAs that positively regulate myogenic lineage differentiation, including eRNA [32], LncMyoD [33], and MUNC [34]. We recently identified a skeletal muscle-specifically expressed and MyoD-regulated lncRNA Linc-RAM (Linc-RNA Activator of Myogenesis) that functionally enhances myogenic cell differentiation by interacting with MyoD to facilitate assembly of the SWI/SNF chromatin-remodeling complex at myogenic gene promoters [35]. However, the upstream triggers and intracellular signaling involved in the MyoD-mediated regulation of Linc-RAM gene expression during muscle cell differentiation remained unexplored. Here, we demonstrate that transcription of the MyoD-regulated Linc-RAM is repressed by FGF2 via the Ras/Raf/Mek/Erk signaling pathway. Furthermore, we provide *ex vivo* and *in vivo* data showing that Linc-RAM is functionally required for the FGF2-controlled differentiation of satellite cells.

Results

Linc-RAM *is negatively regulated by FGF2 in muscle cells*

We recently identified a muscle-specifically expressed and MyoD-regulated lncRNA Linc-RAM and reveal that functional significance in enhancing myogenic cell differentiation [35]. Here, we set out to identify the upstream regulators and intracellular signaling pathways of the MyoD-mediated transcriptional regulation of *Linc-RAM* during muscle cell differentiation. To this end, we grew C2C12 cells in differentiation medium (DM) in the presence or absence of various cytokines, including basic fibroblast growth factor (FGF2), insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF-β), and myostatin (MSTN) [5,12,36,37]. Expressional analysis of *Linc-RAM* in treated cells at various time points revealed that only FGF2 affected the expression of *Linc-RAM* gene expression, which was remarkably reduced in FGF2-treated C2C12 cells cultured in DM (Fig. 1A). The expression levels of *MyoD* and *MyoG*, two downstream targets of FGF2 signaling, were assayed as positive controls (Fig. 1A). To validate the FGF2-mediated down-regulation of *Linc-RAM* in muscle cells, satellite cells were flow cytometrically sorted from the skeletal muscles of *Pax7-nGFP* knock-in mice, and then cultured in the presence or absence of FGF2. Consistent with the data obtained in C2C12 cells, FGF2 treatment significantly decreased the expressions of *Linc-RAM, MyoD*, and *MyoG* while increasing the level of *Pax7* in the tested satellite cells at 24 hr (Fig. 1B) and 48 hr (Fig. 1C) post-treatment. To provide molecular evidence of the ability of FGF2 to down-regulate *Linc-RAM* transcription, we performed luciferase reporter gene assays driven by the *Linc-RAM* promoter [35] in differentiating C2C12 cells cultured in the presence or absence of FGF2. *MyoD* and *MyoG* promoter-reporter genes were used as positive controls. As shown in Fig. 1D, *Linc-RAM* promoter activity was significantly blocked in the FGF2-treated cells. Together, our data indicate that transcription of *Linc-RAM* is negatively regulated by FGF2 in muscle cells.

FGF2 regulates Linc-RAM *gene expression via the Ras/Raf/Mek/Erk signaling pathway*

Next, we investigated intracellular signaling pathway involved in the FGF2-mediated repression of *Linc-RAM* gene expression in muscle cells. Given the critical role of the Ras/Raf/Mek/Erk cascade in the FGF2-regulated expression of myogenesis-specific genes [38,39], we hypothesized that this pathway could control the FGF2-mediated repression of *Linc-RAM* gene expression during myogenic differentiation. To test this possibility, we treated C2C12 cells with the Mek inhibitor, PD98059, followed by FGF2, and analyzed *Linc-RAM* expression. As shown in Fig. 2A, the FGF2-induced phosphorylations of Mek and Erk were significantly blocked by PD98059, as was the FGF2-mediated repression of *Linc-RAM* gene expression (Fig. 2B). The expressions of *MyoD* and *MyoG* were examined as controls (Fig. 2B). To corroborate these observations, we performed promoter-reporter assays in C2C12 cells treated with PD98059 followed by FGF2. As shown in Fig. 2C, FGF2 significantly reduced *Linc-RAM*- and *MyoD*-promoter-dependent luciferase activities, but PD98059 pretreatment significantly rescued these effects of FGF2 (Fig. 2C). Our findings suggest that FGF2 regulates *Linc-RAM* gene expression via the MEK/Erk signaling pathway. To test whether Ras/Raf kinase activity was required for the FGF2-induced repression of *Linc-RAM* expression during differentiation, we treated C2C12 cells with S-farnesylthioacetic acid (FTA, a Ras inhibitor) or GW5074 (GW, a Raf inhibitor) in the presence or absence of FGF2. Indeed, the inhibitions of Ras and Raf both significantly attenuated the FGF2-mediated suppressions of *Linc-RAM, MyoD*, and *MyoG* gene expression (Fig. 2D, E), suggesting that FGF2 negatively regulates *Linc-RAM* expression via the Ras/Raf signaling pathway. Collectively, our experimental data demonstrate that FGF2 represses *Linc-RAM* expression via the Ras/Raf/Mek/Erk signaling pathway during muscle cell differentiation.

MyoD is required for FGF2-mediated Linc-RAM *gene expression*

MyoD, a master transcription factor for myogenesis, has been identified as a downstream target of FGF2 in regulating myogenic gene expression [12], and *Linc-RAM* has been shown to be transcriptionally regulated by the MyoD both *in vitro* and *in vivo* [35,40]. Therefore, we hypothesized that FGF2 might

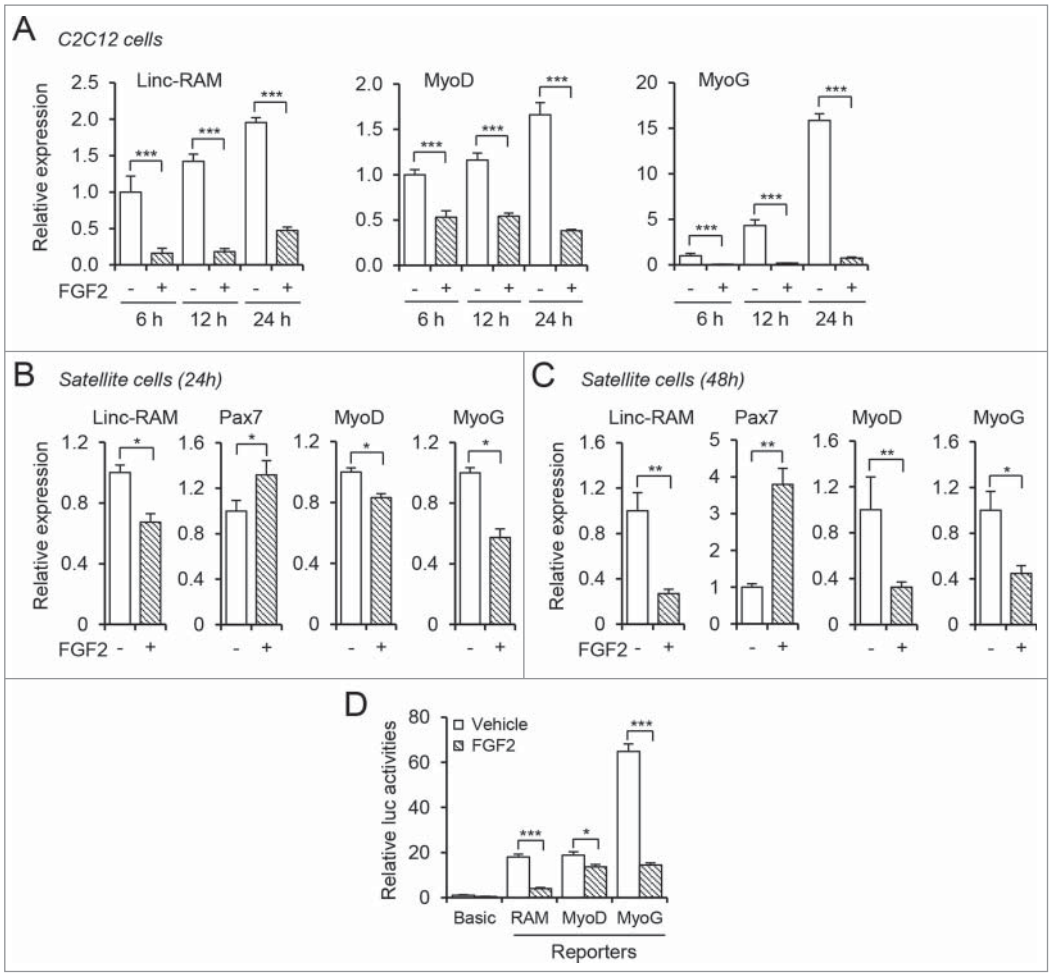

Figure 1. Linc-RAM is negatively regulated by FGF2 in muscle cells. A. C2C12 cells were treated with FGF2 in differentiation medium (DM) for the indicated times. The expressions of *MyoD, MyoG*, and *Linc-RAM* were examined by real-time quantitative RT-PCR (RT-qPCR). B. Satellite cells sorted from *Pax7-nGFP* mice were treated with FGF2 in growth medium (GM) for 24hr, and the expressions of *Pax7, MyoD, MyoG*, and *Linc-RAM* were examined by RT-qPCR. C. Satellite cells sorted from *Pax7-nGFP* mice were treated with FGF2 in GM for 48hr, and the expressions of *Pax7, MyoD, MyoG*, and *Linc-RAM* were examined by RT-qPCR. D. The responsiveness of the *Linc-RAM* gene promoter to FGF2 was examined in C2C12 cells using a luciferase reporter gene assay. C2C12 cells were treated with FGF2 and then transfected with promoter-reporter plasmids for *Linc-RAM* (RAM). The *MyoD* and *MyoG* gene promoters served as positive controls. Promoter activity is expressed as luciferase activity relative to that in the vehicle control group (defined as 1). Values are means ± s.e.m. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All *P* values were generated using the two-tailed Student's *t*-test.

negatively regulate *Linc-RAM* gene transcription by down-regulating MyoD expression in muscle cells. To test this possibility, we first examined the FGF2-mediated repression of *Linc-RAM* expression in satellite cells sorted from the skeletal muscles of MyoD-knockout mice (*MyoD*$^{-/-}$*;Pax7-nGFP*) and their WT littermates (*Pax7-nGFP*). The cells were cultured in the presence or absence of FGF2, and *Linc-RAM* expression was assessed. FGF2 treatment significantly down-regulated *Linc-RAM* expression in satellite cells sorted from *Pax7-nGFP* WT mice, but this effect was abolished in satellite cells sorted from *MyoD*$^{-/-}$*;Pax7-nGFP* mice (Fig. 3A), suggesting that the ability of FGF2 to repress *Linc-RAM* expression is modulated by MyoD in satellite cells. To further confirm this finding, we examined the luciferase activity obtained from a reporter gene driven by the *Linc-RAM* promoter [35] in FGF2-treated C2C12 cells overexpressing MyoD. Indeed, the FGF2-induced suppression of Linc-RAM promoter activity was completely restored by MyoD overexpression (Fig. 3B). Thus, the data obtained from both gain- and loss-of-function experiments demonstrate that MyoD is required for the FGF2-mediated down-regulation of *Linc-RAM* gene expression in muscle cells. Consistent with these findings and the previously reported ChIP-PCR data obtained with a MyoD antibody [35], our ChIP-PCR experiments revealed that FGF2 treatment significantly reduced the enrichments of MyoD protein on the promoters of *Linc-RAM, MyoG*, and *miR-206* in differentiating C2C12 cells (Fig. 3C). Together, our experimental data demonstrate that MyoD is required for FGF2-mediated expression of *Linc-RAM* during muscle cell differentiation.

Overexpression of Linc-RAM rescues the FGF2-induced inhibition of muscle cell differentiation

Given the previous reports that FGF2 is a potent inhibitor of myogenic cell differentiation and Linc-RAM promotes myogenic cell differentiation, together with our present finding that FGF2 down-regulates *Linc-RAM* expression, we questioned whether FGF2 inhibits myogenic differentiation by repressing *Linc-RAM* expression in muscle cells. To examine this possibility, we stably overexpressed *Linc-RAM* in C2C12 cells and

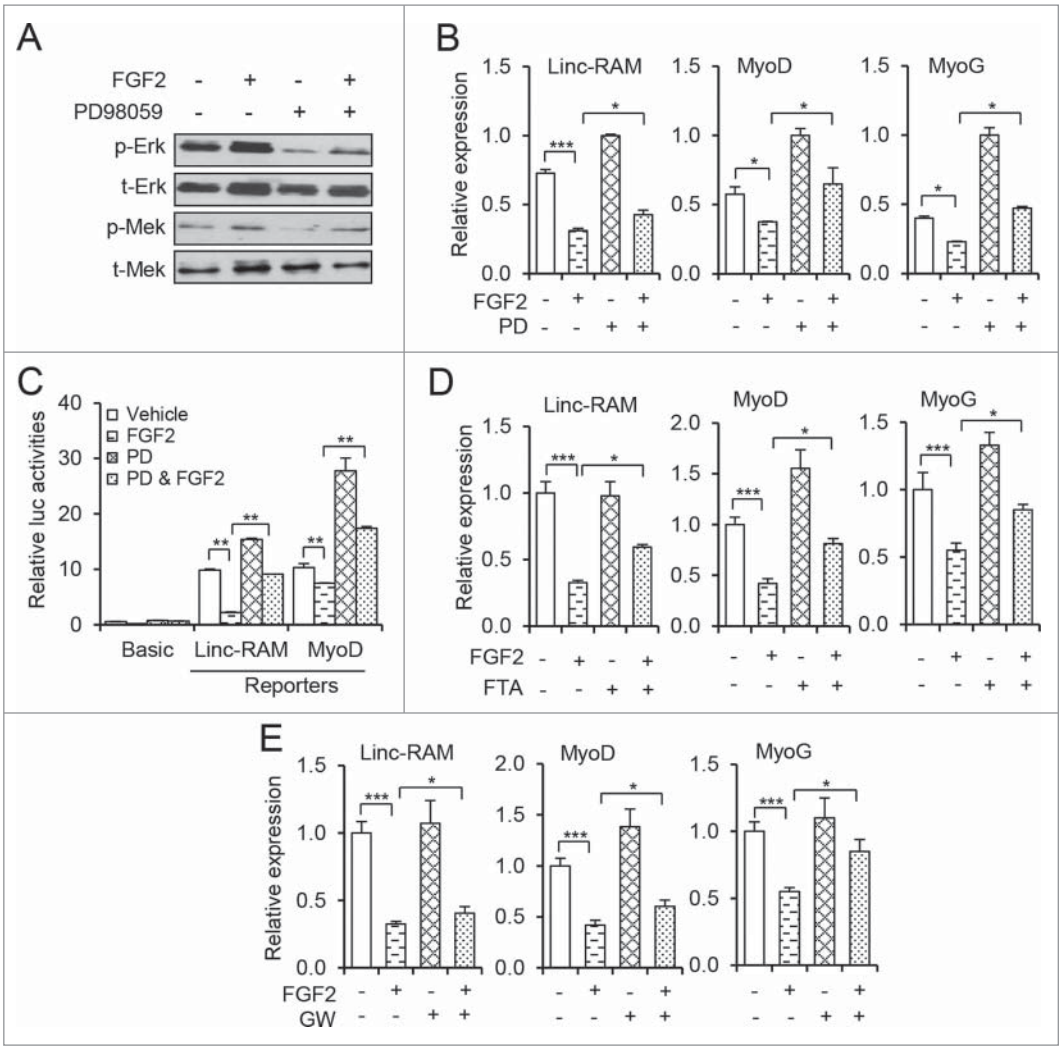

Figure 2. FGF2 regulates *Linc-RAM* gene expression via the Ras/Raf/Mek/Erk signaling pathway. A. C2C12 cells in DM were treated with FGF2, PD98059 (PD, a Mek inhibitor), or PD plus (30 min later) FGF2. After 24 hr, the phosphorylations of Erk and Mek were detected by Western blotting. Total Erk and Mek were examined as controls. The blots shown are representative of the results obtained from three independent experiments. B. The expressions of *Linc-RAM and MyoD, MyoG* in cells treated as described in *a* were examined by RT-qPCR. C. The FGF2 responsiveness of the *Linc-RAM* gene promoter was examined in C2C12 cells by luciferase reporter gene assay. C2C12 cells were treated with FGF2, PD, or PD (for 1 h) plus FGF2, and then transfected with promoter reporter plasmids for *Linc-RAM*. The *MyoD* gene promoter served as positive controls. Promoter activity is expressed as luciferase activity relative to that in the vehicle control group (defined as 1). D. C2C12 cells in DM were treated with FGF2, FTA (a Ras inhibitor), or FTA (for 30 min) plus FGF2. After 24 hr, the expressions of *Linc-RAM* and *MyoD, MyoG* were examined by RT-qPCR. E. C2C12 cells in DM were treated with FGF2, GW (a Raf inhibitor), or GW (for 30 min) plus FGF2. After 24 hr, the expressions of *Linc-RAM* and *MyoD, MyoG* were examined by RT-qPCR. Values are means ± s.e.m. of three independent experiments. $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P < 0.001$, as assessed by the two-tailed Student's *t*-test.

treated the stably overexpressing cells (Linc-OE cells) with either FGF2 or PBS in DM for 24 or 48 hr. The myogenic differentiation of treated cells was assessed by immunostaining with antibodies against MyoG (24 hr) and MHC (48 hr) (Fig. 4A). Consistent with our previous report [35], overexpression of Linc-RAM significantly enhanced myogenic differentiation (Fig. 4A-C). Moreover, we found that *Linc-RAM* overexpression completely abolished the FGF2-mediated reductions of MyoG^{+} (Fig. 4A, B) and MHC^{+} cells (Fig. 4A and C). Together, our data indicate that overexpression of Linc-RAM is sufficient to rescue the inhibitory effect of FGF2 on myogenic differentiation.

Deletion of Linc-RAM *attenuates satellite cell differentiation and primes for self-renewal*

FGF2 plays a critical role in maintaining the self-renewal of satellite cells by inhibiting their differentiation [15]. We previously reported that primary myoblasts isolated from muscle tissues of *Linc-RAM*$^{-/-}$ mice show delayed differentiation than those from wild-type (WT) mice [35]. As our present findings indicated that the FGF2-regulated down-regulation of *Linc-RAM* is required for the ability of FGF2 to inhibit myogenic cell differentiation, we reasoned that satellite cells lacking functional Linc-RAM might exhibit increased self-renewal in the absence of FGF2. We tested this possibility by examining the self-renewal and differentiation of myofiber-associated satellite cells using an *ex vivo* cultured single fiber system [26]. Single fibers isolated from extensor digitorum longus (EDL) muscles were cultured *ex vivo* for 72 hr, and the myofiber-associated satellite cells were immunostained with antibodies against Pax7 and MyoD (Fig. 5A). Typical clusters of satellite cell progeny representing proliferating (Pax7^{+}/MyoD^{+}), differentiating (Pax7^{-}/MyoD^{+}), and self-renewing (Pax7^{+}/MyoD^{-}) cells were counted. Compared to WT control mice, those of the *Linc-RAM*$^{-/-}$ group had significantly more self-renewing cells

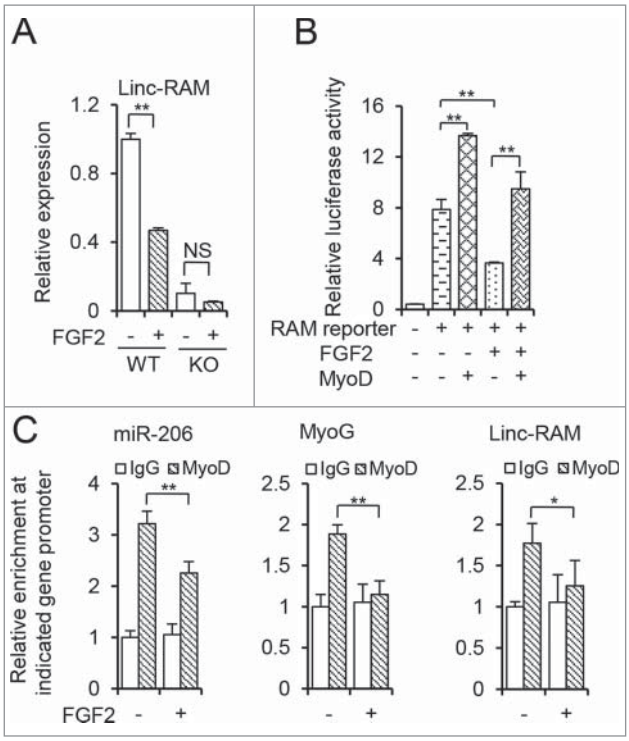

Figure 3. MyoD is required for FGF2-mediated *Linc-RAM* gene expression. A. Expression of *Linc-RAM* in satellite cells isolated from *Pax7-nGFP;MyoD*$^{-/-}$(KO) and *Pax7-nGFP* (WT) mice. Cells were cultured in GM for 48 hr in the presence and absence of FGF2. The expression of *MyoG* served as positive controls. B. The responsiveness of the *Linc-RAM* gene promoter (luciferase activity) was examined in C2C12 cells co-transfected with a MyoD-encoding plasmid and treated with or without FGF2. Promoter activity is expressed as luciferase activity relative to that in the control group (defined as 1). C. The relative enrichment of MyoD in the *Linc-RAM* gene promoter region was assessed by chromatin immunoprecipitation (ChIP) with a MyoD antibody. C2C12 cells cultured in DM for 24 hr in the presence or absence of FGF2 were subjected to ChIP experiments. The *miR-206* and *MyoG* promoters served as positive controls. Values are means ± s.e.m. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as assessed by the two-tailed Student's *t*-test.

(Pax7^{+}/MyoD^{-}) and significantly fewer differentiating cells (Pax7^{-}/MyoD^{+}) (Fig. 5B). These *ex vivo* data reveal that *Linc-RAM*$^{-/-}$ satellite cells exhibit higher self-renewal capabilities and attenuated differentiation in the absence of FGF2. We further confirmed this observation by assessing the differentiation of satellite cells sorted from skeletal muscles of *Linc-RAM*$^{-/-}$*; Pax7-nGFP* mice and their WT littermates (*Pax7-nGFP*) in the presence or absence of FGF2 (Fig. 5C). Consistent with the previous report [35], we found that the differentiation of *Linc-RAM*$^{-/-}$ satellite cells was delayed relative to that of WT cells in the absence of FGF2 (Fig. 5D-F). Remarkably, deletion of *Linc-RAM* exhibited comparable effects on satellite cell differentiation as FGF2 did (Fig. 5D-F), further confirming that Linc-RAM acts as a downstream target of FGF2 in regulating satellite cell function. Together, our results demonstrate that FGF2-regulated Linc-RAM expression is required for the FGF2-mediated self-renewal and differentiation of satellite cells.

Discussion

The widespread application of high-throughput RNA sequencing, with the aid of computational and bioinformatics analysis, has revealed that long noncoding RNAs (lncRNAs) constitute a significant fraction of the transcriptome [41]. Functional screening has implicated many lncRNAs in various cellular events, including cell lineage specification, differentiation, cell proliferation, apoptosis, cellular metabolism, and dosage compensation [42–44]. We recently reported the identification and characterization of a novel long intergenic noncoding RNA, Linc-RAM [35], which is predominantly expressed in skeletal muscle and is transcriptionally regulated by MyoD during myogenic differentiation. *In vivo* functional studies demonstrated that Linc-RAM-knockout mice exhibited an impairment of muscle regeneration that was due to a defect in satellite cell differentiation. Mechanistically, we revealed that Linc-RAM modulates myogenic differentiation by directly interacting with MyoD to facilitate the assembly of the MyoD-Baf60c-Brg1 complex [35]. Although numerous lncRNAs have been described, relatively few have been subjected to functional studies *in vivo* [35,43], and we know very little about the molecular mechanisms underlying their transcriptional regulations. In the present report, we extended our previous work by investigating the transcriptional regulation and intracellular signaling pathway in mediating Linc-RAM gene expression during myogenic cell differentiation. We demonstrate that the FGF2-mediated repression of *Linc-RAM* expression occurs through MyoD via the Ras/Raf/Mek/Erk signaling pathway during myogenic differentiation. Together with our previous findings, our present data provide convincing evidence that Linc-RAM is a lineage-specific lncRNA that plays regulatory roles in modulating muscle cell differentiation via FGF2-mediated signaling.

In skeletal satellite cells, FGF2 potently inhibits myogenic differentiation and activates MAP kinase to promote muscle cell proliferation. This dual function of FGF2, which reflects its regulation of different target genes during myogenic development and muscle regeneration, helps control the balance between cell proliferation and differentiation, and is physiologically and pathologically important for maintaining tissue homeostasis [45]. Various downstream target protein-coding genes involved in the FGF2-mediated behaviors of satellite cells have been identified [12,46,47]. However, no previous work had examined the involvement of non-protein-coding genes, especially long ncRNAs, in the FGF2-mediated inhibition of cell differentiation or promotion of satellite cell self-renewal. In this report, we identify Linc-RAM as the first FGF2-regulated lncRNA and show that FGF2 mediates the self-renewal and differentiation of satellite cells by modulating Linc-RAM in a MyoD-dependent manner. Interestingly, our data show that although overexpression of Linc-RAM promotes differentiation, FGF2 nevertheless inhibits the differentiation in the Linc-RAM overexpression myoblasts, which suggests that FGF2 inhibits myogenic differentiation through both Linc-RAM dependent and independent manner. However, demonstration that FGF2 fails to inhibit myogenic differentiation of the Linc-RAM knockout myoblasts (Fig. 5) excludes the possibility of Linc-RAM independent action of FGF2. The discrepancy between overexpression and knockout experiments suggest the complexity of FGF2 action in regulating myogenic cell differentiation. Taken together, our findings reveal that the transcriptional regulation of a lineage-specific lncRNA can have functional significance in cell fate determination during development.

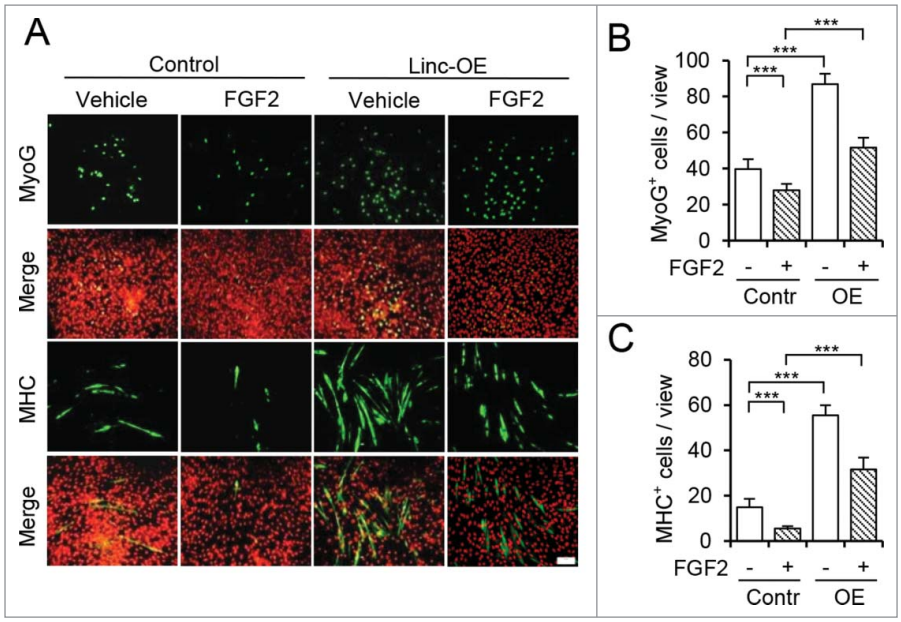

Figure 4. Overexpression of Linc-RAM rescues the FGF2-induced inhibition of muscle cell differentiation. A. C2C12 cells stably overexpressing Linc-RAM (Linc-OE) and control C2C12 cells were treated with FGF2 in DM. After 24 and 48 hr, differentiation was examined by immunostaining for MyoG and MHC, respectively. Scale bar, 100 μm. The images are representative of the results obtained from three independent experiments. B. The numbers of MyoG$^+$ cells per microscopic view in A were counted. C. The numbers of MHC$^+$ cells per microscopic view in A were counted. Values are means ± s.e.m. of three independent experiments. $^{***}P < 0.001$, as obtained using the two-tailed Student's *t*-test.

Given satellite cell plays pivotal role in regulating postnatal muscle growth and tissue homeostasis, muscle regeneration and muscular disease, thus, it has been long a promising target to treat muscle diseases, such as muscular dystrophy. For example, application of FGF2 benefits dystrophic phenotype in mice [21]. Our finding suggests Linc-RAM functions as a downstream target of FGF2 in regulating satellite cell self-renewal and differentiation. It might be intriguing to investigate the pathological phenotype of the *mdx* mice (a human muscular dystrophy mouse model) in absent of Linc-RAM$^{-/-}$ by crossing Linc-RAM$^{-/-}$ mice with the *mdx* mice in the future. The benefit of targeting satellite cells to improve the muscular dystrophy phenotype of *mdx* mice is also supported by additional findings. For example, Wnt7a was shown to considerably ameliorate the muscular dystrophy phenotype of *mdx* mice by stimulating satellite cell self-renewal and expansion [1]. Our group recently reported that acetoacetate acts as a signaling metabolite to alleviate muscular dystrophy in *mdx* mice by stimulating satellite cell proliferation [48]. Finally, our group and other researchers have demonstrated that miRNAs (miR-1, miR-206, miR-431 and miR-127) can functionally improve the pathological phenotype of *mdx* mice by enhancing satellite cell differentiation [26,27,49]. Collectively, the present and previous findings should facilitate the development of stem cell-based treatments that target satellite cell functions in patients with DMD and other muscular diseases.

Materials and methods

Mice and animal care. All animal procedures were approved by the Animal Ethics Committee of Peking Union Medical College (Beijing, China). Mice were housed in the animal facility and had free access to water and standard rodent chow. *Pax7-nGFP* knock-in mice were kindly gifted by Dr. Shahragim Tajbakhsh (Institute Pasteur, France). The *Linc-RAM*$^{-/-}$ mice in the C57BL/6 background were as previously described [35]. The *MyoD*$^{-/-}$ mice (#002523) were obtained from Jackson Laboratory. The *Mdx* mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China).

Cell sorting. FACS was used to obtain satellite cells from 4-week-old gender- and age-matched *Pax7-nGFP*, Pax*7-nGFP; Linc-RAM*$^{-/-}$, and *Pax7-nGFP;MyoD*$^{-/-}$ littermates. Briefly, mononucleated muscle-derived cells were isolated from hindlimb muscles using dispase and collagenase digestion. The GFP$^+$ cells were directly sorted with a BD Aria II Cell Sorting System (BD Biosciences). The sorted cells were cultured on collagen-coated dishes in F10 basal medium (F10 medium containing 20% FBS and 1% penicillin/streptomycin) with/without 10 ng/ml FGF2 (Invitrogen).

C2C12 cell culture, differentiation and treatment. Mouse C2C12 cells (ATCC, CRL-1772) were cultured in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 4.5 g/l glucose, 10% FBS and 1% antibiotics at 37°C in a 5% CO2 atmosphere. At 80–90% confluence, cells were switched to differentiation medium (DM; DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin). For signaling pathway analyses, cells were pretreated with the pharmacological inhibitors, PD98059 (20 μM for 1 hour; Cell Signaling), FTA (5 μM for 30 minutes; Calbiochem) or GW (2.5 μM for 30 minutes; Calbiochem). C2C12 cells stably overexpressing Linc-RAM were generated previously in our laboratory [35].

RT-qPCR analyses. Total RNA was extracted using the TRIzol reagent (Life Technologies) and reverse transcribed using RevertAid reverse transcriptase (Thermo Scientific). qPCR analyses were performed using a Real-Time PCR Detection

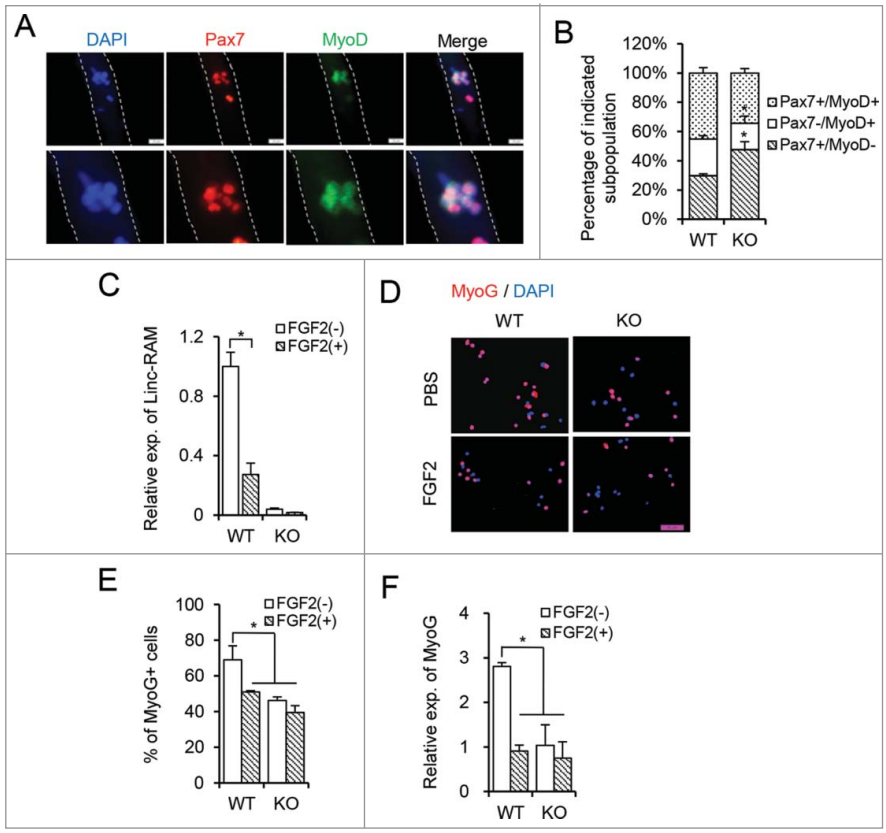

Figure 5. Deletion of *Linc-RAM* attenuates satellite cell differentiation and primes for self-renewal. A. Representative images of satellite cells from *ex vivo*-cultured single fibers immunostained for Pax7 (*red*) and MyoD (*green*). The single fibers were isolated from extensor digitorum longus (EDL) muscles and *ex vivo* cultured for 72 hr. DAPI was used to visualize nuclei (*blue*). Scale bar, 50μm.The lower panel shows magnified views of the upper panels. B. Percentages of Pax7$^+$/MyoD$^+$, Pax7$^-$/MyoD$^+$ and Pax7$^+$/MyoD$^-$ cells immunostained as described in A *Ex vivo*-cultured single fibers were isolated from *Linc-RAM*$^{-/-}$ and WT mice. The data are from three mice of each genotype, and more than 150 myofibers were analyzed per mice. C. The relative expressions of *Linc-RAM* in FACS-sorted satellite cells cultured in the presence or absence of FGF2 were determined by RT-qPCR. The satellite cells were sorted from *Linc-RAM*$^{-/-}$*;Pax7-nGFP*(KO) and *Pax7-nGFP*(WT) control mice (n = 3 animals for each genotype). D. Representative images of MyoG immunostaining (*red*) for FACS-resolved satellite cells cultured in GM for 48 hr in the presence or absence of FGF2. DAPI was used to visualize nuclei (*blue*). Scale bar, 100 μm. E. The percentages of MyoG-positive cells in *d* were calculated from three independent experiments. F. The relative expressions of *MyoG* in the cells described in D were determined by RT-qPCR. Values are means ± s.e.m. $^*P < 0.05$, as assessed by the two-tailed Student's *t*-test.

System (Biorad Connect). The primers were shown in the Table 1.

Luciferase reporter assay. C2C12 cells were transiently transfected with a promoter-luciferase reporter plasmid containing either Linc-RAM promoter [35], or MyoD promoter (2.4 kb upstream of MyoD), or MyoG promoter (gifted by Dr. Zhenguo Wu (Dept. of Biochemistry, Hong Kong University of Science and Technology, Hong Kong), using the FuGene HD transfection reagent (Roche). Twenty-four hours later, the luciferase activity in cell lysates was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Western blot analysis. C2C12 cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet P40, and protease and phosphatase inhibitors. The total proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was performed using primary antibodies against phospho-Mek, Mek, phospho-Erk, and Erk (Cell Signaling Technology). The membranes were washed for 30 min, incubated with HRP-conjugated secondary antibodies (Zhongshanjinqiao Corporation) for 1 hr at room temperature, and then washed with Tris-buffered saline containing 0.1% Tween-20 for 30 min. The membranes were then placed in detection solution (Thermo Scientific), incubated for 1 min at room temperature, and subsequently exposed to X-ray film.

Table 1. Primers used in the present study.

Gene	Sequence	Product
Linc-RAM-F	5′-GGCGGCCATTCCCAGGACTTTG-3′	240bp
Linc-RAM-R	5′-ATCTTCCAGGCTCTGGGGCGAAG-3′	
GAPDH-F	5′-TGGAGAAACCTGCCAAGTATGA-3′	118bp
GAPDH-R	5′-CTGTTGAAGTCGCAGGAGACA-3′	
myogenin-F	5′-GCAATGCACTGGAGTTCGGT-3′	120bp
myogenin-R	5′-GCTGTCCACGATGGACGTAAG-3′	
MyoD-F	5′-CAACGCCATCCGCTACAT-3′	123bp
MyoD-R	5′-GGTCTGGGTTCCCTGTTCT-3′	

ChIP-qPCR assay. C2C12 cells were treated with 10 ng/ml FGF2, chromatin was extracted, and ChIP analyses were performed with the kit (Millipore, Cat. #17–610) according to the manufacturer's standard protocol with MyoD antibodies (Santa Cruz, SC-760). The fold enrichment was quantified by qRT-PCR.

Immunofluorescence staining. Cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 10–15 min, permeabilized with 0.1% Triton X-100, blocked with 3% bovine serum albumen (BSA) in PBS for 10 min, and incubated with antibodies against Pax7, MyoD or MyoG (Developmental Studies Hybridoma Bank [DSHB]) in 3% BSA/PBS (2 μg/mL) for 1–1.5 hr. The cells were then washed with

PBS, incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (10 μg/mL) for 30 min, washed five times with PBS, incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 3 min, washed twice with PBS, and examined by fluorescence microscopy.

Isolation and staining of single myofibers. Single myofibers were isolated from the EDL muscles of 8-week-old male mice by incubation with 3 ml of 0.2% collagenase I (Sigma, C-0130) in serum-free DMEM in a shaking water bath at 37°C for 45–60 min. Digestion was considered complete when the muscle looked less defined and slightly swollen, with hair-like single fibers flowing away from the edges. The digested muscles were placed in a Petri dish, and myofibers were isolated under a microscope. Single fibers were placed in six-well plates precoated with horse serum, and 2 ml/well of fiber medium (DMEM supplemented with 20% FBS and 1% penicillin/streptomycin) was added. The fibers were cultured for 72 hr at 37°C in a 5% CO2 atmosphere, fixed with 4% paraformaldehyde, and stained for Pax7 and MyoD. For statistical analyses, $Pax7^{+}$ and $MyoD^{-}$ cells were counted from at least 40 single fibers per mouse in three pairs of *Linc-RAM*$^{-/-}$ and WT mice.

Statistical analysis. Values are presented as means ± s.e.m. The statistical significance of the difference between two means was calculated with the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

[1] von Maltzahn J, Renaud JM, Parise G, et al. Wnt7a treatment ameliorates muscular dystrophy. Proc Natl Acad Sci U S A. 2012;11;109 (50):20614–9. doi:10.1073/pnas.1215765109

[2] Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. Physiol Rev. 2013; 93(1):23–67. doi:10.1152/physrev.00043.2011

[3] Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell. 2008;10;2 (1):22–31. doi:10.1016/j.stem.2007.12.012

[4] Kuang S, Kuroda K, Le Grand F, et al. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell. 2007;01;129 (5):999–1010. doi:10.1016/j.cell.2007.03.044

[5] DiMario J, Buffinger N, Yamada S, et al. Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. Science. 1989;12;244(4905):688–90. doi:10.1126/science.2717945

[6] Le Grand F, Jones AE, Seale V, et al. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. Cell Stem Cell. 2009;05;4(6):535–47. doi:10.1016/j.stem.2009.03.013

[7] Brack AS, Conboy IM, Conboy MJ, et al. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell .2008;10;2(1):50–9. doi:10.1016/j.stem.2007.10.006

[8] Mousavi K, Jasmin BJ. BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation. J Neurosci. 2006;24;26 (21):5739–49. doi:10.1523/JNEUROSCI.5398-05.2006

[9] Ates K, Yang SY, Orrell RW, et al. The IGF-I splice variant MGF increases progenitor cells in ALS, dystrophic, and normal muscle. FEBS Lett. 2007;12;581(14):2727–32. doi:10.1016/j.febslet.2007.05.030

[10] Miller KJ, Thaloor D, Matteson S, et al. Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. Am J Physiol Cell Physiol. 2000;278(1):C174–81. doi:10.1152/ajpcell.2000.278.1.C174

[11] Bentzinger CF, von Maltzahn J, Rudnicki MA. Extrinsic regulation of satellite cell specification. Stem Cell Res Ther. 2010;26;1(3):27. doi:10.1186/scrt27

[12] Vaidya TB, Rhodes SJ, Taparowsky EJ, et al. Fibroblast growth factor and transforming growth factor beta repress transcription of the myogenic regulatory gene MyoD1. Mol Cell Biol. 1989;9(8):3576–9. doi:10.1128/MCB.9.8.3576

[13] Johnson SE, Allen RE. Activation of skeletal muscle satellite cells and the role of fibroblast growth factor receptors. Exp Cell Res. 1995;219 (2):449–53. doi:10.1006/excr.1995.1251

[14] Kastner S, Elias MC, Rivera AJ, et al. Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. J Histochem Cytochem. 2000;48(8):1079–96. doi:10.1177/002215540004800805

[15] Pawlikowski B, Vogler TO, Gadek K, et al. Regulation of skeletal muscle stem cells by fibroblast growth factors. Dev Dyn. 2017;246 (5):359–367. doi:10.1002/dvdy.24495

[16] Jones NC, Tyner KJ, Nibarger L, et al. The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. J Cell Biol. 2005;11;169(1):105–16. doi:10.1083/jcb.200408066

[17] Bernet JD, Doles JD, Hall JK, et al. p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice. Nat Med. 2014;20(3):265–71. doi:10.1038/nm.3465

[18] Shaoul E, Reich-Slotky R, Berman B, et al. Fibroblast growth factor receptors display both common and distinct signaling pathways. Oncogene. 1995;20;10(8):1553–61

[19] Jones NC, Fedorov YV, Rosenthal RS, et al. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. J Cell Physiol. 2001 Jan;186(1):104–15. doi:10.1002/1097-4652(200101)186

[20] Price FD, von Maltzahn J, Bentzinger CF, et al. Inhibition of JAK-STAT signaling stimulates adult satellite cell function. Nat Med. 2014;20(10):1174–81. doi:10.1038/nm.3655

[21] Lefaucheur JP, Sebille A. Basic fibroblast growth factor promotes in vivo muscle regeneration in murine muscular dystrophy. Neurosci Lett. 1995;29;202(1-2):121–4. doi:10.1016/0304-3940(95)12223-0

[22] Doukas J, Blease K, Craig D, et al. Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. Mol Ther. 2002;5(5 Pt 1):517–27. doi:10.1006/mthe.2002.0579

[23] Simionescu-Bankston A, Kumar A. Noncoding RNAs in the regulation of skeletal muscle biology in health and disease. J Mol Med (Berl). 2016;94(8):853–66. doi:10.1007/s00109-016-1443-y

[24] Chen JF, Tao Y, Li J, et al. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. J Cell Biol. 2010;06;190(5):867–79. doi:10.1083/jcb.200911036

[25] Crist CG, Montarras D, Pallafacchina G, et al. Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc Natl Acad Sci U S A. 2009;11;106(32):13383–7. doi:10.1073/pnas.0900210106

[26] Wu R, Li H, Zhai L, et al. MicroRNA-431 accelerates muscle regeneration and ameliorates muscular dystrophy by targeting Pax7 in mice. Nat Commun. 2015;07;6:7713. doi:10.1038/ncomms8713

[27] Zhai L, Wu R, Han W, et al. miR-127 enhances myogenic cell differentiation by targeting S1PR3. Cell Death Dis. 2017;30;8(3):e2707. doi:10.1038/cddis.2017.128

[28] Ng SY, Bogu GK, Soh BS, et al. The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. Mol Cell. 2013;08;51 (3):349–59. doi:10.1016/j.molcel.2013.07.017

[29] Klattenhoff CA, Scheuermann JC, Surface LE, et al. Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell .2013;31;152(3):570–83. doi:10.1016/j.cell.2013.01.003
[30] Cesana M, Cacchiarelli D, Legnini I, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011;14;147(2):358–69. doi:10.1016/j.cell.2011.09.028
[31] Zhou L, Sun K, Zhao Y, et al. Linc-YY1 promotes myogenic differentiation and muscle regeneration through an interaction with the transcription factor YY1. Nat Commun. 2015;11;6:10026. doi:10.1038/ncomms10026
[32] Mousavi K, Zare H, Dell'orso S, et al. eRNAs promote transcription by establishing chromatin accessibility at defined genomic loci. Mol Cell. 2013;12;51(5):606–17. doi:10.1016/j.molcel.2013.07.022
[33] Gong C, Li Z, Ramanujan K, et al. A long non-coding RNA, LncMyoD, regulates skeletal muscle differentiation by blocking IMP2-mediated mRNA translation. Dev Cell .2015;27;34(2):181–91. doi:10.1016/j.devcel.2015.05.009
[34] Mueller AC, Cichewicz MA, Dey BK, et al. MUNC, a long noncoding RNA that facilitates the function of MyoD in skeletal myogenesis. Mol Cell Biol. 2015;35(3):498–513. doi:10.1128/MCB.01079-14
[35] Yu X, Zhang Y, Li T, et al. Long non-coding RNA Linc-RAM enhances myogenic differentiation by interacting with MyoD. Nat Commun. 2017;16;8:14016. doi:10.1038/ncomms14016
[36] Machida S, Booth FW. Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation. Proc Nutr Soc. 2004;63(2):337–40. doi:10.1079/PNS2004354
[37] McCroskery S, Thomas M, Maxwell L, et al. Myostatin negatively regulates satellite cell activation and self-renewal. J Cell Biol. 2003;15;162(6):1135–47. doi:10.1083/jcb.200207056
[38] Campbell JS, Wenderoth MP, Hauschka SD, et al. Differential activation of mitogen-activated protein kinase in response to basic fibroblast growth factor in skeletal muscle cells. Proc Natl Acad Sci U S A. 1995;31;92(3):870–4. doi:10.1073/pnas.92.3.870
[39] Fedorov YV, Rosenthal RS, Olwin BB. Oncogenic Ras-induced proliferation requires autocrine fibroblast growth factor 2 signaling in skeletal muscle cells. J Cell Biol. 2001;19;152(6):1301–5. doi:10.1083/jcb.152.6.1301
[40] Anderson DM, Anderson KM, Chang CL, et al. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell. 2015;12;160(4):595–606. doi:10.1016/j.cell.2015.01.009
[41] Zhao Y, Li H, Fang S, et al. NONCODE 2016: an informative and valuable data source of long non-coding RNAs. Nucleic Acids Res. 2016;04;44(D1):D203–8. doi:10.1093/nar/gkv1252
[42] Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet. 2014;15(1):7–21. doi:10.1038/nrg3606. PubMe
[43] Sauvageau M, Goff LA, Lodato S, et al. Multiple knockout mouse models reveal lincRNAs are required for life and brain development. Elife. 2013;31;2:e0.1749. doi:10.7554/eLife.01749
[44] Guttman M, Donaghey J, Carey BW, et al. lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature. 2011;28;477(7364):295–300. doi:10.1038/nature10398
[45] Chakkalakal JV, Jones KM, Basson MA, et al. The aged niche disrupts muscle stem cell quiescence. Nature. 2012;18;490(7420):355–60. doi:10.1038/nature11438
[46] Li L, Zhou J, James G, et al. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell. 1992;24;71(7):1181–94. doi:10.1016/S0092-8674(05)80066-2
[47] Hardy S, Kong Y, Konieczny SF. Fibroblast growth factor inhibits MRF4 activity independently of the phosphorylation status of a conserved threonine residue within the DNA-binding domain. Mol Cell Biol. 1993;13(10):5943–56. doi:10.1128/MCB.13.10.5943
[48] Zou X, Meng J, Li L, et al. Acetoacetate Accelerates Muscle Regeneration and Ameliorates Muscular Dystrophy in Mice. J Biol Chem. 2016;29;291(5):2181–95. doi:10.1074/jbc.M115.676510
[49] Liu N, Williams AH, Maxeiner JM, et al. microRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice. J Clin Invest. 2012;122(6):2054–65. doi:10.1172/JCI62656